A Kinetically Competent Thiyl Radical Intermediate in Ribonucleotide Reduction

by

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To my parents
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ABSTRACT
The ribonucleotide reductases (RNRs) catalyze the conversion of ribonucleotides into 2'-deoxyribonucleotides. All known RNRs require metallocofactors for activity. These metallocofactors are proposed to generate a thiyl radical at the active site of the enzyme. This thiyl radical is proposed to initiate nucleotide reduction by abstracting hydrogen from the 3' position of the substrate. The hypothesis that a thiyl radical is a kinetically competent intermediate in ribonucleotide reduction was tested for the ribonucleoside triphosphate reductase (RTPR) of Lactobacillus leichmannii, which requires adenosylcobalamin (AdoCbl) for activity. Homolytic bond cleavage of the carbon-cobalt bond of AdoCbl is required for thiyl radical formation. Rapid freeze quench (RFQ) techniques in combination with electron paramagnetic resonance (EPR) spectroscopy were used to characterize a kinetically competent paramagnetic intermediate. RFQ EPR experiments with RTPR incorporating deuterated cysteine residues showed that this paramagnetic intermediate comprises a protein-based thiyl radical coupled to cob(II)alamin by exchange and dipolar interactions. Carbon-cobalt bond homolysis also takes place in the absence of substrate, during an exchange reaction in which the 5' hydrogens of AdoCbl are exchanged with the solvent. The kinetics and thermodynamics of carbon-cobalt bond homolysis during this exchange reaction were studied using stopped-flow UV-visible (SF UV-vis) spectroscopy. Kinetic and equilibrium isotope effects observed with [5'-2H2]-AdoCbl and/or D2O support mechanism in which carbon-cobalt bond homolysis and thiyl radical formation occur in a concerted fashion. Measurement of the [AdoCbl] and temperature dependence of the rate of carbon-cobalt bond homolysis/thiyl radical formation allows determination of activation parameters for this process, which suggest that entropy, rather than enthalpy, drives the reaction. Evidence that carbon-cobalt bond re-formation follows every turnover was obtained by rapid acid quench studies of the rate of exchange of tritium from [5'-3H]-AdoCbl during the first turnover, as well as from steady state kinetic experiments indicating that AdoCbl can dissociate from RTPR after the first turnover.

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Thesis acknowledgments tend, in general, to be written under less than optimal circumstances. The various demands and stresses of the later stages of thesis writing lend themselves more naturally to bitter, nihilistic ravings or the hallucinatory anti-prose of the sleep-deprived than to the graceful outpouring of gratitude one's mentors, colleagues, and friends deserve. Despite this disadvantage, I will attempt to do justice to the many people who have contributed to this work.

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<tr>
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<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>Ad</td>
<td>adenosine</td>
</tr>
<tr>
<td>AdoCbl</td>
<td>adenosylcobalamin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>βME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>dATP</td>
<td>2´-deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>Ci</td>
<td>curie</td>
</tr>
<tr>
<td>CIUD(T)P</td>
<td>2´-deoxy-2´-chlorouridine di(tri)phosphate</td>
</tr>
<tr>
<td>CTP</td>
<td>cytidine triphosphate</td>
</tr>
<tr>
<td>dA</td>
<td>deoxyadenosine</td>
</tr>
<tr>
<td>DE-52</td>
<td>diethylaminoethyl cellulose from Whatman</td>
</tr>
<tr>
<td>DMB</td>
<td>dimethylbenzimidazole</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>dUTP</td>
<td>2´-deoxyuridine triphosphate</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>ESEEM</td>
<td>electron spin echo envelope modulation</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FUD(T)P</td>
<td>2'-deoxy-2'-'fluorouridine di(tri)phosphate</td>
</tr>
<tr>
<td>G</td>
<td>gravitational acceleration</td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography with mass spectral detection</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>HU</td>
<td>hydroxyurea</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl b-D-thiogalactoside</td>
</tr>
<tr>
<td>K&lt;sub&gt;a&lt;/sub&gt;</td>
<td>association constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>k&lt;sub&gt;obs&lt;/sub&gt;</td>
<td>observed rate constant</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>N&lt;sub&gt;3&lt;/sub&gt;UD(T)P</td>
<td>2'-deoxy-2'-chlorouridine di(tri)phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>O. D.</td>
<td>optical density</td>
</tr>
<tr>
<td>R1</td>
<td>the substrate-binding subunit of the <em>E. coli</em> ribonucleotide reductase</td>
</tr>
<tr>
<td>R2</td>
<td>the diiron cluster-containing subunit of the <em>E. coli</em> ribonucleotide reductase</td>
</tr>
<tr>
<td>RFQ</td>
<td>rapid freeze quench</td>
</tr>
<tr>
<td>RTPR</td>
<td>ribonucleoside diphosphate reductase from <em>E. coli</em></td>
</tr>
<tr>
<td>RP</td>
<td>reversed-phase</td>
</tr>
<tr>
<td>RTPR</td>
<td>ribonucleoside triphosphate reductase from <em>Lactobacillus leichmannii</em></td>
</tr>
<tr>
<td>SCM</td>
<td>S-carboxymethyl</td>
</tr>
<tr>
<td>SF</td>
<td>stopped-flow</td>
</tr>
</tbody>
</table>
tBDMS  $t$-butyldimethylsilyl
TR     $E.~coli$ thioredoxin
TRR    $E.~coli$ thioredoxin reductase
UV-vis ultraviolet-visible
$V_{\text{max}}$ maximal velocity
Chapter 1:

Mechanistic Investigations of Ribonucleotide Reductases
1. Introduction

1.1 General Introduction

Ribonucleotide reductases catalyze the conversion of nucleotides to deoxynucleotides (Eq. 1.1), the monomeric precursors required for DNA biosynthesis. These enzymes are of interest for a variety of reasons and have consequently been the focus of a number of recent reviews. Reductases have been proposed to provide a link between the RNA and the DNA world (Reichard, 1993). Their central role in nucleotide metabolism has made them the successful target for design of antitumor and antiviral agents (Abbruzzese & Plunkett, 1991, Robins et al, 1995, Hertel et al, 1996). Their inducibility by DNA damaging agents suggests that they may play a role in cell cycle regulation (Elledge et al, 1993). The assembly of the essential diferric •Y cofactor of one class of reductases has provided a paradigm for studying and understanding post-translational modification by metal/oxygen-based chemistry and biochemistry. Finally, the observation that reductases possess protein radicals (Stubbe, 1989) has attracted the attention of those interested in understanding how enzymes harness the chemical reactivity of free radicals to execute very difficult chemistry in an exquisitely controlled fashion.

\[
\begin{align*}
\text{E} & \quad \text{SH} + \quad \text{(P)PP0} \quad \text{O} \quad \text{N} \quad \text{H} \quad \text{H} \quad \text{HO} \quad \text{OH} \quad \text{RNR} \\
\text{E} & \quad \text{SH} + \quad \text{(P)PP0} \quad \text{O} \quad \text{N} \quad \text{H} \quad \text{H} \quad \text{HO} \quad \text{OH} \quad \text{H} \quad \text{HO} \quad \text{OH} \\
\text{Thioredoxin, thioredoxin reductase, NADPH} & \quad \text{or} \quad \text{Glutaredoxin, glutaredoxin reductase, NADPH} \\
& \quad \text{or} \quad \text{Formate} \\
\text{+ H2O} & \quad \text{+ H2O}
\end{align*}
\]

Eq. 1.1

This review will focus specifically on the last topic. A detailed chemical mechanism for nucleotide reduction will be presented. The chemical and biochemical evidence in support of each step will be described. Model
reactions providing precedent for each step will be presented and discussed. In some cases, alternative mechanisms for a given step will be presented, and the alternatives evaluated based on biochemical evidence. General principles of free radical-based enzymatic transformations learned from these studies will be put forth with the goal of facilitating future studies on less well-understood enzymatic systems of similar complexity.

Four classes of ribonucleotide reductases have now been isolated and characterized thus far (Fig. 1.1), all of which catalyze the same reaction (Eq. 1.1), although some reductases use nucleoside diphosphates as substrates while others use triphosphates. The class I enzymes are composed of two homodimeric subunits (α2 = R1 and β2 = R2) (Fig. 1.2). The R2 subunit contains a diferric cluster-•Y cofactor, essential for reduction. While it is still controversial, there is approximately one •Y and two diferric clusters per R2. The R1 subunit is the business end of this reductase, and will quite likely have a very similar tertiary fold and active site in all four classes of reductases. It possesses three cysteine residues within the active site, all of which are essential for reduction. The structure of R1 reveals that these three cysteines are within 6 Å of each other (Uhlin & Eklund, 1994, Eliasson et al, 1996). Two additional cysteines in class I and II RNRs, invisible in the structure of the E. coli R1 because the C-terminus of the R1 peptide is disordered, are required for in vivo reduction, shuttling reducing equivalents into the active site by the physiological reducing system thioredoxin or glutaredoxin reducing systems (Mao et al, 1992b, Booker et al, 1994).

The class II enzymes require adenosylcobalamin (AdoCbl) as a cofactor. Three of the members of this class are composed of monomers (Panagou et al, 1972, Booker & Stubbe, 1993, Yang et al, 1994, Tauer & Benner, 1997), while the fourth has an α2β2 subunit composition.(Tsai & Hogenkamp, 1980) Despite the availability of sequences for all of these proteins and the recent X-ray structure of the adenosylcobalamin-requiring methylmalonyl-CoA mutase (Mancia et al, 1996), an AdoCbl binding motif has not yet been identified.

The class III reductases are composed of two homodimeric subunits (α2β2). The cofactor-generating subunit, β2, bears sequence homology to the pyruvate formate lyase activating enzyme (Fleischmann et al., 1995,
Fig. 1.1. Metallocofactors used by ribonucleotide reductases.

*E. coli*, mammalian, HSV

**L. leichmannii**

*B. ammoniagenes*

S-Adenosylmethionine
Fe-S Cluster
anaerobic *E. coli*
Fig. 1.2. The class I reductase from *E. coli*. The R2 subunit contains the diferric cluster and the •Y. The R1 subunit contains the substrate binding site, the thiol that becomes the putative thiol radical (C439), the cysteines that provide reducing equivalents to the nucleotide (C462 and C225), the cysteines that shuttle reducing equivalents from an external reducing system (C754 and C759), and a glutamate proposed to act as a general base (E441).
Rödel et al., 1988, Sun et al., 1995, Tomaszewski & Rüger, 1987). Recent data suggests that both of these enzymes possess a single iron-sulfur cluster at their subunit interface and bind S-adenosylmethionine (SAM). The iron sulfur cluster in the presence of SAM generates a glycyl radical on the α2 subunit, in a sequence context similar to the glycyl radical generated on pyruvate formate lyase (Rödel et al., 1988) (Tomaszewski & Rüger, 1987, Fleischmann et al., 1995, Sun et al., 1995). During glycyl radical generation, SAM is converted to 5'-deoxyadenosine (5'-dA) and methionine. The possible analogy between this unprecedented chemistry and the reactivity of AdoCbl has not gone unnoticed (Knappe & Schmitt, 1976, Wong & Kozarich, 1994). This enzyme differs from the class I and II reductases in that formate, rather than thioredoxin or glutaredoxin, provides the reducing equivalents for nucleotide reduction and, in the process, it is oxidized to CO₂. It is probably not a coincidence that formate is also one of the products of pyruvate formate lyase.

Based on growth requirements, the loss of enzymatic activity in the presence of hydroxyurea and the UV-visible spectrum of the purified protein, the class IV reductases are proposed to contain a dinuclear manganese cluster and a •Y (Willing et al., 1988, Auling & Follmann, 1994). Recent EPR studies support the latter proposal (Griepenburg et al., 1996). Few details on this protein, however, have yet emerged.

Despite the apparent diversity in cofactor requirement, subunit organization, and primary sequence, a unifying mechanistic theme for RNRs has emerged. The domains or subunits in which nucleotide reduction occurs in each class of RNR are predicted to have similar secondary and tertiary structures and to catalyze the reduction process through similar radical-based chemical mechanisms. What is unique to each class is the metallo-cofactor that functions as the radical chain initiator of the reduction process. Each cofactor has been proposed to generate a thiyl radical in the active site of the subunit in which reduction occurs and this thiyl radical is proposed to initiate the radical-dependent reduction process. In the first half of this review, the postulated mechanisms by which each cofactor generates a thiyl radical will be presented and discussed. In the second half of the review, a detailed mechanism for the radical-mediated nucleotide reduction process is presented. Data in support of this mechanism, including detailed studies with a variety of
mechanism-based nucleotide inhibitors of RNR and studies of site-directed mutants of RNR, which must be accomodated by any proposed alternative mechanisms, will be presented and discussed.

1.2 Outline of the Proposed Mechanisms for Thiol Radical Formation, Nucleotide Reduction, and Mechanism-Based Inactivation by Substrate Analogs

What we know at present about the generation of the putative essential thiol radical for RNRs (Fig. 1.1) will be discussed in detail for the the class I E. coli and class II L. leichmannii enzymes. In the case of the class I reductases, the •Y on the R2 subunit, formed during the assembly of the diferric cluster,(Atkin et al, 1973, Bollinger et al, 1991a, Bollinger et al, 1991b, Bollinger et al, 1994a, Ravi et al, 1994, Tong et al, 1996) is proposed (Nyholm et al, 1993, Sahlin et al, 1995, Ekberg et al, 1996, Larsson et al, 1996) to initiate a chain of proton-coupled electron transfers between amino acid residues (Fig. 1.3) that results in a net intersubunit electron transfer and formation of a thiol radical on the R1 subunit. The pathway for this process has been proposed based on the recent structures of the R1 and R2 subunits and on biochemical studies, which suggest a mode of docking of these subunits. In addition sequences of 40 class I RNRs are now available and all of the indicated residues are conserved (van der Donk & Stubbe, 1998). Finally, recent studies from the Sjöberg, Thelander, and Gräslund laboratories have shown that mutation of any of these residues destroys the ability of the enzymes to catalyze nucleotide reduction (Nyholm et al, 1993, Sahlin et al, 1995, Ekberg et al, 1996, Larsson et al, 1996). A distance of ~35 Å is proposed between Y122 radical on R2 and the thiol of C439 on R1. The mechanism of communication between these subunits is a major focus of effort in many laboratories. The thermodynamics and kinetics of •Y reduction will be discussed, as will the possible significance of proton transfer.

The class II RNRs utilize AdoCbl as a radical chain initiator. The L. leichmanii reductase catalyzes homolysis of the carbon-cobalt bond of AdoCbl. The unpaired spin generated at the 5′ position of the axial adenosine ligand of the cofactor is proposed to generate an active site thiol radical on residue C408. Thus, AdoCbl has been proposed to serve as the functional equivalent of the R2 subunit in the class I reductase (Fig. 1.4).
Fig. 1.3. Residues proposed to be involved in electron transfer between the R2 and R1 subunits of the *E. coli* RNR. Y122 of R2 is the stable •Y. C439 of R1 is proposed to form the catalytically essential thiol radical. Other residues shown represent a proposed electron/proton transfer pathway between the two proteins. Adapted from Ekberg, et al, 1996.
Fig. 1.4. The class II reductase from *L. leichmannii*. Shown are contains the cysteine believe to be oxidized to a thiol radical (C408), the cysteines proposed to provide reducing equivalents to the nucleotide (C119 and C419), the cysteines proposed to shuttle reducing equivalents from an external reducing system (C731 and C736), and a glutamate proposed to act as a general base (E440).
Functional Equivalent of *E. coli* R2
The major difference between these two classes, as will be described in detail subsequently, is that the cofactor is directly involved in hydrogen atom abstraction from cysteine 408, thus requiring the cofactor to bind in a region close to the active site cysteines. The factors influencing catalysis of carbon-cobalt bond homolysis, the question of whether carbon-cobalt bond homolysis and thyl radical formation are concerted or stepwise, and the issue of whether the carbon-cobalt bond is re-formed after each nucleotide reduction event will be addressed.

The proposed mechanism for nucleotide reduction (Stubbe, 1990, Stubbe & van der Donk, 1995) is shown in Scheme 1.1. In the first step, the metallo-cofactor generates the essential thyl radical, the detailed mechanism of which is cofactor-dependent. Once the thyl radical is generated, the mechanisms of all RNRs are proposed to become congruent. The thyl radical is proposed to initiate catalysis by abstraction of the 3′ hydrogen of the nucleotide substrate to generate a 3′-nucleotide radical intermediate. This intermediate is proposed to rapidly lose its 2′ hydroxyl group as water, and the hydrogen of its 3′-hydroxyl group to glutamate, forming a 3′-keto-2′-deoxynucleotide radical. Reduction is proposed to proceed via a single electron transfer from one of the cysteines located on the α face of the nucleotide concomitant with rapid proton transfer to generate a 3′-keto-2′-deoxynucleotide intermediate and a disulfide radical anion. A second one-electron reduction from the resulting disulfide radical is proposed to generate a 3′-ketodeoxynucleotide radical and a disulfide, and regenerate the anionic form of glutamate. Re-abstraction of hydrogen atom from the cysteine on the β face of the nucleotide by the 3′ deoxyketyl radical completes the first turnover and regenerates the thyl radical. In order for multiple turnovers to take place, the disulfide must be re-reduced. Thermodynamic and kinetic considerations related to each of the proposed steps will be described, with a special emphasis on the influence of protonation state on the stability of radical intermediates. Alternative mechanisms recently proposed by others will also be examined.

Since the initial report of Eckstein, Thelander and their coworkers in 1976 that the *E. coli* RNR could be inactivated by 2′-chloro and 2′-azido-2′-deoxynucleotides, these and similar compounds have played an important role in understanding the catalytic capabilities of the RNRs. Any
Scheme 1.1. General mechanism for nucleotide reduction by the ribonucleotide reductases. Although the diphosphate is pictured here, the same mechanism is proposed for di- and triphosphate-reducing enzymes.
Scheme 1.2. General mechanism for mechanism-based inactivation of ribonucleotide reductases by 2'-substituted nucleotide analogs ($X =$ halo, azido, etc.). Adapted from Stubbe and van der Donk, 1995.
Reduction from bottom (α) face

Reduction from top (β) face

Loss of tyrosyl radical (for class I reductases)
mechanism proposed for the nucleotide reduction process must also explain the unusual chemistry observed with 2′-substituted nucleotide analogs. Over the years a mechanistic paradigm (Scheme 1.2) has unfolded to explain the observed inactivation of ribonucleotide reductases by 2′-substituted-2′-deoxynucleotides (Stubbe and van der Donk, 1995). Almost all of these nucleotides are mechanism-based inhibitors in which, as for the normal substrate, the reaction is initiated by abstraction of the 3′ hydrogen by the active site thyl radical. In addition, i.e. the case of the 2′-chloro- and fluoro- derivatives, inactivation is also accompanied by deoxynucleotide production. To explain the difference in the results observed with these inhibitors in comparison with the normal substrate, the 2′ substituent is proposed to depart without the requirement for protonation of the leaving group. Thus, the same 3′-keto-2′-deoxynucleotide radical is present in the active site as is observed during the normal reduction process, but both thiols on the α face of the nucleotide are proposed to remain protonated. The 3′-keto-deoxynucleotide radical can then be reduced via hydrogen atom transfer, not electron transfer as proposed with the normal substrate. Reduction from the β face regenerates the thyl radical, allowing active cofactor to be regenerated as well. Reduction from the α face forms a disulfide radical. In both cases, the product is the 3′-keto-deoxynucleotide, which has been identified by trapping with sodium borohydride (Ator & Stubbe, 1985, Ashley et al, 1988). Appropriate labeling studies using [2′- or 3′-3H]- labeled nucleotide analogs has provided strong support for this proposal. Reduction from the top face is tantamount to a 1, 2 hydrogen shift and is markedly similar to reactions catalyzed by enzymes using AdoCbl to mediate rearrangement reactions.

Regardless of which thiol provides the reducing equivalents, a 3′-keto-deoxynucleotide is generated. This compound is released from the enzyme active site into solution, where its chemical instability results in release of base (N), loss of inorganic pyrophosphate or tripolyphosphate, and generation of 3-methylene-3-2H-furanone. This sugar analog is highly activated toward nucleophilic attack, and can alkylate and inactivate the R1 subunit or its equivalent. Remarkably, the normal nucleotide substrate can become a mechanism-based inhibitor when either of the cysteines in the active site are mutated to serines, disrupting the normal reduction process,
or when the cysteines are oxidized to a disulfide (Mao et al, 1989, Mao et al, 1992a, Mao et al, 1992b, Booker et al, 1994, van der Donk et al, 1996b). In addition, in recent studies with R1 mutants of the conserved glutamate within the active site, the normal substrate again acts as a mechanism-based inhibitor (van der Donk et al, 1996a, Persson et al, 1997). Similarities and differences between chemical model systems for the inactivation processes and the inactivation chemistry observed in enzymatic systems will be described. Whatever model is proposed, it must be able to account for a wealth of biochemical data that has been accumulated with these mechanism-based inhibitors.

2. Generation of Thiyl Radicals at an RNR Active Site by Adenosylcobalamin

2.1 Evidence for a Thiyl Radical

The cofactor whose role in catalysis is best understood is AdoCbl in the class II RNRs, so we will begin with this system. The RNR from L. leichmannii (RTPR) has been studied most extensively of the class II enzymes. Evidence will be presented that homolytic bond cleavage of the carbon-cobalt bond of adenosylcobalamin (AdoCbl) allows abstraction of a hydrogen atom active site cysteine, C408 (Scheme 1.3). This system has provided the only direct spectroscopic evidence for thiyl radicals in RNRs to date. However, the biochemical and chemical similarities between this enzyme and the E. coli class I RNR strongly suggest that thiyl radicals are intermediates in this enzyme, as well.

A key to understanding the function of the cofactor using presteady state stopped flow (SF) UV-vis spectroscopy and rapid freeze quench (RFQ) EPR spectroscopy came with the discovery of a second reaction catalyzed by this enzyme. It was reported in the 1960s by two groups that if [5'-3H]-AdoCbl was incubated with RTPR, allosteric effector, and reductant, that the 5'-hydrogens of AdoCbl could exchange with solvent (Beck et al, 1966a, Abeles & Beck, 1967, Hogenkamp et al, 1968). A proposed mechanism for this process is shown in Scheme 1.3. The proposal is that the cofactor generates, in a stepwise or a concerted fashion, a thiyl radical, 5'-deoxyadenosine (5'-dA) and cob(II)alamin and that this process is reversible. Based on the relatively fast rate of this process in comparison to
Scheme 1.3. Generation of a thyl radical at the active site of the *L. leichmannii* class II reductase and its role in nucleotide reduction and exchange of the 5' hydrogens of AdoCbl with solvent. Isotopic label is denoted by an asterisk.
Initiation of Nucleotide Reduction
the nucleotide reduction process, we have argued in detail that this exchange reaction offers a glimpse of the mechanism by which the cofactor generates the thyl radical, and hence reproduces the key first step in the nucleotide reduction process (Licht et al, 1996).

Historically, the first evidence that the *L. leichmannii* RTPR catalyzes the cleavage of the carbon-cobalt bond of AdoCbl came from the characterization of this exchange reaction. Incubation of RTPR with [5'-3H]-AdoCbl labeled non-specifically in both the 5'-pro-R and pro-S positions was found to cause exchange all of tritium in the cofactor with the solvent, indicating that the two 5' methylene hydrogens become equivalent in the course of this reaction (Frey et al, 1967). This observation suggests that the enzyme catalyzes formation of 5'-dA and cob(II)alamin from AdoCbl.

The hypothesis that RTPR catalyzes the formation of cob(II)alamin was confirmed in the 1970s by spectroscopic characterization of intermediates formed during the exchange reaction (and nucleotide reduction) (Tamao & Blakley, 1973, Orme-Johnson et al, 1974). SF UV-vis spectroscopy showed that under the conditions of the exchange reaction, using DTT as a reductant, RTPR catalyzes the conversion of AdoCbl into cob(II)alamin with an apparent first order rate constant of ~40 s⁻¹ (Tamao & Blakley, 1973). Carbon-cobalt bond cleavage is thus kinetically competent to be on the pathway for nucleotide reduction, which occurs with a rate constant of 1.5 s⁻¹. RFQ EPR experiments revealed that the intermediate formed on carbon-cobalt bond homolysis is unique and distinct from cob(II)alamin bound to the enzyme in the presence of 5'-dA when turnover is not taking place. In the latter case, the spectrum exhibits a $g_{\perp}$ of 2.23, $g_{\parallel}$ of 2.0, and a cobalt nuclear hyperfine interaction of 110 gauss (Hamilton et al, 1971). In the former case, the freeze-quenched intermediate has a $g$ value of 2.12 and a cobalt hyperfine of 54 G (Orme-Johnson et al, 1974, Licht et al, 1996). This intermediate appears with a rate constant ~40 s⁻¹, the same as the rate constant for cob(II)alamin formation measured by SF (Orme-Johnson et al, 1974, Licht et al, 1996). The EPR signal was originally hypothesized to result from a cob(II)alamin/5'-dA• radical pair. However, the repetition of the experiment with [5'-2H₂]- and [5'-13C]-AdoCbl did not alter the EPR spectrum of this intermediate, as would have been predicted for an
intermediate with unpaired spin density on the 5′ position of 5′-dA. Recent experiments to establish the identity of this intermediate provide strong evidence that it is an enzyme-based thyl radical exchange-coupled to cob(II)alamin, and that this thyl radical mediates both nucleotide reduction and the exchange of the 5′ hydrogens of AdoCbl.

Evidence that the species that gives rise to the observed EPR signal is a thyl radical comes from several different types of experiments. Cloning and overexpression of RTPR (Booker and Stubbe, 1993) has allowed a number of mechanistically informative site-directed mutagenesis studies. The x-ray structure of the R1 subunit of the E. coli RNR, in conjunction with many biochemical studies, strongly suggested that C439 of R1 is the cysteine that is converted to a thyl radical via the \( \bullet Y \). Based on a short, statistically insignificant, sequence homology with the E. coli RNR, C408 was predicted to act as the corresponding thyl radical in RTPR (Fig. 1.3).

\[ \text{L. leichmannii RTPR: TNPC408GEISLA} \]
\[ \text{E. coli RDPR: SNLC439LEIAP} \]

When this residue was mutated to a serine, the mutant enzyme was unable to catalyze either nucleotide reduction or the exchange reaction, consistent with the proposed role for C408 (Booker et al., 1994). To test this hypothesis further, RTPR was prepared in which all of the cysteines contained deuterium at their \( \beta \) positions (\( [\beta-^{2}H_{2}] \)-cysteine), and this labeled RTPR was used in RFQ EPR experiments. This isotopic substitution resulted in a pronounced narrowing of the cobalt hyperfine features of the EPR spectrum of the intermediate, establishing its predicted identity (Licht et al., 1996). Computer simulation of the observed EPR signals at both 9 and 35 GHz, further confirmed its identification, and allowed an estimate of the distance between the thyl radical and cob(II)alamin to be 5.5-6 Å (Gerfen et al, 1996).

Although the experiments described above were carried out in the absence of substrate, experiments in the presence of substrate indicate that an enzyme-based thyl radical with very similar spectroscopic properties is also generated in a kinetically competent fashion. When reaction mixtures containing RTPR, substrate, allosteric effector, and TR/TRR/NADPH are freeze-quenched during turnover, the EPR-active intermediate trapped is
similar in lineshape and effective g value to the intermediate trapped in the exchange reaction. It also exhibits cobalt hyperfine narrowing on substitution of the β hydrogens of cysteine residues with deuterium (Licht et al., 1996). This species, generated with k_{obs} of >200 s^{-1}, is kinetically competent to be involved in turnover. These results support our contention that the exchange reaction provides an excellent model for how AdoCbl acts as a radical chain initiator.

While direct spectroscopic observation of a thyl radical intermediate has so far only been established with the *L. leichmannii* class II RNR (although similar results are observed with the *T. acidophilus* RNR, J. Wu and J. Stubbe, unpublished results), the striking biochemical similarities between this enzyme and *E. coli* class I RNR strongly imply that a thyl radical intermediate is also formed with this RNR during nucleotide reduction as well. Both enzymes react with 2'-chloro-2'-deoxynucleotidase mechanism-based inactivators, forming PP (PPP), base, and 2-methylene-3-(2H)-furanone which reacts with the protein to inactivate the enzyme and form a chromophore at 320-nm chromophore (Scheme 1.2) (Stubbe & Kozarich, 1980, Stubbe et al, 1983, Harris et al, 1984, Ator and Stubbe, 1985, Ashley et al, 1988). In both enzymes, the phenotypes of five conserved cysteine to serine site-directed mutants, including the cysteine proposed to form the thyl radical in the *L. leichmannii* reductase (C408), are strikingly similar, consistent with a commonality of mechanism. (Stubbe et al, 1983, Mao et al, 1989, Mao et al, 1992a, Mao et al, 1992b, Mao et al, 1992, Booker et al, 1994) What is known mechanistically about the normal reduction process is also very similar in both RNRs. In both cases, the 2' hydroxyl group of the nucleotide is replaced, with retention of configuration, by a solvent-derived hydrogen during the normal reduction process (Batternam et al, 1967, David & Eustache, 1971). Isotope effects on 3' C-H bond cleavage are comparable in the two enzymes (Stubbe & Ackles, 1980, Stubbe et al, 1981), consistent with a common mechanism for 3' hydrogen abstraction. The allosteric regulation patterns for both enzymes are similar (Beck et al, 1966b, Beck, 1967) (Lammers & Follmann, 1983). Finally, both enzymes react with 2'-difluoro-2'-deoxynucleotides (F2CDP or F2CTP, respectively) and 2'-fluoromethylene cytidine 5'-di or triphosphates (FMCDDP or FMCTTP) to form new radical species shown in one case to be substrate-derived (van der Donk et al, 1996a). Since the mechanisms of nucleotide
reduction appear to be similar in the two enzymes, and the cysteine residues involved in the mechanism appear to be performing the same functions in both enzymes, it is likely that the thiol radical intermediate will be present in the *E. coli* RNR as well as the *L. leichmannii*.

2.2 *Catalysis of Carbon-Cobalt Bond Cleavage*

One of the critical questions in the mechanism of RTPR is how the enzyme catalyzes homolytic carbon-cobalt bond cleavage, and how this bond homolysis leads to formation of a thiol radical. Answering this question must begin with a measurement of the bond dissociation energy of the carbon-cobalt bond in AdoCbl. Studies from the Finke (Finke & Hay, 1984, Hay & Finke, 1988) and Halpern (Halpern et al, 1984) laboratories have established that this bond dissociation energy is ~30 kcal/mol in neutral aqueous solution, with the rate constant determined to be 10^{-9} s^{-1} at room temperature (Hay & Finke, 1986). Activation parameters for this reaction have also been determined, both in neutral aqueous solution \( (\Delta H^\ddagger = 31.8 \pm 0.7 \text{ kcal/mol, } \Delta S^\ddagger = 6.8 \pm 1.0 \text{ cal/mol K}) \) (Hay and Finke, 1986) and in ethylene glycol, where where viscosity makes cage effects more important (Koenig et al, 1988) \( (\Delta H^\ddagger = 34.5 \pm 0.8 \text{ kcal/mol, } \Delta S^\ddagger = 14 \pm 1.0 \text{ cal/mol K}) \) (Hay and Finke, 1988).

The observation of a rate constants for cob(II)alamin formation on the order of 200 s^{-1} for RTPR (under turnover conditions) requires a rate acceleration of \(-10^{11}\) over the uncatalyzed homolysis,(Hay and Finke, 1986) which corresponds to a \( \Delta G^\ddagger \) of 16 kcal/mol. There is also evidence that the thermodynamics of the carbon-cobalt bond cleavage reaction are highly perturbed when the cofactor is bound to RTPR. Up to 50% of bound cobalamin is in the form of cob(II)alamin under the conditions of the exchange reaction (Tamao and Blakley, 1973), suggesting an equilibrium constant on the order of unity (\( \Delta G \sim 0 \)) between AdoCbl and its homolysis products. This perturbation of the equilibrium thermodynamics of carbon-cobalt bond homolysis is likely to be required for catalysis. The activation parameters for carbon-cobalt bond homolysis in solution indicate that the transition state is not much higher in energy than the products, as expected for a highly endergonic reaction. Thus, lowering the activation barrier by \(-16 \text{ kcal/mol} \) would not be possible by lowering the energy of the transition
state alone. Enzymatic destabilization of the reactant ground states and/or stabilization of product is also required.

2.3 Chemical Models of the Role of Steric Strain and Basicity of the Axial Ligand in Acceleration of Carbon-Cobalt Bond Homolysis

As outlined below, model systems have inspired a variety of mechanisms for possible enzymatic mechanisms of catalysis of carbon-cobalt bond homolysis. To date, there is no general consensus on which, if any, of these mechanisms are actually used. One mechanism for the modulation of the energetics of carbon-cobalt bond cleavage has been proposed to be through the use of steric effects of the trans axial ligand of AdoCbl (Halpern, 1985) (Pratt, 1982, Randaccio et al, 1989, Banerjee, 1997). Studying heterolytic dealkylations of alkylcobalamins, Grate and Schrauzer observed that the presence of the nitrogen base coordinating to cobalt increased the dealkylation rate (Grate & Schrauzer, 1979). They suggested that the axial nitrogen ligand causes an upward bending of the corrin, applying steric strain to the axial alkyl substituent of cobalt and accelerating bond cleavage. Although this study examined heterolytic cleavage, the same principle would apply to homolytic bond cleavage. For cobaloximes, studies on the effects on carbon-cobalt bond strength of bulky phosphine axial ligands support this hypothesis (Ng et al, 1983), as does the increased carbon-cobalt bond strength observed for benzylcobalt complexes of a porphyrin compared with the analogous cobaloxime, which is less rigid (Geno & Halpern, 1987).

A second mechanism suggests that the basicity of the axial ligand can affect the homolysis rate, with lower basicity making the carbon-cobalt bond more labile. The bond dissociation enthalpies of a series of cobaloximes with substituted pyridines or imidazole as trans-axial ligands were found to increase with increasing basicity of the trans-axial ligand. These results are consistent with electron donation by the ligand stabilizing the cobalt (III) oxidation state of the intact cobaloxime over the cobalt (II) state resulting from homolytic bond cleavage (Ng et al, 1982).

Work on the lability of neopentylcobalamin (a derivative of AdoCbl in which a neopentyl group replaces the 5'-deoxyadenosyl moiety) supports the importance of both steric and electronic factors. Neopentylcobalamin undergoes uncatalyzed carbon-cobalt bond cleavage up to $10^6$ times faster.
than does AdoCbl (Waddington & Finke, 1993). In addition, the base-on form of neopentylcobalamin is $\sim 10^3$ times more reactive than the base-off form (Chemaly & Pratt, 1980, Brown & Brooks, 1991).

Other studies on this system emphasize the effect of the entropy due to mobility of the acetamide side-chains on the energetics of carbon-cobalt bond homolysis (Kim et al, 1988, Brown and Brooks, 1991, Waddington and Finke, 1993, Brown et al, 1994, Brown et al, 1995). While the activation enthalpies are approximately the same for the base-on and base-off forms, the activation entropies differ by $\sim 10$ cal/mol K in aqueous solution (Kim et al, 1988) and $\sim 20$ cal/mol K in ethylene glycol (Waddington and Finke, 1993), indicating that the destabilization is entropic. This observation led to the suggestion that steric interactions between the neopentyl group and one or more of the acetamide side-chains cause neopentylcobalamin to be entropically destabilized relative to the products of carbon-cobalt bond homolysis (Brown and Brooks, 1991). In support of this hypothesis, epimerization at C13 of the corrin, which puts another acetamide side chain in a position to interact with the neopentyl group, increases the entropy of activation of carbon-cobalt bond homolysis (Brown et al, 1994), as does modification of the $c$ side chain to the bulkier $N$-methyl, -dimethyl, and -isopropyl derivatives (Brown et al, 1995).

Structural studies have also been used to analyze the factors that contribute to carbon-cobalt bond strength. X-ray crystallographic studies are consistent with a role for the trans-axial ligand in governing the length and, by implication, the strength of the carbon-cobalt bond (Randaccio et al, 1989). Comparison of the structure (determined both by crystallography and by NMR) of a cobalamin with imidazole and cyanide as axial ligands with that of cyanocobalamin (dimethylbenzimidazole and cyanide as axial ligands) showed that the bulkier dimethylbenzimidazole causes a greater "upward" distortion of the corrin ring than does imidazole (an angle between the "northern and "southern" halves of the corrin ring of $18\pm 0.3^\circ$ for the cobalamin containing dimethylbenzimidazole vs. $11.3\pm 0.2^\circ$ for the cobalamin containing imidazole) (Kräutler et al, 1994).

However, resonance Raman experiments indicate that the carbon-cobalt bond stretching frequency is the same for the base-on and base-off forms of AdoCbl, suggesting that the trans ligand does not alter the bond strength of the carbon-cobalt bond itself in cobalamins (Dong et al, 1996).
Nonetheless, the base-off form of AdoCbl has a bond dissociation energy that is ~5 kcal/mol larger than the base-on form (which is also in contrast to what would be predicted from the electronic effects outlined above) (Hay & Finke, 1987). One explanation for these results is that the base affects the stability of cob(II)alamin without having a large effect on the stability of AdoCbl. Thus, while model systems have demonstrated how the energetics of a carbon-cobalt bond might be modulated, the specific factors that determine the bond dissociation energy for the physiologically relevant cobalamins remain an active area of investigation.

2.4 Molecule-Induced Homolysis as a Model for Acceleration of Carbon-Cobalt Bond Cleavage

In the case of RTPR, the chemistry may be distinct from the model studies due to the involvement of a protein radical. In the enzymatic reaction, we have proposed that carbon-cobalt bond cleavage occurs in a concerted fashion with thyl radical formation (Scheme 1.3) (Licht et al., 1996). This process would be less endergonic than carbon-cobalt bond homolysis alone. Using 30 kcal/mol as the homolytic bond dissociation enthalpy of the carbon-cobalt bond of AdoCbl (Hay and Finke, 1986), 88-91 kcal/mol as the homolytic bond dissociation enthalpy of the RS-H bond (Benson, 1978, McMillen & Golden, 1982), and 98 kcal/mol as the homolytic bond dissociation enthalpy of the C-H bond (Lide, 1996), the enthalpy for a concerted reaction can be estimated to be 15-20 kcal/mol, as opposed to 30 kcal/mol for the carbon-cobalt bond homolysis alone.

This concerted reaction may be analogous to the class of radical reactions known as molecule-induced homolyses, in which homolytic cleavage of a non-radical species is accelerated by interaction with another non-radical species (Pryor, 1966). The radical chain halogenation of styrene by t-butyl hypochlorite proceeds rapidly and exothermically in the dark, even though the bond dissociation energy of the oxygen-chlorine bond in t-butyl hypochlorite is ~40 kcal/mol (Walling et al, 1965). Observing 1,2-dichlorophenylethane among the products of this reaction, Walling and co-workers hypothesized that radical initiation requires a concerted O-Cl bond homolysis and addition of chloro radical to styrene (Scheme 1.4a). Since the latter reaction is estimated to be exothermic by 49 kcal/mol, the
concerted reaction would be expected to be exothermic (Walling et al., 1965). A similar molecule induced homolysis has been reported for the radical addition of iodine to styrene (Scheme 1.4b), in which formation of radical species is $10^6$ times faster than the homolysis of iodine in the absence of styrene (Fraenkel & Bartlett, 1959).

a.

\[ \text{O-Cl} \xrightarrow{\text{\textbullet}} \text{O} \cdot \]

b.

\[ \text{I} \cdot \]

Scheme 1.4. Molecule-induced homolyses. a. Radical chain halogenation of styrene by t-butyl hypochlorite. b. Radical addition of iodine to styrene.

Although concerted reactions can be favorable from an enthalpic standpoint, they are generally entropically less favorable than the corresponding stepwise reactions due to the formation of a more ordered transition state. In an enzymatic system, the binding energy of AdoCbl would be expected to compensate for the entropy cost of pre-organization of the reacting species at the enzyme active site (Jencks, 1969). In this regard, it may be significant that the concentration dependence of the observed rate constants for cob(II)alamin formation under both exchange reaction and turnover conditions, in combination with the failure to detect binding of AdoCbl to enzyme in equilibrium binding experiments on the catalytically inactive C408S mutant, suggest that the $K_d$ for binding of AdoCbl to RTPR is $>100 \mu$M (S. Licht and J. Stubbe, unpublished results). Given the number of binding determinants on AdoCbl, a much lower $K_d$ should be possible and might have been expected. Perhaps unfavorable entropic factors associated with pre-organization of the enzyme and the cofactor contribute to this relatively high $K_d$.

In non-enzymatic systems, a favorable enthalpy change of a reaction can facilitate concerted reactions even when the entropy change of the reaction is very negative. The oxidative addition of methane to rhodium
(II) porphyrin complexes (Fig. 1.5) is another example of how molecule-assisted homolysis can allow rapid homolytic cleavage of strong bonds. Wayland and co-workers have shown that this reaction is second order in rhodium complex, exhibits a sizable deuterium isotope effect ($k_{\text{CH}_4}/k_{\text{CD}_4} = 8.6$), and has a relatively small activation enthalpy ($\Delta H^\ddagger = 7.1 \pm 1.0 \text{ kcal/mol}$) and a large negative activation entropy ($\Delta S^\ddagger = -39 \pm 5 \text{ kcal/mol}$). They proposed a concerted reaction with a four-centered transition state to account for these observations (Fig. 1.5b). In this mechanism, C-H bond breaking occurs in concert with Rh-H and Rh-CH$_3$ bond formation (Wayland et al, 1991). The oxidative addition of methane to a diporphyrin dirhodium complex with the porphyrin units tethered by a diether spacer occurred $\sim 10^2$-fold faster than the termolecular reaction, consistent with pre-organization of the rhodium units reducing the entropy cost of the four-centered transition state (Fig. 1.5c) (Wayland et al, 1991). The thermodynamics of these model reactions are roughly analogous to the thermodynamics expected for concerted carbon-cobalt bond cleavage and thiol radical formation. In the case of the rhodium complexes, the formation of two weaker bonds (Rh-H and Rh-CH$_3$, both $\sim 60$ kcal/mol) "pays for" the homolytic cleavage of a stronger bond (CH$_3$-H, 105 kcal/mol) (Wayland et al, 1991). In the case of a concerted carbon-cobalt bond cleavage, the formation of a stronger bond (the CH$_2$-H bond of 5'-dA, $\sim 100$ kcal/mol) partially "pays for" the homolytic cleavage of two weaker bonds (Co-C, $\sim 30$ kcal/mol and S-H, $\sim 90$ kcal/mol).

2.5 Structural Information on Cobalamin Enzymes and Its Relevance to the Problem of Carbon-Cobalt Bond Cleavage

As summarized above, data from model systems suggests that the identity and conformation of the trans axial ligand of cobalamin bound to an enzyme could affect the carbon-cobalt bond strength, although the precise causes of trans effects in model systems are still incompletely understood. One of the reasons attention has focused on the trans axial ligand is the unusual 5,6-dimethylbenzimidazole (DMB) moiety that serves this purpose in AdoCbl. This ligand might affect the structure and reactivity of the cofactor through both the steric effects mentioned previously (Kräutler et al, 1994) (Section 2.3) and electronic effects. The
pK_a of the dimethylbenzimidazole moiety in AdoCbl has been measured to be 3.7 (Brown & Hakimi, 1984), compared with a pK_a of 7 for imidazole,
a.

\[
2 \text{ Rh(II)} + \text{CH}_4 \rightarrow \text{CH}_3 \text{Rh(II)}
\]

b.

c.

Fig. 1.5. a. Oxidative addition of methane to rhodium porphyrins. b. Four-centered transition state for the oxidative addition of methane. c. Covalently linked porphyrin rhodium dimer.

consistent with different σ donor properties for these two ligands.

Recent crystallographic and EPR studies of the cobalamin-containing enzymes methionine synthase (which catalyzes carbon-cobalt bond heterolysis) (Drennan et al, 1994) and methylmalonyl-CoA mutase (which catalyzes carbon-co:alt bond homolysis) (Padmakumar et al, 1995, Mancia et al, 1996) indicate that the trans axial ligand is likely to be very important for establishing the reactivity of the cofactor, albeit in a surprising way. In both cases, the DMB base is not coordinated to cobalt in the enzyme-bound cobalamin, and a histidine residue from the enzyme serves as the trans axial ligand in its place.

In the case of methylmalonyl-CoA mutase, the cobalamin in the crystal is mixture of cobalt(II) and cobalt(III) forms, and no electron density
corresponding to the 5'-deoxyadenosyl moiety is observed (Marcia et al., 1996). These observations suggest that the crystal structure is close to the structure the enzyme assumes after carbon-cobalt bond cleavage. The crystallographic data has been interpreted as indicating a long histidine-cobalt bond (2.5 Å vs. 1.95–2.2 Å in free cobalamins) (Marcia et al., 1996). It was hypothesized that this unusual Co-N bond length promotes carbon-cobalt bond homolysis by stabilizing the cobalt(II) oxidation state of the homolysis products over the cobalt(III) AdoCbl state. This view is at odds with Spiro and Banerjee's interpretation of their resonance Raman data, which posits that for cobalamins, coordination of benzimidazole alters the stability of cob(II)alamin (the homolysis product) rather than AdoCbl (Dong et al., 1996). In addition, the existing crystallographic data does not rule out a role for steric interactions in catalysis. Unfavorable steric interactions between the enzyme and AdoCbl might be relieved after homolysis, resulting in the observed structure, which is consistent with a flat, rather than a "flexed," corrin ring. The mechanism of labilization of the carbon-cobalt bond by this enzyme thus remains an interesting and controversial question. Nonetheless, the structural data rule out the possibility that the unusual DMB cofactor, long postulated to be the mediator of steric weakening of the carbon-cobalt bond at enzyme active sites, interacts directly with either cobalt or the corrin ring to weaken the carbon-cobalt bond in methylmalonyl-CoA mutase-bound AdoCbl.

While the methylmalonyl-CoA mutase structure has been extremely thought-provoking for the field of cobalamin biochemistry, the L. leichmannii RTPR is likely to activate the carbon-cobalt bond for homolytic cleavage by a different mechanism. EPR studies on unlabeled (14N in DMB) cob(II)alamin bound to [U-15N]-RTPR in the presence of an allosteric effector and a mechanism-based inactivator, 2'-methylene-2'-deoxycytidine, have shown 14N hyperfine interaction with cobalt. These results demonstrate that the enzyme does not provide the trans axial nitrogen ligand and strongly suggest that the DMB is bound to cobalt (C. Lawrence, S. Licht, J. Stubbe, unpublished results). Thus, this enzyme may use the added steric bulk of the DMB for catalysis in ways that appear to be unnecessary in methylmalonyl-CoA mutase. Further speculation must await determination of a three-dimensional structure of RTPR.
2.6 Mechanistic Studies on the L. leichmannii RNR Provide Evidence for a Concerted Mechanism in which Carbon-Cobalt Bond Homolysis is Entropy-Driven

To address the question of how RTPR accomplishes catalysis of carbon-cobalt bond homolysis, the kinetics and thermodynamics of carbon-cobalt bond homolysis/thiyl radical formation have been studied for the exchange reaction. Efforts were initially focused on establishing if this reaction proceeds in a stepwise or a concerted fashion. One piece of evidence favoring the concerted model is the inability of C408S RTPR to catalyze carbon-cobalt bond homolysis (Booker, 1994).

To make a distinction between the two mechanisms, kinetic and equilibrium isotope effects on cob(II)alamin formation were measured using unlabeled AdoCbl in D₂O, [5'-²H₂]-AdoCbl in H₂O, and with [5'-²H₂]-AdoCbl in D₂O (Licht, 1998). Comparison of the k_{obs}s in these experiments with that measured with unlabeled AdoCbl in H₂O revealed k_{H₂O}/k_{D₂O} of 1.7, 1.6, and 2.7, respectively. An equilibrium isotope effect was also measured. Twice as much cob(II)alamin was generated with [5'-²H₂]-AdoCbl in D₂O than with AdoCbl in H₂O. The kinetic isotope effects on k_{obs}, which contains contributions from both forward and reverse rate constants, can be ascribed to primary isotope effects on both abstraction of hydrogen from thiol to form the thiyl radical and abstraction of hydrogen from 5'-dA to regenerate the thiol of C408. The equilibrium isotope effect is consistent with the low fractionation factor (0.4–0.5) expected for thiols (Schowen & Schowen, 1982). Because the heavier isotope will fractionate onto the carbon center of 5'-dA in preference to the cysteine thiol, deuteration of the thiol group drives the reaction toward formation of thiyl radical and cob(II)alamin.

Quantitative modeling of the kinetics was required to make a distinction between the two mechanisms. Kinetic simulations were carried out taking into account a variety of constraints imposed by factors such as the fractionation factor associated with thiols and the statistical factors expected for hydrogen abstraction from mixed isotopomers of 5'-dA. In addition, global analysis (Kuzmic, 1996) of the isotope effect data was performed, placing no constraints on possible rate constants. Both of these approaches favored the concerted mechanism, and thus provided the
framework for interpreting the temperature dependent studies on this process.

The kinetics of cob(II)alamin formation in the exchange reaction have also been measured as a function of [AdoCbl] and temperature. The [AdoCbl] dependence of the kobs allowed assignment of microscopic rate constants both for carbon-cobalt bond homolysis/thiyl radical formation and for carbon-cobalt bond re-formation/thiol regeneration. The variation of these microscopic rate constants with temperature provided the activation enthalpies and entropies (∆H‡ and ∆S‡) for both forward and reverse steps. A similar analysis of the amount of cob(II)alamin formed at equilibrium as a function of [AdoCbl] and temperature provided an independent check on the enthalpy and entropy of this reaction (∆H and ∆S).

The results of these studies provide further evidence that the enzyme makes the reactant (AdoCbl-bound) state and the product (cob(II)alamin/5’-dA/thiyl radical) state approximately equal in energy. This dramatic thermodynamic perturbation, in addition to transition-state state stabilization, is required to account for the large rate acceleration observed on this process, as discussed above. The ΔH measured is consistent with the calculated net ΔH for non-enzymatic carbon-cobalt bond homolysis, S-H bond homolysis, and C-H bond formation. This observation suggests that weakening of the carbon-cobalt bond by strain or electronic effects, proposed from model studies, is not the predominant factor in the observed thermodynamic perturbation. The free energy of the enzymatic reaction (∆G) is dominated by a large T∆S term. Thus, entropic effects, rather than enthalpic effects, appear to be most important in determining the thermodynamics of carbon-cobalt bond homolysis/thiyl radical formation.

The ΔH‡ is measured to be ~45 kcal/mol, which is likely to represent contributions from carbon-cobalt bond cleavage, thiyl radical formation, protein conformational changes, and solvent reorganization. This large ΔH‡ is significant in that, like ΔH, its magnitude suggests that the enzyme does not function by weakening the carbon-cobalt bond. As observed for ΔG, ΔG‡ is dominated by a favorable T∆S‡, which brings ΔG‡ to ~15 kcal/mol. While the source of this large entropic term is not yet known,
solvent release and protein conformational entropy are likely to be critical in the entropy changes that drive catalysis.

Comparable experiments in the presence of substrate will be difficult to interpret, due to the complexity of the kinetics (Tamao and Blakley, 1973). However, neither the rate of carbon cobalt bond homolysis (~200 s⁻¹ vs 40 s⁻¹), nor the amount of cob(II)alamin in the steady state are dramatically perturbed in the presence of substrate relative to the exchange reaction conditions. Thus, large entropic effects will likely play a role a major role in the turnover process as well. While the mechanisms for thyl radical formation will be different for enzymes containing different metallo-cofactors, these studies on the L. leichmannii RlTPR show that a metallocofactor at an enzyme active site can effect rapid conversion of a cysteine residue to a thyl radical.

2.7 Carbon-Covert Bond Re-formation Follows Each Turnover

The exchange reaction, which has been so useful as an avenue for studying the mechanism and energetics of carbon-cobalt bond homolysis, has also shed light on the issue of whether the carbon-cobalt bond is re-formed after every turnover during the normal reduction process (Scheme 1.3). Since it requires thyl radical abstraction of tritium from 5'-dA, the rate of tritium release to the solvent provides a lower limit for the rate of carbon-cobalt bond reformation.

To investigate the chain length of the nucleotide reduction process, rapid acid quench experiments were carried out with RTPR, [5'-³H]-AdoCbl, effector, and [¹⁴C]-NTP. The relative rates of formation of tritiated water and [2-¹⁴C]-dNTP are indicative of the chain length for nucleotide reduction. In one limiting case, where every dNTP is accompanied by reformation of the carbon-cobalt bond, the chain length would be 1. However, due to the kinetic isotope effect on this process and the statistical effect (associated with the three equivalent hydrogens in the methyl group of 5'-dA), not every carbon-cobalt bond re-formation will result in tritiated water release. Assuming a selection effect against tritium abstraction of 10 and a statistical effect of 2, a chain length of 1 should result in 1/20 of an equivalent of tritiated water released for every turnover. In the case of multiple t turnovers, the number should be much lower.
The rapid acid quench experiments showed that after 300 ms, 0.6 equivalent of [2-\textsuperscript{14}C]-dATP is formed and 0.06 equivalent of tritiated water is released. No lag phase was evident in the formation of either product. Furthermore, the ratio of $^3\text{H}_2\text{O}$ to dATP increased as a function of time. These results indicate that most of the tritiated water is released after nucleotide reduction and suggest that reformation of the carbon-cobalt occurs after every turnover. Additional data supporting this model comes from steady state kinetic analysis of dNTP formation when [RTPR]>>[AdoCbl] (S. Licht and J. Stubbe, unpublished results). The short chain length can be easily rationalized chemically as a method of protecting the reactive thiyyl radical. If carbon-cobalt bond re-formation did not follow each turnover, the thiyyl radical would be present during dissociation of the product and binding of another molecule of substrate. It would thus be exposed to the solvent, rendering it vulnerable to reaction with oxygen or other reactive species in solution. The cob(II)alamin under these conditions would also have an enhanced probability of oxidation.

Knowledge of the chain length of the reaction is also critical in defining the rate acceleration mediated by RTPR (Halpern, 1985). If, for example, one carbon-cobalt homolysis event catalyzed formation of many dNTPs, then the actual rate acceleration catalyzed by RTPR could be much less than the $10^{11}$ predicted based on $k_{\text{obs}}$. Thus the chain length of approximately 1 suggests that $10^{11}$ is the actual rate acceleration.

This section has described evidence for the existence of a thiyyl radical intermediate in nucleotide reduction by the \textit{L. leichmannii} class II RNR, and offered hypotheses as to how this radical might be formed rapidly with complete control. In the next section, formation of a thiyyl radical in the \textit{E. coli} class I reductase will be discussed.

3. Proposed Role of Protein-Based Tyrosyl Radical in Catalysis and Model Studies in support of the Proposed Role

3.1 Tyrosyl Radicals are Proposed to Generate Active Site Thiyl Radicals

While there is extensive biochemical and structural data available on the \textit{E. coli} RNR, and the •Y was shown many years ago to be essential for the nucleotide reduction process (Ehrenberg & Reichard, 1972), the function of the •Y in catalysis has so far eluded experimental verification.
What is amazing about the Class I RNRs is that their small subunit (R2) contains this remarkably stable •Y (located on residue 122 in *E. coli* RNR, and having a $t_{1/2}$ of 4 days at 4 °C) (Atkin et al, 1973), yet it is this radical that is proposed to serve as the initial electron acceptor in a series of coupled electron and proton transfer reactions that lead to the generation of putative thyl radical at the active site of the large subunit (R1) of the enzyme (Fig. 1.3).

Analysis of the structures of R2 and R1, in conjunction with extensive biochemical analysis of this system has allowed Uhlin and Eklund to dock the two subunits together, with the resulting structure suggesting that the distance between the Y122 on R2 and the active site C439 of R1 is ~35 Å (Fig. 1.3) (Nordlund & Eklund, 1993, Uhlin and Eklund, 1994, Logan et al, 1996). The R2 environment must allow generation of this •Y from Y122 by the diferrous form of R2 in the presence of O$_2$ and reductant. It further must allow stabilization of this radical, which would normally have a $t_{1/2}$ in solution of 10 ms (Prütz et al, 1983), so that it is not quenched adventitiously by external reductants or amino acid residues surrounding the cofactor site. In addition, this radical, in the presence of the second subunit, R1, and substrate, NDP, must react with other protein residues to accomplish a net electron transfer over a very long distance to generate a single specific thyl radical. As described in detail (Fig. 1.3), a specific electron transfer pathway comprised of conserved amino acid residues with extensive hydrogen bonding interactions has been proposed based on the structures of R1 and R2 (Sjöberg, 1994, Ekberg et al, 1996, Katterle et al, 1997). Site-directed mutagenesis experiments on R2 (Climent et al, 1992, Rova et al, 1995) and R1 (Ekberg et al, 1996) (D. Silva and J. Stubbe, unpublished results) have shown that mutation of any of these residues causes reduction of enzymatic activity to the level of contaminating wt activity in all the mutant preparations.

In order to understand the unique role that the protein plays in the reactivity of the R2 •Y, it is necessary to understand the thermodynamics and kinetics of the reversible oxidation and reduction of Y to •Y. This can be studied most easily using tyrosine and tyrosine-containing model peptides. How the R2 environment can modulate these properties may then be examined based on the structure of R2 and a variety of R2 mutants.
3.2 Thermodynamics and Kinetics of Tyrosyl Radical Formation: Dependence on Protonation State

The diferrous iron center of R2 catalyzes the oxidation of the Y122 to a neutral tyrosyl radical, •Y122, in the presence of oxygen and reductant. In contrast to many reports in the literature, it is clear from thermodynamic arguments that no tyrosyl radicals of biological importance are in the protonated state (that is, exist as cation radicals), since the pKₐ's of these species are on the order of -2 (Dixon & Murphy, 1975, Holton & Murphy, 1979). Both ENDOR (Bender et al, 1989) and resonance Raman spectroscopy (Backes et al, 1991) provide direct support for this conclusion. The detailed analysis of the mechanism by which the diferrous form of R2 generates the •Y is beyond the scope of this review. However, analysis of the effects of pH on the kinetics and thermodynamics of tyrosine oxidation can furnish insight into how the protein environment could influence the redox properties of Y122.

Pulse radiolysis experiments have established that oxidation of a neutral tyrosine to a •Y by an azide radical occurs with a rate constant of \( \sim 10^8 \text{ M}^{-1}\text{s}^{-1} \) (Land & Prütz, 1979, DeFilippis et al, 1989), while deprotonated tyrosine is oxidized ten times faster (Land and Prütz, 1979). Modulation of the pKₐ of the phenol of the tyrosine residue is thus one way that the protein can affect the rate of hydrogen atom or electron transfer. Even if the electron transfer step is not rate limiting, such a modulation of rates might be important to ensure the specificity of such a process; for example, if a tyrosine residue is close to a tryptophan residue, formation of the tryptophan radical might be kinetically favored if the tyrosine residue was protonated, but less favored if the tyrosine residue was deprotonated.

The protonation state of a tyrosine residue is also important in determining the thermodynamics of the oxidation of tyrosine. Klapper and co-workers have used cyclic voltammetry and pulse radiolysis methods (DeFilippis et al, 1989) to obtain a midpoint potential of 0.93 V for oxidation of tyrosine to a •Y at pH 7 (Harriman, 1987, DeFilippis et al, 1991). Furthermore, only modest differences in pKₐ and oxidation potential between the free amino acid and the amino acid incorporated into di- or tripeptides have been reported (DeFilippis et al, 1991). The issue of whether the protein environment perturbs this potential thus remains to
be elucidated. Nonetheless, these model studies suggest that modulation of the protonation states of tyrosine could be an important mechanism for maintaining specificity of proton and electron transfer within a protein.

3.3 Spectroscopic Approaches to Determining the Hydrogen Bonding State of the R2 Tyrosyl Radical

While Y122 is a neutral radical in the active form of the cofactor of R2, it has been proposed that the ability of this residue to mediate oxidation of an adjacent amino acid side chain via hydrogen atom transfer (Fig. 1.3) might be related to its hydrogen-bonding environment of this residue (Babcock et al., 1997). Electron paramagnetic resonance spectroscopy (EPR) and related spectroscopies (ENDOR, ESEEM) have provided a way of looking selectively at •Y and the distribution of its spin density. Accordingly, these techniques have provided a probe of the structural and electronic properties of tyrosyl radicals in class I RNRs from a variety of sources.

ENDOR spectroscopy in D₂O (Bender et al., 1989), EPR spectroscopy of R2 incorporating [¹⁷O]-labeled tyrosine (Hoganson et al., 1996), and high field EPR spectroscopy (139.5 (Gerfen et al., 1993) and 245 GHz (Un et al., 1995)) all provide evidence that the •Y in E. coli R2 is not hydrogen bonded to another protein residue or to bulk water. In the latter cases (Gerfen et al., 1993, Un et al., 1995), the g-anisotropy, especially in g₁, has been proposed to be an important indicator of hydrogen bonding status. Interestingly, the X-ray structure of R2 shows that Y122 is hydrogen-bonded to D84, a ligand to the proximal iron in diferrous R2 (Logan et al., 1996), but that after cluster assembly and •Y formation, this proton is no longer present (Nordlund et al., 1990). It could in fact be transferred to the putative hydroxide of intermediate X in the assembly process, generating the water observed on the iron adjacent to the •Y in the resting state of the protein (Nordlund et al., 1990, Nordlund and Eklund, 1993). This same water molecule might also provide a proton to the phenoxy oxygen of Y122 on reduction of the •Y during turnover (Fig. 1.3). Tyrosyl radicals in other class I RNRs have also been examined using EPR. The details of these studies have recently been summarized (Gräslund & Sahlin, 1996). Surprisingly the spin density in almost all tyrosyl radicals appears to be the same regardless of their environment. While it was initially thought that
the *E. coli* •Y would serve as the prototype of all class I tyrosyl radicals, it is becoming clear that this is not the case. Recent high field EPR experiments on the mouse RNR have revealed a g anisotropy consistent with a hydrogen bond to the •Y (Schmidt et al, 1996). This interpretation needs to be confirmed by other spectroscopic methods, however. While the hydrogen bonding of the •Y in the resting enzyme appears to vary from species to species, the change in hydrogen bonding of this residue during electron transfer remains unknown and is most probably of importance in electron transfer. Unfortunately, experiments to test this hypothesis are very difficult in biological systems. The next section describes model systems for the coupling of proton and electron motion.

3.4 Model Studies on Proton-Coupled Electron Transfer

Model studies are required to gain a basic understanding of how coupling of proton motion to electron transfer might operate. Nocera and co-workers have reported several systems for studying proton-coupled electron transfer. One is based on formation of a hydrogen-bonded interface between an electron donor (a zinc porphyrin) and an electron acceptor (3,4-dinitrobenzoic acid), both bearing carboxylic acid moieties (Fig. 1.6a) (Turro et al, 1992). Transient absorption spectroscopy shows that the rate constant for forward electron transfer is \( \sim 5 \times 10^{10} \) s\(^{-1}\). Thus, electron transfer in this system is fast, and comparable in rate to electron transfer in a similar system in which a zinc porphyrin is covalently linked to a quinone acceptor (Wasielewski et al, 1985). The observation of kinetic isotope effects on forward and back electron transfer (\( k_{H}/k_{D} = 1.7 \) and 1.6, respectively) when the acid groups are deuterated demonstrates that proton motion accompanies electron transfer. This study suggests that while the dicarboxylic acid bridge is the medium for electron transfer, it is not a static medium; protons in the bridge rearrange in response to the transfer of an electron.

In order to investigate whether changes in charge and polarity associated with proton motion affect the rate of electron transfer, Nocera and co-workers devised systems containing donors and acceptors linked by an asymmetric hydrogen-bonded interface, an amidinium-carboxylate salt bridge (Kirby et al, 1995, Roberts et al, 1995). One such system is a zinc
Fig. 1.6. Models for proton-coupled electron transfer.
porphyrin donor linked to 3,4-dinitrobenzoic acid through an amidinium-
carboxylate salt bridge (Fig. 1.6b) (Kirby et al., 1995). Measurement of the
excited state lifetime of the zinc porphyrin when complexed to the acceptor
gave a rate constant of \(~8\times10^8\) s\(^{-1}\), slower than that observed for the system
in which donor and acceptor were linked through a dicarboxylic acid
bridge. Similarly, a Ru-(bpy)\(_3\)^{2+} donor linked to a 3,5-dinitrobenzene
acceptor through an amidinium carboxylate salt bridge (Fig. 1.6c)
exhibited an electron transfer rate constant \(~2\)-fold smaller than the
analogous system containing a dicarboxylic acid bridge, even though the
driving force for the latter system is 0.07 V smaller (Roberts et al., 1995).
Again, a kinetic isotope effect was observed on substitution of exchangeable
hydrogens for deuterium (\(k_H/k_D = 1.34\)). These observations suggest that
proton-coupled electron transfer also occurs in systems with an asymmetric
hydrogen-bonded bridge, but that it is less efficient than in symmetric
systems. Nocera and co-workers suggest that the charge rearrangement
that accompanies proton motion in the asymmetric systems gives rise to
solvent reorganization, which manifests itself in a slower electron transfer
rate (additional Franck-Condon factors).

These model studies are consistent with the hypothesis that electron
transfer in proteins can be mediated through hydrogen bonds (Langen et al,
1996). Furthermore, they suggest that the rate of electron transfer through
a hydrogen bond will depend on whether proton motion can be coupled to
electron motion without large reorganization of solvent or of neighboring
amino acid residues. Perhaps hydrogen bonding to amino acid residues
involved in electron transfer not only modulates the driving force of
electron transfer reactions, but also minimizes charge rearrangements that
would increase the reorganization energy of electron transfer.

3.5 Thermodynamic and Kinetic Studies of Formation and Reduction of
the R2 Tyrosyl Radical

The reduction potential of the •Y122 of E. coli R2 has been estimated
to be 1.0 ± 0.1 V (Silva et al., 1995). The inability of redox mediators to
equilbrate with the radical in a reasonable amount of time has made
precise determination of this number thus far impossible. However, these
results are similar to the model studies (\(~ 1.0\) V at pH 7) (Harriman, 1987,
DeFilippis et al., 1991), suggesting that the protein has not dramatically perturbed the reduction potential, at least in the *E. coli* R2.

While the thermodynamic properties of •Y122 appear to be relatively unperturbed by the protein environment of R2, this is not the case for its chemical reactivity. The lifetime of days for •Y122 in the protein in comparison with milliseconds for •Y in solution is consistent with the buried and hydrophobic environment of •Y122 established crystallographically (Nordlund et al., 1990). The detailed structural basis for this kinetic stability has been a focus of much effort (Karlsson et al., 1992, Örmo et al., 1992).

Evidence for the role of the protein in maintaining the kinetic stability of Y122• comes from experiments using hydroxyurea (HU), a known reductant of the •Y. Karlsson et al. showed that the •Y of R2 reacts with hydroxyurea at least 10 times faster in the presence of R1, ATP (an allosteric effector), and CDP (a substrate) or dCDP (a product) than it does in the presence of R1 alone, suggesting that the conformation of the holoenzyme governs the kinetic accessibility of the •Y (Karlsson et al., 1992). Analogous experiments with mutant R1s in which the three active site cysteines that interact directly with the substrate are mutated to alanines (C225A, C462A, and C439, Fig. 1.2) demonstrated that these mutations had differing effects on the stability of the •Y. While the C225 and C462 R1 mutants (mutated in the cysteines delivering the reducing equivalents) had effects on the reactivity of the R2 •Y comparable to that of the wild-type, the C439 mutant (the site of the putative thyl radical) effectively abolished the increase in reactivity observed with the wild type R1, increasing the rate of •Y loss by only ~1.4 fold over the rate in the presence of bovine serum albumin, ADP, and dCDP. This effect is not due to a simple inability of C439A R1 to bind to R2, as this mutant functions as an inhibitor of ribonucleotide reduction with a low μM K_i (Mao et al., 1992).

These results are consistent with a specific role for the active site cysteines in maintaining a holoenzyme conformation that modulates the ability of HU to participate in a chain of hydrogen atom abstractions that ultimately results in quenching of the •Y. This interpretation assumes a hydrogen atom transfer mechanism for quenching by HU, a point that has not been experimentally determined. Regardless of the detailed
mechanism, the ability of subtle alterations in the R1 subunit to affect the
HU reactivity of the R2 subunit suggests that the conformation of R1
effects electron transfer/proton transfer within R2 and between R1 and R2.

3.6 Attempts at Direct Observation of Electron Transfer Between R1 and R2

One area that requires further effort is obtaining direct evidence for
kinetically competent electron transfer within R2 and between R1 and R2.
Efforts to observe reduction of the •Y during substrate (NDP) reduction by
means of RFQ EPR and SF UV-vis spectroscopy have been unsuccessful
(Stubbe, 1990). However, the inability to observe •Y reduction is not at all
inconsistent with this step being on the catalytic pathway. One way in
which •Y reduction might escape direct detection is if a step preceding its
reduction were rate-limiting, and all the steps subsequent to that step were
relatively fast. In that case, the only observable species would contain the
•Y, even under pre-steady state conditions. A conformational change,
perhaps triggered by substrate binding, that is required for intersubunit
electron transfer could be such an early rate-limiting step.

The need for further investigation of the hypothesis that
intersubunit electron transfer is essential for nucleotide reduction is
emphasized by a recent report from Cooperman and coworkers that the
Y177F M2 (the mouse homolog of R2), is able to support nucleotide
reduction.(Henriksen et al, 1994) They report that Y122F M2 catalyzes
nucleotide reduction with a turnover number ~0.5% that of the wt
enzyme. Control experiments show that any amount of •Y177 that may be
present is insufficient to account for the enzymatic activity. The
observation that a nonapeptide corresponding to the C-terminus of M2
(which is essential for interaction with M1) inhibits this activity, but the C-
terminus peptide of E. coli R2 does not is also consistent with the
assignment of the enzymatic activity to M2 rather than a ribonucleotide
reductase in the host strain. Cooperman and coworkers ascribe this activity
to a putative transient radical species generated during the assembly and
disassembly of the required diferric-•Y cofactor. In contrast to the E. coli R2,
the iron center and tryosyl radical in M2 can be reduced by DTT, present in
their reaction mixtures as a reductant to generate deoxynucleotides, and
can be reoxidized in the presence of O2. Transient intermediates in the
reoxidation, such as the Fe3+/Fe4+ intermediate X in the assembly of the
active cofactor (Bollinger et al, 1991a, Bollinger, 1993, Bollinger et al, 1994b, Bollinger et al, 1994a), are capable of generating alternative protein radicals (Örmo et al, 1992, Sahlin et al, 1994, Sahlin et al, 1995) which could eventually generate the required thyl radical on R1. Interestingly, HU also inhibits the observed enzymatic activity, although it is unknown whether this inhibition is due to reduction of a transient radical or to reaction of HU with the iron center (McClarty et al, 1990, Nyholm et al, 1993). The quantification of the number of equivalents of O₂ and ferrous iron consumed relative to the amount of dNDP produced would establish the validity of this intriguing model. It is of interest to note that Bollinger et al., when studying the assembly of the cofactor of Y122F R2 from E. coli, observed a species (10% of the protein) that had a UV/vis spectrum identical to a •Y (Bollinger et al, 1991a). It was suggested at that time that it might be associated with Y356 (Fig. 1.3), one of the residues on the putative electron transfer pathway. To date, however, no group has been able to associate this transient absorption feature with an EPR-active species (Tong et al, 1996). A similar experiment should be carried out with Y177F M2. If a transient intermediate is observed, then a presteady state three syringe experiment could be attempted. Rapid mixing of R1 and NDP with R2 at the time of maximum formation of the transient •Y should lead to dNDP formation.

3.7 Indirect Evidence for Electron Transfer and Its Pathway

Despite much effort from Sjöberg and her colleagues (Nyholm et al, 1993, Sahlin et al, 1995, Ekberg et al, 1996, Larsson et al, 1996), there is as of yet no direct evidence to support the proposed electron transfer pathway between C439 on R1 and Y122 on R2 (Fig. 1.3). Their effort to test this model has focused on the generation of a mutant of each of the residues in the proposed pathway. While all of the mutants are "inactive", no build-up of any transient radical has been observed. The x-ray structure of the R1 double mutant Y730,731F in the absence of R2 suggests no major disruptions of R1 that would lead to loss of activity (Ekberg et al, 1996). Unfortunately, the overall phenotype of inactivity is difficult to interpret. We have carried out several similar experiments with Y730F and Y731F mutants of R1. The model suggests that for Y730F R1 in the presence of substrate, allosteric effector and R2, reduction of •Y122 R2 might be

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detected concomitant with transient generation of •Y on 731 of R1, assuming similar oxidation potentials for these two residues. Disappointingly, no such a transient radical is observed using RFQ EPR methods. The loss of activity in this mutant has been rationalized in terms of the disruption of the putative H-bonding network, proposed to be crucial in this process (Ekberg et al., 1996). This is a case where unnatural amino acids, with the same H bonding network and minimal structural perturbation but altered oxidation perturbation might allow build up of a radical in the second subunit, concomitant with loss in the •Y.

While no direct evidence for the required reversible e- transfer pathway has yet been obtained, several indirect methods demonstrating irreversible e- transfer have provided strong support for reduction of the •Y122 on R2 and concomitant oxidation of a nucleotide substrate analog or an amino acid residue on R1. The most compelling evidence has resulted from the study of the interaction of 2′-vinylfluoro-2′-deoxyctydine 5′-diphosphate (VFCDP) with E. coli RNR (van der Donk et al., 1996a). As shown in Scheme 1.5, VFCDP is a stoichiometric mechanism-based inhibitor of RNR. Inactivation of the enzyme results from destruction of the •Y on the R2 subunit and covalent stoichiometric labeling, most probably of E441 (Fig. 1.2) of R1. Loss of the •Y is accompanied by formation of a new radical which recent isotopic labeling studies have established is allylic and nucleotide-derived (van der Donk et al., 1998). Thus, reduction of •Y122 on R2 results in formation of a second radical in the active site of R1. These results establish that long-range electron transfer is feasible, but say nothing about the pathway for this process.

A second example of electron transfer between R1 and R2 comes from the studies of the stoichiometric mechanism-based inhibitor 2′-azido-2′-deoxyuridine diphosphate (N3UDP) (Scheme 1.6). Inactivation by this compound results from destruction of the essential •Y122 on R2 (Thelander et al., 1976, Ator et al., 1984) Loss of this radical is accompanied by formation of a second radical (Sjöberg et al., 1983). Nitrogen gas release, uracil and diphosphate formation, and, eventually, alkylation of R1 with 2-methylene-3-(2H)-furanone also occur in the course of mechanism-based inactivation (Thelander et al., 1976, Ator et al., 1984). Studies using
Scheme 1.5. Mechanism of inactivation by VFCDP. The major pathway, which does not regenerate the thyl radical, leads to irreversible loss of the \( \cdot Y \).
Scheme 1.6. Mechanism of inactivation by N₃UDP. Release of hydrazoic acid, formation of a thyl radical, and reaction to form N₂ and a nitrogen-centered radical. The further reaction of the nitrogen-centered radical is illustrated in Scheme 1.15.
[\textsuperscript{15}N]-N\textsubscript{3}UDP and [\textbeta{}-\textsuperscript{2}H-cysteine]- RDPR have shown that a new nitrogen-centered radical derived from the azido group of the inhibitor is generated (~0.4 eq. of new radical formed per equiv of •Y122 lost) (Salowe et al, 1987, Salowe et al, 1993), and that this radical covalently attached to a cysteine of R1 (van der Donk et al, 1995). Site-directed mutagenesis studies suggest that C225 (Fig. 1.2) is the modified residue (Salowe et al, 1993, van der Donk et al, 1995). Furthermore, studies of the inactivation of RDPR with [3'-\textsuperscript{2}H]-N\textsubscript{3}UDP reveal an isotope effect on loss of the •Y122 (Salowe et al, 1993). While the chemistry is obviously complex, the data strongly suggests that the radical on R2 can be transferred to R1 via the nucleotide.

Finally, two R1 active mutants, C225S R1 and E441Q R1, provide additional evidence for electron transfer between the subunits. When C225S R1 is incubated with UDP and R2, the •Y122 on R2 is lost and the normal substrate is converted to a mechanism-based inhibitor (Mao et al, 1992a). Loss of this radical on R2 results in polypeptide cleavage on R1 (Eq. 1.2). The unusual products (a C-terminal peptide with a carboxamide terminus and a formylated N-terminal peptide) of this cleavage between S224 and S225 suggest that the radical has been transferred from one subunit to the second (van der Donk et al, 1996b). Using [3'-\textsuperscript{2}H]-UDP, V\textsubscript{max} and V/K isotope effects of ≈ 2.0 have been detected on loss of the •Y (Mao et al, 1992a). These effects are likely to arise from a coupled pair of reactions: reduction

\[
\begin{align*}
\text{C225S R1} \xrightarrow{\text{O}_2} & \text{\textldots SVGRTPRTQFSS -NH}_2 \xrightarrow{\text{O}} \text{\textldots VLECGDSLDSINAT}\end{align*}
\]

Eq. 1.2

of the •Y to form a thyl radical at C439 of R1, and abstraction of the 3' hydrogen by the thyl radical. Recent studies with E441Q R1, CDP, and R2 also result in the conversion of the normal substrate into a mechanism-based inhibitor (van der Donk et al, 1996a, Persson et al, 1997). Under one set of conditions, the •Y on R2 is reduced and several new radicals, thought to be nucleotide based, are detected. While the details of these systems remain to be elucidated, both suggest that once again electron transfer is occurring between the two subunits.
4. Generation of a Thiyl Radical on R1 from the Tyrosyl Radical on R2

4.1 Precedent for the Formation of Thiyl Radicals from Phenoxy Radicals

Reactions of thiols with phenoxy radicals have previously been reported to produce thiyl radicals and disulfide radical anions derived from these thiyl radicals (Ross et al, 1984, Rao et al, 1990). Moldéus and co-workers have used spin trap reagents to obtain evidence for generation of thiyl radicals in a system containing glutathione or cysteine and an oxidizing system consisting of acetaminophen, horseradish peroxidase, and hydrogen peroxide (at pH 8.0) (Ross et al, 1984). In rapid-flow EPR experiments, Mason and co-workers found that the same system (at pH 7.5) produces the disulfide radical anion of glutathione or cysteine. (Rao et al, 1990) They propose that the phenoxy radical generated via the oxidation of acetaminophen by horseradish peroxidase reacts with glutathione to form a thiyl radical, which in turn can react with another molecule of glutathione (in the thiolate form) to form the disulfide radical anion. These model studies show that a phenoxy radical can generate a thiyl radical, although the yield of this process was not reported. In addition, in the model system, in contrast to the reductase system, the reaction could be driven to the right uniquely by the experimental conditions, such as the presence of a spin trap. The proposed mechanism for RNR requires that a reasonable amount of thiyl radical be formed without a very exergonic step immediately following the generation of this radical. Nevertheless, this model system illustrates how an oxidizing system might generate a thiyl radical through the intermediacy of a phenoxy radical, in analogy with the diferric cluster assembly of R2 generating a •Y that functions to form a thiyl radical.

Pulse radiolytic experiments also provide support for the formation of thiyl radicals from tyrosyl radicals. Prütz and coworkers have obtained evidence for this reaction in several different contexts. They demonstrated that the presence of thiols inhibits a tyrosine dimerization proposed to occur through •Y intermediates, suggesting that the •Y was reduced by hydrogen atom from the thiol (Prütz et al, 1983). They also generated a •Y on the Trp-Tyr dipeptide, and showed that the presence of glutathione (2 mM) at pH 8.1 accelerated the decay of the •Y, allowing estimation of a rate constant of ~2x10^5 M^{-1} s^{-1} for this process (Prütz et al, 1989). A rate constant
for the reverse reaction, abstraction of a hydrogen from tyrosine by a thiyl radical, was measured to be $5.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for the Gly-Tyr dipeptide and glutathione at pH 8.1. These studies suggest that the equilibrium between •Y and thiyl radical lies toward the former, but not so far that generation of thiyl radical from •Y is thermodynamically unfeasible. At pH 8.1, a significant fraction of the glutathione is deprotonated, allowing for the possibility of a thermodynamically favorable single electron transfer between a •Y and the thiolate.

4.2 Thermodynamics of Thiyl Radical Formation

Critical to the proposed mechanism for the *E. coli* RNR is the •Y-mediated thiyl radical formation. Model studies described above suggest that this is, in fact, possible. The midpoint potential for the tyrosine/•Y couple in neutral solution has been measured as -0.93 V (Harriman, 1987). However, this potential is dependent on the protonation state of the tyrosine. The tyrosinate is clearly more easy to oxidize than neutral tyrosine (at pH 11, the oxidation potential is -0.7 volts). The midpoint potential for oxidation of protonated β-mercaptoethanol or penicillamine to a thiyl radical has been measured to be -1.33 V at pH 7 (Surdhar & Armstrong, 1987), while that for one-electron oxidation of glutathione is 0.91 V at pH 5.0 (Tamba & O'Neill, 1991). Cysteine might be expected to have a similar oxidation potential, and hence its oxidation via tyrosine would be thermodynamically unfavorable. One-electron oxidation of cysteinate, on the other hand, has a midpoint potential of -0.73 V (Surdhar and Armstrong, 1987). Thus, depending on the protonation state of the C439 and Y122, the equilibrium amounts of thiyl radical and •Y could vary substantially.

4.3 Kinetics of Thiyl Radical Formation

Model systems indicate that the abstraction of hydrogen from a thiol by many different radical species occurs at diffusion-controlled rates. Phosphite radicals abstract a hydrogen atom from penicillamine with a rate constant of $3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ (Schäfer & Asmus, 1981). The thiyl radical derived from β-mercaptoethanol can abstract a hydrogen atom from dithiothreitol with a rate constant of $1.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Akhaq & von Sonntag, 1987). Carbon-centered radicals derived from ethylene glycol and D-ribose abstract
hydrogen atoms from dithiothreitol with rate constants of 2.6x10^7 M^{-1} s^{-1} and 9x10^7 M^{-1} s^{-1} respectively (Akhlaq et al, 1987). Formation of thyl radicals by electron transfer has also been reported to be rapid, with the OH• adduct of deoxyguanosine reacting with cysteine with a rate constant of 8.4x10^8 M^{-1} s^{-1} at pH 9.5 (where cysteine will be predominantly in the thiolate form) (O'Neill, 1983). Thus, while structural data suggests that C439 will be protonated in R1 in the resting state (Uhlin and Eklund, 1994), thyl radical formation would be expected to be kinetically facile by either hydrogen atom abstraction or by electron transfer, if the cysteine thiol is transiently deprotonated. Thus, the success of the oxidation might be governed by proton mediated electron transfer, with Y730 being one, of potentially many (Fig. 1.3), important residues.

5. A Glycyl Radical May Generate an Active Site Thyl Radical in the Anaerobic E. coli Ribonucleotide Reductase

The anaerobic E. coli ribonucleotide reductase is composed of two homodimeric subunits (α2β2). The β2 subunit is an activating enzyme that binds S-adenosylmethionine (SAM) (Eliasson et al, 1990, Harder et al, 1992), flavodoxin (Bianchi et al, 1993), and flavodoxin reductase. It contains a single [4Fe-4S] cluster at the dimer interface (Sun et al, 1993, Ollagnier et al, 1996). This activating enzyme catalyzes formation of an essential glycyl radical on the α2 homodimeric subunit that is required for nucleotide reduction (Sun et al, 1993). The details of the mechanism of this reaction remain to be determined. One of many possibilities currently being considered is that SAM is reductively cleaved to form methionine and 5'-deoxyadenosyl radical (5'-dA•), which abstracts a hydrogen from glycine. Pyruvate formate lyase activating enzyme is the prototype for the β2 chemistry catalyzed by RNR. It is required to generate the glycyl radical of pyruvate formate lyase (PFL) that is required for formation of acetyl-CoA and formate from pyruvate and coenzyme A (Wong and Kozarich, 1994). SAM is converted to 5'-deoxyadenosine (5'-dA) and methionine during this reaction (Knappe et al, 1984), and recent studies have shown that using [α-2H] glycine labeled PFL, deuterium is found in the 5' position of 5'-dA (Frey et al, 1994). Determination of the thermodynamics of the reductive cleavage of SAM will be an important step in evaluating the mechanism of
this process and the roles of the enzyme and the iron sulfur cluster in catalyzing the formation of glycyl radical.

Using the mechanistic information about the *E. coli* and *L. leichmannii* reductases as a guide, we have postulated that the role of the glycyl radical in the anaerobic *E. coli* reductase is to generate a thyl radical that initiates nucleotide reduction by abstracting the 3'-hydrogen of the substrate (Stubbe and van der Donk, 1995). Evidence that the protein radical that initiates nucleotide reduction is not the glycyl radical is provided by a study in which nucleotide reduction was studied in D₂O (Eliasson et al, 1995). When the deoxynucleotide product was isolated and examined by NMR spectroscopy, in addition to deuterium located in the pro-R position at C2', 1-2% deuteration was also observed at the 3' position. This important result demonstrates that the 3'-hydrogen atom is abstracted during the course of the reaction. Moreover, it suggests that the protein radical that abstracts the 3'-hydrogen, and presumably also returns it, can exchange with the solvent. Although the glycyl radical in pyruvate formate lyase has been shown by EPR to exchange with solvent (Unkrig et al, 1989, Parast et al, 1995), the rate of this process is 10⁻⁵ the normal turnover rate. Furthermore, the glycyl radical in the anaerobic *E. coli* reductase does not exchange with solvent (Mulliez et al, 1993, Sun et al, 1996). Thus, the observation of deuterium incorporation from solvent suggests that the glycyl radical does not abstract hydrogen from the 3' position of the substrate, and is consistent with the hypothesis that a thyl radical serves that purpose.

Model studies have provided information about the thermodynamics and kinetics of the abstraction of hydrogen from a thiol by a glycyl radical. The reduction potential for the alanyl radical (unpaired spin on the α carbon)/alanine couple has been estimated to be 1.22 V (Zhao et al, 1994b), based on the reduction potential of ethylamine radical (Armstrong et al, 1993) and the estimated effect of an α carboxyl group on the C-H bond energy (Merenyi & Lind, 1994), using Benson's group additivity rules (Benson, 1976). Using the reduction potential of 1.33 V measured for the thyl radical/thiol couple (Surdhar and Armstrong, 1987), an equilibrium constant of 200 was estimated for the equilibrium between a thyl radical and an alanyl radical (Zhao et al, 1994a). The corresponding value for a glycyl radical is likely to be similar, although the inductive
effect of the methyl group will probably make alanine easier to oxidize than glycine. In the same paper, the rate constant for abstraction of hydrogen from the α position of glycine by a cysteine thyl radical was determined to be 3.2x10^5 M⁻¹ s⁻¹ (Zhao et al., 1994a). The thermodynamics and kinetics of this model reaction indicate that hydrogen abstraction by the putative enzyme-based thyl radical to regenerate the glycyl radical should be both thermodynamically favorable and rapid. However, this model system also suggests that hydrogen abstraction by a glycyl radical to form a thyl radical would be thermodynamically unfavorable. The enzymatic system is likely to alter the energetics of the redox chemistry to make both the forward and reverse reactions reasonably facile.

Theoretical quantum mechanical studies predict that a planar, extended conformation will be highly favored for the glycyl radical (Barone et al., 1995), making it difficult for the enzyme to modulate the reactivity of the glycyl radical through a conformational change (e.g., making the glycyl radical more reactive by enforcing a conformation in which captodative stabilization was minimal). It is thus more plausible that the enzyme modulates the reactivity of the thyl radical. As discussed earlier (Section 4.1), the protonation state of a cysteine residue has a large effect on the redox potential, with the reduction potential for the thyl radical/thiolate couple being 0.73 V, compared to 1.33 V for the thyl radical/thiol couple. While full deprotonation of the putative active site cysteine would preclude hydrogen atom abstraction, hydrogen bonding that weakened the S-H bond might shift the redox potential of this residue enough that formation of the thyl radical would be thermodynamically favorable, or at least energetically neutral.

6. The Mechanism of Nucleotide Reduction (Scheme 1.1): Abstraction of the 3'-Hydrogen by a Thyl Radical

6.1 Thermodynamics of Hydrogen Abstraction by Thyl Radicals

The model postulated in Scheme 1.1 is that the thyl radical abstracts a hydrogen atom from the 3'-position of the nucleotide to initiate the nucleotide reduction process. The initial question that has been raised by many critics of this model is whether this reaction is thermodynamically favorable. The homolytic bond dissociation energy of an S-H bond
(derived from heats of formation) is between 88-91 kcal mol\(^{-1}\) (Benson, 1978, McMillen and Golden, 1982), while the bond dissociation energy of the hydrogen of a deoxyribose sugar is \(\sim 91\) kcal mol\(^{-1}\) (Schöneich et al, 1990). Thus, the \(\Delta H\) of this reaction allows for the possibility of a favorable \(\Delta G\).

Experimentally, however, equilibria in solution greatly favor thyl radicals over carbon centered radicals. Asmus and co-workers measured the forward and reverse rate constants for the reaction of penacillamine thyl radicals with 2-propanol to be \(1.4 \pm 0.3 \times 10^4\) M\(^{-1}\) s\(^{-1}\) and \(1.2 \pm 0.3 \times 10^8\) M\(^{-1}\) s\(^{-1}\), respectively (Schöneich et al, 1989). This corresponds to an equilibrium constant of \(\sim 10^{-4}\), or a \(\Delta G^o\) of \(\sim 5\) kcal/mol. For abstraction of hydrogen from dimethyltetrahydrofurran by the thyl radical, the analogous equilibrium constant is \(\sim 10^{-5}\) (Akhlaiq et al, 1987), while for hydrogen atom abstraction from HCO\(_2^-\), the equilibrium constant is \(\sim 5 \times 10^{-4}\) (Surdhar et al, 1989).

Deprotonation of the 3′-hydroxyl of the substrate is one way to perturb the energetics of 3′-hydrogen atom abstraction. Theoretical studies of Evans and co-workers have shown that deprotonation of the hydroxyl group of methanol lowers the C-H bond dissociation energy by \(\sim 15\) kcal mol\(^{-1}\) (Steigerwald et al, 1979). Although full deprotonation of the 3′ hydroxyl of the substrate is unlikely to occur at the enzyme active site, this study suggests that hydrogen bonding of the 3′ hydroxyl (for example to the 2′ hydroxyl) could lower the bond dissociation energy of the 3′ C-H enough that hydrogen abstraction by a thyl radical would be exergonic.

Hydrogen bonding from groups within the active site of the enzyme could also contribute to the net energetics of this step. While the 3′ hydrogen of the substrate would not participate in hydrogen bonding, the thiol resulting from this abstraction could function as a hydrogen bond donor. While the primary role of enzymes is usually thought to be lowering the transition state energy of a given process, in a multi-step mechanism, equalizing the relative ground state energies of intermediates can also make a significant contribution to catalysis (Albery & Knowles, 1976).
6.2 Kinetics of Hydrogen Abstraction by ThiyI Radicals

As noted above, the rates of hydrogen abstraction by thiyl radicals are typically quite fast. The thiyl radicals of cysteine, glutathione, and penicillamine abstract hydrogen from 2-propanol with rate constants of \( \sim 10^4 \text{M}^{-1}\text{s}^{-1} \) (Schöneich et al., 1989, Schöneich et al., 1990). With even moderate effective concentrations of thiyl radical at the active site, therefore, these rate constants would allow for rates of hydrogen atom abstraction greatly in excess of the overall rate constant for turnover to produce deoxynucleotide (\( \sim 5 \text{s}^{-1} \)).

The most reasonable mechanism to accommodate an unfavorable equilibrium is the coupling of this equilibrium to a subsequent fast, irreversible step in the mechanism. This type of reaction with thiyl radicals has ample precedent in the literature through the work of Huyser and Kellog (Huyser & Kellogg, 1966), which in fact provided the experimental model for this mechanistic proposal made many years ago. As will be discussed in detail in Section 7, the loss of water from the 2' position of the nucleotide, proposed to follow 3' hydrogen abstraction (Scheme 1.1) is fast and irreversible, and model studies suggest that the coupling of this step to the abstraction of the 3' hydrogen could easily give rise to kinetically competent formation of the 2' ketyl radical intermediate even if the equilibrium between thiyl radical and nucleotide radical is unfavorable.

6.3 Evidence for 3' Hydrogen Atom Abstraction in the Enzymatic Reaction

Strong evidence for 3' hydrogen abstraction comes from studies of kinetic isotope effects on the reduction of 3'-tritium and deuterium labeled nucleotides. When RDPR reduces [3'-\(^3\text{H}\)]-NDPs, tritium isotope effects on V/K are typically on the order of 2, with values ranging from 1.4 to 4.7 depending on the pH and the allosteric effector (Stubbe and Ackles, 1980, Stubbe et al., 1983). Deuterium substitution at the 3'-position of the substrate does not produce and isotope effect on \( V_{\text{max}} \), but has the expected value for a V/K isotope effect based on the tritium isotope effect and the Swain-Schaad relation. Substitution of tritium at other positions on the sugar of the nucleotide does not give rise to selection effects. In addition, a small but significant amount of tritium (\( \sim 0.5\% \) of total tritium at 50% conversion) is released to the solvent on incubation of [3'-\(^3\text{H}\)]-NDP with
RDPR. This tritium washout is pH dependent as well, with slightly more tritium being volatilized at more basic pH. These results require that hydrogen be abstracted from the 3' position of the nucleotide, that this hydrogen abstraction take place before the first irreversible step, and that the hydrogen abstraction not be the overall rate-limiting step in nucleotide reduction.

The magnitude of the \( T(V/K) \) is relatively small, but a comparison of the \( T(V/K) \) isotope effect with the \( D(V/K) \) isotope effect shows that these effects are consistent with a primary kinetic isotope effect on C-H bond cleavage. Using the \( T(V/K) \) and \( D(V/K) \) isotope effects to calculate the intrinsic deuterium isotope effect on C-H bond cleavage by the method of Northrup (Eq. 1.3) (Northrup, 1982),

\[
\frac{DV/K - 1}{TV/K - 1} = \frac{D_k - 1}{D_k^{1442} - 1}
\]

Eq. 1.3

where \( D_k \) is the intrinsic deuterium isotope effect, gives a value of \( \sim 5 \) for \( k_H/k_D \), a reasonable primary isotope effect. The intrinsic deuterium isotope effect in turn allows calculation of the sum of the forward and reverse commitment factors (Northrup, 1982).

\[
D(V/K) - 1 = \frac{D_k - 1}{1 + C_f + C_r}
\]

Eq. 1.4.

In this case, the forward commitment represents the tendency of the thiyl radical to abstract the 3' hydrogen, rather than to partition back to the previous intermediate, while the reverse commitment is the tendency of the 3' nucleotide radical to re-abstract a hydrogen from C439 to regenerate the thiyl radical as opposed to undergoing dehydration. The sum of the commitment factors is calculated to be \( \sim 3 \). Assuming that the dehydration that follows the hydrogen atom abstraction is fast and irreversible (Section 7), the reverse commitment will be zero, so the value of \( \sim 3 \) would represent the forward commitment. This analysis suggests that the
observed V/K isotope effects are small because the commitment to catalysis is high, not because the intrinsic isotope effect is low.

Kinetic isotope effects have also been observed with mechanism-based inhibitors labeled with deuterium or tritium at the 3′ position. During inactivation of RDPR by [3′-3H]-N3UDP, approximately 0.2 eq. of tritiated water is released per equivalent of enzyme inactivated (Salowe et al., 1987). This indicates that 3′ C-H bond cleavage is required for this reaction. The partitioning between tritiated water release and inactivation corresponds to a five-fold selection effect against abstraction of tritium. As mentioned previously, with [3′-2H]-N3UDP, an isotope effect is observed on loss of the •Y (Salowe et al., 1993). The kinetics of •Y loss are multiphasic, and only the fast phase is isotope sensitive. Analyzing only the fast phase, a \( DV_{\text{max}} \) isotope effect of 1.5 and a \( D(V/K) \) isotope effect of 2.2 were calculated. The kinetics of inactivation are also isotope sensitive, although the multiphasic nature of these kinetics complicates quantitative interpretation. These observations are consistent with abstraction of hydrogen being partially rate-limiting in inactivation by N3UDP.

Site-directed mutagenesis of C225 (Fig. 1.2) in the E. coli class I R1 to a serine produces a mutant enzyme which catalyzes its own cleavage into two polypeptides when incubated with R2 and a nucleotide substrate (Eq. 1.2) (Mao et al., 1989, Mao et al., 1992a, van der Donk et al., 1996b). This self-inactivation is proposed to occur through the intermediacy of a substrate-based radical. Consistent with this hypothesis, this self-inactivation exhibits kinetic isotope effects similar to those observed with N3UDP. Using [3′-3H]-UDP, the rate of tritium washout to solvent was compared to the rate of uracil release to calculate a selection effect of 3.2 against tritium abstraction (Mao et al., 1992a). The •Y loss that accompanies self-inactivation also exhibits a kinetic isotope effect when [3′-2H]-UDP is used as the substrate: \( DV \) and \( D(V/K) \) are 2.0 and 2.0, respectively (Mao et al., 1992a). These observations are consistent with hydrogen atom abstraction being partially rate-limiting in self-inactivation as well as inactivation by N3UDP.

It appears that while hydrogen atom abstraction is not rate-limiting in substrate reduction by wild-type RDPR, it is partially rate-limiting in mechanism-based inactivation of wt and mutant enzymes. One explanation for this is that the inactivation reactions bypass a slow step in
the reduction chemistry that occurs after 3' hydrogen atom abstraction. The proposed reduction of the 3' keto intermediate by an enzyme-based disulfide radical anion might be that slow step, as model reactions suggest it is a difficult transformation (Section 8.3).

7. Loss of the 2' Hydroxyl Group

7.1 Model Studies on Dehydration of Ethylene Glycol

Following abstraction of the 3' hydrogen, the nucleotide substrate is proposed to lose the 2' hydroxyl as water. The original proposal focused on a cation radical intermediate generated by this dehydration, although based on model systems, both cation radical and anion radical, as outlined subsequently, could feasibly account for the data. We still feel that a cation radical mechanism can best accommodate all of the available data on the enzymatic system, although we have also recently considered the possibility of a concerted mechanism as well (Stubbe and van der Donk, 1995, Zipse, 1995). Rapid deprotonation of the 3'-hydroxyl by the E441, would yield a ketyl radical (Scheme 1.1). One-electron oxidation of ethylene glycol and derivatives, an excellent model for the 2', 3', cis diol of the ribose of nucleotides, provided the basis for our original working hypothesis for this transformation on the enzyme.

EPR studies on the one-electron oxidation of ethylene glycol (Scheme 1.7) have shown that two radical species can be produced (Buley et al, 1966, Livingston & Zeldes, 1966). When ethylene glycol is oxidized in the absence of acid using •OH produced from photolysis of H₂O₂, a 24-line spectrum arising from the ethylene glycol alkyl radical is observed. In the presence of acid (pH <2.5) (Gilbert et al, 1971), however, a spectrum consisting of three broad lines is observed in place of the 24-line spectrum and assigned as the aldehyde radical, based on the g-value of 2.0046. This g value is significantly higher than the values of 2.0024 typically observed for 2-hydroxylalkyl radicals and is close to the g value of 2.00443 observed for the ketyl radical derived from acetone. A structure in which the oxygen formed a three-membered ring was ruled out based on the inequivalency of hyperfine couplings (Livingston and Zeldes, 1966). Similar results were obtained when ethylene glycol was oxidized by H₂O₂/titanous ion in experiments monitored by rapid flow (Buley et al, 1966). These results are
consistent with a mechanism in which a 2-hydroxyalkyl radical is
generated initially, then undergoes acid-catalyzed dehydration to form the
formylmethyl radical (Scheme 1.7).

Rate constants for hydrogen atom abstraction and dehydration have
been measured by taking advantage of the ability of the hydroxyl radical
formed in the Fenton reaction to initiate the one-electron oxidation of
ethylene glycol (Walling & Johnson, 1974). s The stoichiometry of H$_2$O$_2$
consumption (moles of H$_2$O$_2$ consumed/mole of Fe$^{+2}$ oxidized) depends
on the partitioning between two pathways of dehydration of an
intermediate radical, one requiring Fe$^{+3}$ and regenerating Fe$^{+2}$, the other
requiring H$^+$ (Scheme 1.8). The chain length of the reaction thus depends
on the concentrations of acid and Fe$^{+3}$ present. Quantitative analysis of the
acid and Fe$^{+3}$ dependence of the partitioning allows estimation of the rate
constant for dehydration to form the radical cation. At pH 1.3, this rate
constant is estimated to be $1.3 \times 10^8$ s$^{-1}$. This large rate constant suggests that
this reaction could occur very rapidly at the active site of RDPR if the
enzyme could effect this acid catalysis.

Pulse radiolytic studies have also been used to characterize these
reactions and to assign rate constants. The observation of acetaldehyde and
derivatives after pulse radiolysis of unbuffered aqueous ethylene glycol
solutions (von Sonntag & Thoms, 1970) supports the sequence of reactions
in Scheme 1.7. Elimination of water from sugar radicals has also been
invoked to explain why deoxy products and malondialdehyde are formed
on irradiation of ribose and other pentoses, especially under alkaline
conditions (Scherz, 1970). Kinetic analysis using optical absorption and
polarographic measurements of radical formation during the pulse
radiolysis of aqueous ethylene glycol solutions (Bansal et al, 1973) shows
that from pH 3 to pH 7, only the 1,2-dihydroxyethyl radical is formed. This
radical decays with second order kinetics ($1.1 \times 10^6$ M$^{-1}$ s$^{-1}$), consistent with
dimerization of the radical leading to this decay. At pH 1.4, a fast, first
order decay is observed in addition to a slower second order decay. Fitting
the observed kinetics to a mechanism in which a rapid equilibrium
protonation is followed by dehydration of the protonated 1,2-
dihydroxyethyl radical (Scheme 1.7) yields an equilibrium constant of 0.18
M$^{-1}$ for the protonation equilibrium ($pK_a = 0.74$) and a rate constant for
dehydration of $8.6 \times 10^5$ s$^{-1}$. This rate constant is lower than that obtained by
Scheme 1.7. Mechanism of decomposition of ethylene glycol and derivatives following one-electron oxidation: a. ethylene glycol; b. 2-chloroethanol; c. acetic acid 2-hydroxyethyl ester.

Scheme 1.8. Oxidation of ethylene glycol by hydroxyl radical formed in the Fenton reaction.
analysis of the stoichiometry of oxidant consumption (Walling and Johnson, 1974), but much larger than the rate constant for enzymatic turnover. The unimolecular dehydration step in the acid-catalyzed dehydration of the radical derived from erythritol has also been reported to proceed with a rate constant of $3.5 \times 10^5 \text{ s}^{-1}$, suggesting that ethylene glycol is a good model system for the kinetics of this reaction in sugars and nucleotides (Steenken et al., 1986).

The dehydration of the 1,2-dihydroxyethyl radical can also be base-catalyzed (Bansal et al., 1973, Steenken, 1979). Pulse radiolytic studies show that OH-reacts with the neutral 1,2-dihydroxyethyl radical to form the radical anion (simple deprotonation is unlikely, given that the pK$_a$ of this radical is likely to be greater than 10) (Bansal et al., 1973). The rate constant for elimination of OH$^-$ has also been studied by pulse radiolysis, and measured to be $3 \times 10^6 \text{ s}^{-1}$ (Steenken, 1979). In contrast, the rate constant for the uncataylzed dehydration (i.e., in the absence of acid or base) has been measured to be $\sim 10^4 \text{ s}^{-1}$ (Steenken et al., 1986).

The reactions observed with ethylene glycol also take place with nucleoside radicals, as shown by a recent model study from Giese's laboratory (Lenz & Giese, 1997). Photolysis of a ribonucleoside derivatized with a selenol ester at the 3' position generates a 3' nucleotide radical specifically (Scheme 1.9), thus providing the closest model yet available for the enzymatic system. Rate constants for the dehydration were measured by competition kinetics. Release of base from the nucleoside was used as a measure of the dehydration reaction in this experiment, as the dehydration leads to a 3'-ketodeoxynucleoside which undergoes loss of the base under the experimental conditions (Binkley et al., 1978, Hansske et al., 1984). Direct reduction of the 3' nucleoside radical by tributyltin hydride present in the reaction mixture causes formation of the ribo- or xylo-nucleoside, so that comparison of the amounts of nucleoside product with the amount of base release allows determination of the rate of elimination relative to the rate of hydrogen atom abstraction. With this method, a rate constant of $2 \times 10^3 \text{ s}^{-1}$ was measured for the dehydration in the absence of buffer, while in the presence of 0.1 M triethylammonium acetate (pH 7), the rate constant was $1.5 \times 10^6 \text{ s}^{-1}$.

Lenz and Giese also examined the pH dependence of the rate of dehydration (Lenz and Giese, 1997). The rate was found to increase from
Scheme 1.9. Model reaction for the dehydration of nucleotide substrates during ribonucleotide reduction (Lenz and Giese, 1997). Boxed species are the products detected.

pH 5–7 in 0.1 M phosphate buffer, but to decrease with decreasing pH in the pH range of 3–6 with citrate buffer. These results were interpreted to favor base catalysis over acid catalysis for the dehydration of this model system (Scheme 1.10). This model reaction was proposed to support a radical anion mechanism for ribonucleotide reductases. In this mechanism, E441 (a conserved residue in both class I and II reductases) (van der Donk et al., 1996a, Persson et al., 1997) was proposed to act as a base, generating the radical anion leading to the putative ketyl radical. This interpretation is consistent with the three dimensional structure of R1, which places E441 in the active site, close to where the 3’ hydroxyl of the substrate is proposed to bind (Uhlin and Eklund, 1994, Lenz and Giese, 1997). However, the earlier studies with ethylene glycol suggest that pH <2 would be required to observe acid catalysis, as the pKₐ for the hydroxyl group of the radical has been determined to be 1.4 (Steenken et al., 1986) (although an earlier measurement puts this value at 0.74 (Bansal et al., 1973)). It is thus anticipated that if the studies of Lenz and Giese were extended over a greater pH range (to 1.5), specific acid catalysis would also be observed for this nucleoside model system.
Scheme 1.10. Proposed radical anion mechanism for dehydration of nucleosides after abstraction of hydrogen from the 3’ position.

Both the ethylene glycol model system and the nucleoside model system suggest that acid or base catalysis can accelerate the dehydration by up to 1000-fold relative to the uncatalyzed reaction. As has been noted previously (Stubbe, 1990, Lenz and Giese, 1997), acceleration of this step may be critical in driving the reaction, given the unfavorable equilibrium for the previous proposed step, abstraction of the 3’ hydrogen by a thyl radical. In fact, model studies now allow a prediction of the rate constants of the hydrogen atom abstraction and radical dehydration steps in the enzymatic mechanism. Experiments from the Asmus and von Sonntag laboratories (Akhalq et al., 1987, Schöneich et al., 1989) have demonstrated that while the equilibrium between thyl radical and carbon centered radical greatly favors the thyl radical ($K_{eq}$ of $10^{-4}$–$10^{-5}$), the rate of approach to equilibrium (i.e., the sum of the forward and reverse rates) is large, since the forward rate constant is $\sim 10^8$ M$^{-1}$ s$^{-1}$. From the model studies on ethylene glycol (von Sonntag and Thoms, 1970, Bansal et al., 1973, Walling and Johnson, 1974, Steenken et al., 1986) and the nucleoside model of Giese and co-workers (Lenz and Giese, 1997), the rate constant for acid- or base-catalyzed dehydration of the 3’-nucleotide radical can be estimated to be $\sim 10^6$ s$^{-1}$. If the enzyme active site enforces a reasonably high effective concentration ($\geq 1$ M) for the thyl radical and the 3’ nucleotide radical, the rate of approach to equilibrium for the hydrogen abstraction step will be greater than the rate of the dehydration step by a factor of at least 100. In this case, the observed rate of formation of the 3’-ketodeoxynucleotide radical at the enzyme active site will be equal to the product of the equilibrium constant for the hydrogen atom abstraction step and the rate constant for the dehydration step ($K_{eq}k_{dehyd}$). For the acid- or base-
catalyzed dehydration, then, the observed rate should be \( \sim 10-100 \text{ s}^{-1} \), fast enough for this sequence of reactions to be kinetically competent for ribonucleotide reduction. Since model studies (Steenken et al., 1986, Lenz and Giese, 1997) predict that the uncatalyzed dehydration of the 3' nucleotide radical would be \( \sim 1000 \)-fold slower than the catalyzed reaction, enzymatic catalysis of this step would be required for the hydrogen abstraction/dehydration sequence to be kinetically competent unless the enzyme acted to perturb the thermodynamics of the hydrogen abstraction equilibrium (Section 6.1).

Both the acid- and base-catalyzed mechanisms that have been proposed for the enzyme would require sizable perturbations in pK\(_a\)s of substrate and/or enzyme moieties involved in catalysis. Using a pK\(_a\) of 4.3 as the value for a glutamate carboxylate in solution (Serjeant & Demprey, 1979), and a pK\(_a\) of 9.8 for the 3' hydroxyl of the 3' radical derived from 2'-deoxyribose (Hayon & Simic, 1973) (the pK\(_a\) for ribose is likely to be lower, due to hydrogen bonding from the 2' hydroxyl), the pK\(_a\)'s of the glutamate and the radical would have to be perturbed by a total of \( \sim 6 \) pK units for the glutamate (E441) to deprotonate the 3' hydroxyl effectively. In order for a cysteine (C225 or C462) to protonate the 3' nucleotide radical, the enzyme would have to perturb the pK\(_a\)'s of the cysteine and the radical by a total of 7 pK units, taking a pK\(_a\) of 8.3 for the cysteine thiol in solution (Serjeant and Demprey, 1979). Shifts of up to 4 pK units have been reported for a number of enzymatic systems, and a perturbation of the pK\(_a\) of histidine in the active site of serine proteases may be as large as 5.5 (Fersht, 1985).

However, site-directed mutagenesis experiments offer indications that the mechanism of catalysis of dehydration might be more complex than a single enzyme residue acting as a general acid or a general base. If dehydration were required to drive the hydrogen atom abstraction to completion and either C225 or C462 were absolutely required as a proton donor for this dehydration, mutation of the critical cysteine to a serine would be expected to prevent both dehydration and hydrogen atom abstraction. The C225S and C462S RDPR mutants (and the C119S and C419S mutant RTPRs (Booker et al., 1994) are in fact able to catalyze both 3' hydrogen atom abstraction and formation of the 3'-ketodeoxynucleotide intermediate, as judged by release of the base from the sugar, formation of a 320 nm-absorbing species on the enzyme, and loss of \( \cdot Y. \)(Mao et al., 1989,
Mao et al, 1992a, Mao et al, 1992b, Mao et al, 1992) If, on the other hand, E441 was absolutely required as a proton acceptor, the E441Q mutant would also be expected to be inactive with respect to hydrogen atom abstraction and dehydration of the radical. However, our preliminary studies (van der Donk et al, 1996a) and more recent studies of Sjöberg and coworkers (Persson et al, 1997) indicate that this mutant is able to catalyze release of base from nucleotide substrates, meaning that it can catalyze hydrogen atom abstraction and formation of the 3'-ketodeoxynucleotide.

Several explanations might reconcile the model studies with the phenotypes of site-directed mutants. First, both general acid and general base catalysis might be in effect, so that no single mutation could abolish catalysis. Alternatively, as discussed earlier (Section 6.1), the equilibrium between thiyl radical and carbon-centered radical might be perturbed on the enzyme, making catalysis of the dehydration step less important for the overall reaction. Finally, Zipse has suggested that deprotonation of the 3' hydroxyl and elimination of water might be concerted (Zipse, 1995). This suggestion is based on ab initio calculations on the interaction of the trans-butene radical cation with water, which indicate that the addition of water to this radical cation is unfavorable. Zipse interprets these calculations to suggest that the analogous proposed intermediate in the ribonucleotide reductase mechanism (Scheme 1.1) will be relatively high in energy and that a concerted formation of the relatively stable 3' ketyl radical is likely. Although Zipse invokes a hydrogen bond acceptor that can interact with both the 3' hydroxyl group and a protonated 2' hydroxyl simultaneously, a concerted mechanism might also occur with two different hydrogen bond acceptors acting simultaneously, or with the 3' hydroxyl acting as a hydrogen bond acceptor for the 2' hydroxyl.

Working out the sequence of proton transfers that ribonucleotide reductases actually use to effect the hydrogen atom abstraction/water elimination sequence will be extremely challenging. The combination of model reactions, quantum chemical simulations, and mechanistic studies on wt and mutant enzymes allows several conclusions to be drawn. First, this sequence of reactions could be kinetically competent for ribonucleotide reduction. Second, deprotonation of the 3' hydroxyl is likely to be important for this sequence of reactions to be kinetically competent for turnover, although it is unclear whether this would take place before
hydrogen atom abstraction (in order to render this process less thermodynamically unfavorable (Steigerwald et al, 1979)) or after (to provide base catalysis (Lenz and Giese, 1997)). Third, a single residue furnishing general acid or general base catalysis is unlikely to account for the acceleration of this sequence of reactions by the enzyme. Studies on mechanism-based inhibitors, described in the next section, have begun to address the proton transfer question as it relates to the protonation state of the 2′ hydroxyl and the enzymatic group(s) that could be involved in this protonation.

7.2 Model Systems for Reactions of RDPR with Mechanism-Based Inhibitors

Replacement of one of the hydroxyl groups of ethylene glycol with a good leaving group allows the loss of the leaving group from the radical without acid or base catalysis. When 2-chloroethanol is oxidized (titanous ion/hydrogen peroxide), the formylmethyl radical is observed (Scheme 1.7) (Buley et al, 1966, Gilbert et al, 1971). The alkyl radical derived from ethylene glycol and its gem diol regioisomer (Scheme 1.7b) are also detected under these conditions. This radical is assigned the structure •CH₂CH(OH)₂ based on its g value of 2.0025, which indicates that oxygen is not bound to the atom bearing the unpaired spin, and its hyperfine coupling constants which are similar to the radical •CH₂CH(OH)(OMe) (Gilbert et al, 1971). These products could result from hydration of the protonated formylmethyl radical that would be the direct product of chloride loss from the 2-chloroethanol alkyl radical. These results are consistent with the loss of chloride to give the formylmethyl radical occurring without acid catalysis, in contrast to loss of water from ethylene glycol, which would require acid or base catalysis. Significantly, the radical •CH(OH)CH₂Cl was not detected, although a small amount of •CHClCH₂OH was observed (Gilbert et al, 1971). The failure to detect •CH(OH)CH₂Cl suggests that this species may eliminate chloride too rapidly to be observed.

Similarly, when unbuffered aqueous solutions of acetic acid 2-hydroxyethyl ester (Scheme 1.7c) are subjected to pulse radiolysis, acetic acid is eliminated with a rate constant estimated to be 5×10⁵-5×10⁶ s⁻¹ (Matsushige et al, 1975). This estimated range of rate constants for acetate
loss is significant for understanding the mechanism of action of 2'-deoxy-2'-halonucleotide mechanism-based inhibitors of ribonucleotide reductases. It shows that when the hydroxyl in ethylene glycol is replaced by a good leaving group (in this case, an acetate group), elimination does not require acid or base catalysis to be rapid ($\sim10^6 \text{ s}^{-1}$). This contrasts to the elimination of water, which requires acid or base catalysis to be accelerated to this extent (Steenken et al., 1986, Lenz and Giese, 1997). As discussed previously (Section 7.1), a rate constant of $\sim10^6 \text{ s}^{-1}$ for the dehydration step in ribonucleotide reduction may be required to drive the 3' hydrogen atom abstraction.

The principle that elimination of acetic acid from a 3' nucleoside radical can be rapid even in the absence of acid or base catalysis is illustrated more directly in experiments from Giese's laboratory on a ribonucleoside derivatized with a selenol ester at the 3' position and an acetate group at the 2' position (Scheme 1.9) (Lenz and Giese, 1997). Photolysis of this compound (an analogue of the model for the normal reduction process discussed in Section 7.1) (Lenz and Giese, 1997) led to rapid elimination of acetic acid even in the absence of acid or base. This conclusion was drawn from the observation that in the presence of tributyltin hydride as a radical scavenger, the products observed from the photolysis were derived entirely from elimination of acetic acid (e.g., acetic acid, the 3'-ketonucleoside), with no products from abstraction of hydrogen from tributyltin hydride observed. A small amount ($\sim10\%$) of nucleoside product derived from hydrogen abstraction was observed when butyl thiol was used as the radical trap. Although the lack of products derived from hydrogen atom abstraction prevents a detailed kinetic analysis using the competition methods described above, examination of the one set of conditions published by Lenz and Giese that did afford a measurable amount of hydrogen atom abstraction product (see Lenz and Giese, 1997, Table 2, entry 4) suggests that the rate constant for elimination of acetic acid is $\sim10^5 \text{ s}^{-1}$. This model system thus provides strong support for the idea that a good leaving group at the 2' position of a nucleotide can obviate the need for acid or base catalysis for elimination.

Model systems of Robins and co-workers offer another perspective on the elimination of leaving groups from the 2' position of mechanism-based inactivators of ribonucleotide reductases. Robins has proposed that
2’-azido and -chloro substituents, among others, will be expelled as radicals, rather than anions (Gilbert et al., 1971, Robins et al., 1996, Robins et al., 1997). In order to generate radicals at the 3’ position of 2’-substituted nucleosides, Robins and co-workers treated protected 3’-O-(phenoxythiocarbonyl)-2’-substituted nucleosides (Scheme 1.11) with radical initiators (either tributyltin hydride/AIBN or triphenyl-silane/dibenzoyl peroxide) in refluxing toluene. The 2’-iodo, chloro, methylthio- and azido-substituted compounds reacted to give the 2’,3’-didehydro-2’,3’-dideoxynucleoside elimination products. In contrast, the 2’-fluoro, -mesyloxy, and -tosyloxy derivatives reacted under the same initiation conditions to give the 3’-deoxy-2’-substituted nucleosides. This difference in reactivity was interpreted as arising from differences in homolytic bond dissociation energy within this series of compounds. While the homolytic bond dissociation energies for the carbon-iodine, carbon-chlorine, and carbon-azide bonds are low, allowing facile loss of iodo-, chloro-, and azido-radicals, the 2’-fluoro, -mesyloxy, and -tosyloxy derivatives would be expected to eliminate the 2’ substituent as an

Scheme 1.11. Reaction of 3’-O-(phenoxythiocarbonyl)-2’-substituted nucleosides with radical initiators as a model for mechanism-based inactivation by 2’-substituted nucleotide analogs.

anion (Curran, 1988b, Curran, 1988a). Thus, the observation that the iodo-, chloro-, and azido-substituted nucleosides underwent elimination, while the fluoro-, mesyloxy-, and tosyloxy- derivatives did not is consistent with elimination proceeding through a radical mechanism. A key difficulty in applying these results to the mechanism of ribonucleotide reductase action on the di- or triphosphates of 2’-halo- or azido-substituted inactivators is that the model compounds studied lack the crucial 3’ hydroxyl group. The
observation of base catalysis for elimination of water from ethylene glycol (Steenken et al., 1986), the elimination of the 2'-hydroxyl when a radical is generated at the 3' position of a protected nucleoside (Lenz and Giese, 1997), and the failure of monohydric alcohols to undergo facile dehydration on titanous ion oxidation (Dixon & Norman, 1963) all suggest that the 3' hydroxyl is essential to the chemical reactivity of the 2'-hydroxyl (Stubbe, 1990).

More recent work from the Robins group introduces a model system which addresses this concern. Treatment of homoadenosine and homouridine derivatives bearing nitro groups at the 6' position with tributyltin hydride and the radical initiator AIBN produces an alkoxy radical at the 6' position (Lopez et al., 1989) (Scheme 1.12). This radical is proposed to react with the 3' hydrogen of the nucleoside to form a 3' radical (Barton et al., 1961, Kabasakalian et al., 1962). If the 2' position has a chloro- or tosylxy- substituent, its elimination followed by β elimination of uracil results in formation of the α,β unsaturated ketone (Scheme 1.12).

In order to determine whether the 2'-chloro substituent is lost as chloride anion or as a chloro radical, Robins and co-workers treated 2'-chloro-2'-deoxy-6'-nitrohomouridine with tributyltin deuteride and AIBN (Scheme 1.12). Deuteration incorporation at C4 of the final product was not observed, consistent with the hypothesis that the 2'-chloro substituent is lost as chloro radical. These results thus support the proposal that no long-lived nucleoside-based radical intermediate exists subsequent to hydrogen atom abstraction from the 3' position (Robins et al., 1997). If a radical intermediate were present, it would be reduced by tributyltin deuteride. In addition, no 2'-chloro-2'-deoxy-6'-homouridine was isolated from this reaction (Robins et al., 1996), indicating that abstraction of hydrogen from tributyltin deuteride by the putative 3' radical is slow compared to elimination of chlorine.

In contrast, treatment of 2'-O-tosyl-6'-nitrohomoadenosine with tributyltin deuteride and AIBN led to ~30% incorporation of deuterium at the C4 position of the product (Scheme 1.12b), consistent with a mechanism that includes loss of tosylate anion and formation of a 3'-ketone-2'-deoxynucleoside radical that can abstract a deuterium from tributyltin deuteride (selective transfer of deuterium to the α face accounts for the 30% incorporation) (Robins et al., 1997). These results were interpreted to
Scheme 1.12. Reaction of \( 2'-O\)-tosyl-6'-nitrohomoadenosine derivatives with radical initiators as a model for mechanism-based inactivation by \( 2'\)-substituted nucleotide analogs. a. \( 2'\)-chloro derivative. b. \( 2'\)-tosyl derivative.
suggest that RNR interaction with 2′-chloro-2′-deoxynucleotides might proceed by loss of Cl• rather than Cl− as proposed many years ago (Stubbe et al, 1983, Ator and Stubbe, 1985, Harris et al, 1987). The mechanistic possibility of Cl• loss was considered in the original formulation of a hypothesis to account for the observations with the enzyme. As outlined below, however, the studies with the enzymatic system cannot be accommodated by loss of Cl•. Thus, while the photolysis of 2′-chloro-2′deoxy-6′-nitrohomouridine provides a chemically feasible model for mechanism-based inactivation of ribonucleotide reductases, it appears that the enzymatic reaction proceeds by a different mechanism.

7.3 Mechanism-Based Inhibition of Ribonucleotide Reductases by 2′-Halo-Substituted Nucleosides

In 1976, Thelander, Eckstein, and co-workers first reported that incubation of E. coli RDPR with CIUDP resulted in its inactivation and that this process was accompanied by chloride ion and uracil release. (Thelander et al., 1976) Since these important early studies, this reaction has been studied in detail using [5′-3H], [β-32P], [3′-3H], [2′-3H]- and [14C]-labeled CIUDP and CIUTPs with both class I and II RNRs, respectively. Studies of the products of these reactions has provided much information about catalytic capabilities of RNRs, and has allowed formulation of a detailed mechanism for this process. Any mechanism for this process must provide a reasonable explanation for why these compounds function as inactivators and, under conditions in which the enzymes are protected against inactivation, substrates. The mechanistic model that explains available data has been presented earlier (Scheme 1.2).

Evidence is presented that has led to formulation of this model, in which Cl−, rather than Cl•, is lost through a cation radical, rather than anion radical, is presented. Incubation of [3′-3H]-ClND(T)Ps with RNRs leads to release of 3H2O to the solvent, suggesting that inactivation is mechanism-based, beginning with abstraction of hydrogen from the 3′ position of the inactivator as is the case with the normal substrate (Scheme 1.1). (Stubbe et al., 1983, Harris et al., 1984, Ator and Stubbe, 1985) As summarized in Scheme 1.13, this inactivation is accompanied by initial formation of a 3′-ketodeoxynucleotide which dissociates from the enzyme and decomposes into nucleic acid base, pyrophosphate (or tripoly-
phosphate), and 2-methylene-3(2H) furanone. (Ator and Stubbe, 1985) The latter species alkylates the R1 subunit (or its equivalent in other RNRs) in the presence of the thioredoxin reducing system. However, if DTT is used as reductant, this furanone is trapped at the exocyclic methylene, leading to protection against inactivation (Ator and Stubbe, 1985, Harris et al, 1987). The ability to effect multiple turnovers results in the ability to detect production of deoxynucleotides.

Our working model (compare Schemes 1.1 and 1.2) predicts that the 3'-nucleoside radical is a common intermediate in the inactivation and reduction mechanisms and that partitioning between these two processes occurs through the 3'-ketyl radical intermediate. The requirement for protonation of the leaving group was proposed to govern whether two cysteines or a cysteine and cysteinate were present in this form of the enzyme. If both dNDP and inactivation products (Scheme 1.13) are generated through a common intermediate subsequent to 3'-hydrogen atom abstraction, they both could exhibit the same isotope effect on this process. Unfortunately, it has not yet been possible to demonstrate this. In

![Scheme 1.13](image)

a double label experiment using [3'-3H]- and [β-32P]-ClUDP, a V/K effect of < 1.17 was determined (Harris et al, 1984). However, a similar experiment could not be carried out on dNDP produced in this same experiment because the amount generated is too low for accurate dual label analysis. This number is less than that measured (k_H/k_T = 3.3) using the [3'-3H]-UDP (Stubbe et al, 1983). This might be the expected result, as V/K isotope
effects measure the chemistry up to and through the first irreversible step. In our proposed mechanism this step would be Cl\textsuperscript{-} vs. H\textsubscript{2}O release. Based on model studies, Cl\textsuperscript{-} release would be expected to be faster, not requiring acid or base catalysis, resulting in a larger commitment to catalysis and hence a lower isotope effect.

In support of this proposal, studies with a variety of 2\textsuperscript{-}ara or ribo halogenated nucleotides (F, Cl, Br, I) have been carried out (Harris et al., 1987). ara-CIATP and ara-BrATP inactivate the \textit{L. leichmannii} reductase through a 3\textsuperscript{-}ketodeoxynucleotide at rates comparable to the ribo isomers. These results suggest that interaction of the leaving group with the enzyme is not important for inactivation (that is, general acid catalysis is not required). An analogous experiment using ara-ATP (which has a hydroxy group at the 2\textsuperscript{-} position) results in no inactivation, demonstrating the requirement for protonation of the leaving group in elimination of H\textsubscript{2}O. (Similar experiments with the \textit{E. coli} RNR and the diphosphate forms of these inactivators provide no evidence for halide release, suggesting that this enzyme does not tolerate the increased steric bulk at the \(\beta\) face of the nucleotide). These observations must be accomodated by any proposed mechanism. Finally, it is also important to note that 2\textsuperscript{-}fluoro-2\textsuperscript{-}deoxynucleotides (FUD(T)P) are also substrates and inactivators. It is unlikely that F\textsuperscript{•} would be involved in either process.

If one makes the assumption that a 3\textsuperscript{-}nucleotide radical is a common intermediate in the reactions of the various 2\textsuperscript{-}halo-substituted inactivators, the dependence on the pK\textsubscript{a} of the leaving group of the observed partitioning between turnover and inactivation can be interpreted as evidence for a mechanism in which the 2\textsuperscript{-} halogen substituents are eliminated as halide ion, rather than as the radical. In experiments performed on the \textit{L. leichmannii} RTPR, the enzyme was found to catalyze the production of approximately 1 eq. of dUTP per 1.5 eq. of fluoride released when incubated with FUTP in the presence of 3 mM DTT at pH 7.8 (Harris et al., 1987). In contrast, incubation of RTPR with CIUTP results in 1 eq. of dUTP per 220 turnovers (at pH 7.4 in the presence of 3 mM DTT) (Harris et al., 1987). The partitioning thus correlates with the pK\textsubscript{a} of the leaving group. When the leaving group is hydroxyl (pK\textsubscript{a} = 16), all turnovers result in deoxynucleotide formation. When the leaving group is fluoride (pK\textsubscript{a} = 3.2) both reduction and inactivation occur, with
reduction predominating. When the leaving group is chloride (pK_a = -7), however, inactivation predominates over reduction. The simplest interpretation of this data, assuming a commonality of mechanism, is that at least one of the cysteines proposed to interact with the α face of the substrate (C119 and C419 for the L. leichmannii enzyme, C225 and C462 for the E. coli enzyme) must be deprotonated for reduction to occur. If the leaving group is not protonated, deprotonating the appropriate thiol in the active site, the 3'-keto-2'-deoxynucleotide radical intermediate will be reduced by hydrogen atom transfer either from the β face (C439) or the α face (C225 or C462), forming a 3'-keto-2'-deoxynucleotide (Scheme 1.2). C225 and C462 will thus either be in the form of a dithiol or a neutral disulfide radical. In either case, C225/C462 will not be in the disulfide radical anion state proposed to be required for reduction of the 3'-keto-2'-deoxynucleotide (Section 8.3). Thus, the 3'-keto-2'-deoxynucleotide will not be reduced rapidly, and dissociation of this intermediate from the active site to form the furanone species in solution competes effectively with reduction.

Supporting this hypothesis is the observation that the partitioning of CIUTP between reduction and inactivation is sensitive to the pH when DTT is used the reductant. At pH 5.5, the partitioning is 200 turnovers per reduction, while at pH 8.3, reduction occurs once in every 70 turnovers (Harris et al., 1987). The partitioning is also sensitive to the concentration of exogenous reductant; changing the DTT concentration from 3 mM to 30 mM increases the ratio of reduction events to turnovers from 1 in 220 to 1 in 120 (Harris et al., 1987). The DTT dependence suggests that when the redox environment does not allow the α face cysteines to be fully reduced, the rate of reduction is diminished, and furanone formation competes more effectively. Taken together, these data support the hypothesis that halide ion is eliminated from the 2' position, rather than halo radical.

The strongest evidence against halo radical elimination mechanism comes from the modes of inactivation that are observed with 2'-halo-substituted inactivators. The observation that the •Y in the E. coli class I RNR is not reduced during inactivation (Thelander et al., 1976) requires that one of the intermediates be able to re-abstract a hydrogen from C439, regenerating the thyl radical which can, in turn, regenerate the •Y. The 3'-ketyl radical intermediate could perform this function if Cl- is eliminated.
However, if Cl• is eliminated, then no nucleotide radical species would be available to abstract a hydrogen from C439 unless Cl• could migrate from the α face of the nucleotide to the β face. This would be an unlikely scenario given the steric constraints of the enzyme active site. Thus, elimination of Cl• would trap C439 in the thiol form, preventing reformation of the R2 •Y, contrary to experimental observations.

Experiments defining the fate of the 3′ hydrogen in the course of inactivation by ClUDP (CIUTP) provide unambiguous support for loss of halide ion. The radiolabeled 2′-deoxy-3′-ketoUDP formed on incubation of RDPR with [3′-3H]-ClUDP was trapped using sodium borohydride. Chemical analysis showed that the tritium originally at the 3′ position had migrated to the 2′ position on the β face (Ator and Stubbe, 1985). This result requires a 3′ to 2′ shift of hydrogen and can be explained as abstraction of the 3′ hydrogen from CIUDP by the thyl radical (C439), followed by elimination of chloride ion to form the 3′-ketyl radical and re-abstraction of the hydrogen on C439 (Scheme 1.2) by the 3′-ketyl radical to place the hydrogen originally at the 3′ position at the 2′ position. This result is not consistent with chloro radical formation, as chloro radical would have to migrate to the β face of the nucleotide, abstract tritium from C439, and deliver it to the 2′ position of the intermediate. Thus, in the enzyme-catalyzed reaction, it is unlikely that the mechanism of C-X bond cleavage is homolytic, in contrast to the model studies of Robins (Robins et al., 1996, Robins et al., 1997).

7.4 Summary

Both simple model systems based on generation of radicals from ethylene glycol (von Sonntag and Thoms, 1970, Bansal et al., 1973, Walling and Johnson, 1974, Steenken, 1979) and more complex models that allow generation of radical at specific positions on nucleosides (Robins et al., 1996, Lenz and Giese, 1997, Robins et al., 1997) have provided insight into how ribonucleotide reductases catalyze elimination of the 2′ substituent of nucleotide substrates and inactivators. Both base catalysis (deprotonation of the 3′-hydroxyl) and acid catalysis (protonation of the 2′-hydroxyl) may be involved, although the enzymatic groups involved and the timing of protonation and deprotonation remain to be determined.
The rates of dehydration measured for both ethylene glycol-based and nucleoside-based systems suggest that the acid- or base-catalyzed dehydration is fast enough to drive the potentially unfavorable hydrogen atom abstraction that precedes it. The enzyme may employ some combination of stabilization of the carbon-centered radical (to make the hydrogen atom abstraction less thermodynamically unfavorable) and acceleration of dehydration to make this sequence of reactions kinetically competent for nucleotide reduction.

Model systems predict that elimination of 2'-halo substituents in the course of mechanism-based inactivation of ribonucleotide reductases does not require acid or base catalysis, and might occur either through elimination of halide ion or halo radical. A variety of biochemical studies establish loss of halide from C2. The differences between the reactions with mechanism-based inactivators and reduction of the normal substrate have been rationalized as arising from a difference in protonation state of the cysteines interacting with the α face of the substrate. Deprotonation of these cysteines is proposed to be critical for nucleotide reduction to proceed normally. The next section will discuss model chemistry which supports the hypothesis that deprotonation of active site thiols is required for nucleotide reduction.

8. Reduction of the 3'-Keto-2'-Deoxynucleotide Radical by Two Single Electron Transfers and Formation of 2'-Deoxynucleotide Product

8.1 One-Electron Reduction of the 3'-Keto-2'-Deoxynucleotide Radical: Model Studies

The next step proposed in the ribonucleotide reductase mechanism is the one-electron reduction of the ketyl radical by a pair of cysteine residues at the active site, forming the enolate anion of the 3'-keto-2'-deoxynucleotide and a protonated disulfide radical. The protonated disulfide radical should rapidly protonate the enolate, forming a 3'-keto-2'-deoxynucleotide and a disulfide radical anion (Scheme 1.1). Once again, model systems have provide a basis for understanding what reactions might be thermodynamically favorable and kinetically competent for nucleotide reduction. As has been the case for many of the proposed reactions in the ribonucleotide reductase mechanism, model studies
suggest that the protonation state of enzyme side chains is a critical
determinant of enzymatic reactivity.

Pulse radiolytic studies from the von Sonntag laboratory have
allowed determination of rates of reduction of formylmethyl radicals by
thiolate anions (Scheme 1.14) (Akhlaq et al, 1987).

\[
\begin{align*}
\text{HC} \dot{\text{CH}}_2 + \text{RS}^- & \rightarrow \text{H} \equiv \text{CH}_2 + \text{RS}^* \\
\end{align*}
\]

Scheme 1.14. One-electron reduction of formylmethyl radical by thiolate
anion.

The rate of reduction by dithiothreitol of the formylmethyl radical derived
from ethylene glycol was measured to be $1.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ at pH 10, and
$3.5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ at pH 11.1. In contrast, the rate constant at pH 8.4 (below the
first pK_a of dithiothreitol) is $<<10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Tamba & Quintilliani, 1984).
These results have been interpreted by von Sonntag and co-workers as
indicating that the dithiothreitol thiolate anion (or, at pH 11.1, the even
more reactive dianion) is responsible for the reduction, which would thus
occur by electron transfer rather than hydrogen atom transfer. Evidence for
electron transfer from thiols to organic radicals has also been obtained in
studies of the repair of OH• adducts of dGMP by monothiols (O’Neill,
1983). The pH dependence of this reaction indicated that the thiolate,
rather than the thiol, is the reactive species, suggesting that the reaction
proceeds by electron transfer rather than hydrogen atom transfer.

These results are in accord with the hypothesis that the protonation
state of the "bottom face" cysteines determine the partitioning between
normal turnover and inactivation, as discussed in the previous section. If
one of these cysteines can be deprotonated, it can reduce the 3'-keto-2'-
deoxynucleotide radical by electron transfer (followed by rapid proton
transfer), by analogy with the reduction of formylmethyl radical. If the
leaving group at the 2' position is not a strong enough base to deprotonate
one of these cysteines, the reduction will be slow, and the pathway leading
to inactivation will begin to predominate.

The initial product of an electron transfer between a cysteine thiolate
adjacent to a cysteine thiol and the 3'-keto-2'-deoxynucleotide radical will
be the enolate anion and a neutral, protonated disulfide radical (Scheme 1.14). However, the pKa of a neutral disulfide radical is 5.2 (Akhlaq and von Sonntag, 1987). The enolate should therefore be rapidly protonated, resulting in the formation of 3'-keto-2'-deoxynucleotide and disulfide radical anion. Protonation of an enolate by protonated disulfide radical from the α face would also account for the observation that the 2'-hydroxyl group of the substrate, in all classes of RNRs, is replaced by a solvent-derived hydrogen atom with retention of configuration (Batterham et al., 1967, David and Eustache, 1971).

These model studies suggest that this reduction will not be rate-limiting for turnover. With a reasonable effective concentration for the reducing thiolate at the active site (>1 M), the rate of this first electron transfer should be >10^8 s⁻¹, much faster than the overall rate of turnover.

8.2 Evidence from Mechanism-Based Inhibitors for Reduction from the α Face of the Substrate

As discussed in section 3.6, N₃NDP is a stoichiometric inactivator of class I RNRs in which 3' hydrogen atom abstraction initiates the inactivation process (Scheme 1.6). Formation of hydrazoic acid could proceed by one of several mechanisms. Based on model studies with 2-azido ethanol and the titanous ion/H₂O₂ oxidizing system, azide radical could be released subsequent to hydrogen atom abstraction. Thus, one possibility is that azide radical could be reduced in the enzyme active site by a cysteine residue to generate a thyl radical and hydrazoic acid (Cartwright et al, 1976, Staros et al, 1978). Alternatively, hydrazoic acid could be generated directly, through a mechanism analogous to that proposed for the substrate reduction (van der Donk et al., 1995). The 3'-keto-2'-deoxynucleotide radical is proposed to abstract a hydrogen from a cysteine at the α face of the nucleotide, forming a thyl radical (Salowe et al., 1993, van der Donk et al., 1995). Addition of the resulting thyl radical to hydrazoic acid (which, despite its pKₐ of ~5.7, must remain protonated for reaction to occur), followed by loss of N₂, is hypothesized to produce a nitrogen-centered radical, which is then proposed to react with the 3'-keto-2'-deoxynucleotide to form a radical that can be trapped and observed by EPR. Detailed analysis of the EPR spectra of this radical at 9 and 140 GHz has allowed full assignment of all three principal g values and partial
assignment of the components of the nitrogen hyperfine tensor (van der Donk et al., 1995). This information, in conjunction with EPR spectroscopy of the nitrogen centered radical generated using β-deuterium labeled cysteine-R1 (van der Donk et al., 1995) and the use of ESEEM data on selectively deuterated variants of the inhibitor (Salowe et al., 1993) allowed the proposal that the nitrogen centered radical has one of the structures shown in Scheme 1.15. The proposed initiation of this chemistry by a nucleotide-based radical abstracting a hydrogen from a thiol (C225) (Salowe et al., 1993, van der Donk et al., 1995) (Scheme 1.15a) is analogous to the one-electron reduction of an α-keto radical proposed to take place in the normal catalytic mechanism, with the exception that a hydrogen atom, rather than an electron, is transferred. However, at present the alternative mechanism (Scheme 1.15b) in which azide radical is lost initially, cannot be ruled out as an initial event required for the inactivation process. In fact, this pathway is favored based on model chemistry discussed above.

Direct experimental discrimination between the proposed electron transfer/proton transfer sequence and a hydrogen atom transfer from a "bottom face" cysteine is likely to be quite difficult. Nevertheless, the combination of model studies and the reactivity of different 2'-halo-substituted mechanism-based inhibitors provides substantial indirect evidence for this hypothesis. The model studies also suggest that this step will not be rate limiting for turnover.

8.3 One Electron Reduction of 3'-Keto-2'-deoxynucleotides: Model Studies

Protonation is also predicted to be critical for the second proposed electron transfer, from disulfide radical anion to the 3'-keto-2'-deoxynucleotide (Scheme 1.1). The reverse reaction, reduction of a disulfide to a disulfide radical anion by a ketyl radical, is highly exergonic. The one-electron reduction potential of dithiothreitol is -1.6 V, while the one-electron reduction potential of acetone is ~-2 V (Steenken, 1985, Schwarz & Dodson, 1989). Using pulse radiolysis, the reduction of oxidized DTT by the α-hydroxyalkyl radical anion derived from 2-propanol was shown by von Sonntag and co-workers to proceed with a rate constant of 4x10^8 M^{-1} s^{-1} (Akhaq et al., 1989). These results suggest that the proposed
Scheme 1.15. Mechanism of nitrogen-centered radical formation with N₃UDP. Formation of the radical observed in frozen reaction mixtures. Both of the boxed structures represent viable hypotheses for the structure of the radical.
electron transfer would not be observed if the product were the ketyl radical anion.

However, as Giese has noted (Lenz and Giese, 1997), the reduction of a ketone to a protonated ketyl radical (i.e., an α-hydroxyalkyl radical) would be thermodynamically favorable. Using the reduction potential of acetone and the pKₐ of the neutral α-hydroxyalkyl radical of 2-propanol, Schwarz and Dodson calculated the reduction potential for conversion of acetone and a proton to a protonated ketyl radical to be -1.4 V (Schwarz and Dodson, 1989). These results suggest that a disulfide radical anion can reduce a ketone, as long as the product ketyl radical is protonated. Steady-state radiolytic studies of von Sonntag (Akhalq et al., 1989), in combination with the work of Schwarz and Dodson (Schwarz and Dodson, 1989), allow an estimate of the rate constant for this process. The overall rate constant for reduction of oxidized DTT by neutral alcohol radicals is estimated to be ~100 s⁻¹ (Akhalq et al., 1989). The reduction of acetone by a disulfide radical anion is predicted to have an equilibrium constant of ~2400, based on the difference of 0.2 V between the reduction potentials of disulfide radical anion and acetone (Schwarz and Dodson, 1989). The rate constant for reduction of acetone to a neutral radical by disulfide radical anion can thus be calculated to be ~2x10⁵ M⁻¹ s⁻¹.

These results suggest that a glutamate in the active site of the enzyme is necessary to accomplish the second electron transfer. Giese has suggested that E441 of R1 serves this purpose (Lenz and Giese, 1997). This is consistent with the phenotype of the E441Q mutant R1, which catalyzes base elimination preferentially over nucleotide reduction (van der Donk et al., 1996a, Persson et al., 1997). If E441 is required to donate a proton to allow this reduction to take place, mutating this glutamate to a glutamine will allow the chemistry to proceed through the formation of the 3′-keto-2′-deoxynucleotide, but prevent the reduction of that intermediate, allowing elimination of base and polyphosphate. The phenotype of E441D R1, which produces deoxynucleotide, albeit at a rate much lower than wt R1, is also consistent with this model.
9. Abstraction of Hydrogen from a Cysteine Thiol by the 3'-deoxynucleotide Radical: Completion of Nucleotide Reduction and Regeneration of the Thiyl Radical

The final step in the proposed mechanism is the re-abstraction of hydrogen by the 3'-deoxynucleotide radical to complete nucleotide reduction and re-generate the thiyl radical for another catalytic cycle. Extensive model studies have been carried out on abstraction of hydrogen from alcohol radicals by thiols (Chatgilialoglu & Asmus, 1990). The rate constant for abstraction of hydrogen from β-mercaptopethanol by the α-hydroxyalkyl radical derived from 2-propanol is ~5x10⁸ M⁻¹ s⁻¹ (von Sonntag, 1990). The hydrogen re-abstraction step in the ribonucleotide reductase mechanism is thus expected to be rapid. Finally, while we have shown in the case of the class II RNR that reformation of the starting form of the cofactor (AdoCbl, in that case) is regenerated on every turnover, in the case of the class I enzymes, the number of turnovers effected per thiyl radical formation has not yet been established.

10. Summary and Conclusions

Mechanistic studies on enzymatic ribonucleotide reduction and on small molecule model systems for the steps in this reaction support the mechanism in Scheme 1.1. In the enzymatic systems, both the proposed thiyl radical (in the presence of physiological substrates) and nucleotide based radicals (derived from mechanism-based inhibitors) have been observed spectroscopically. Kinetic and product analysis studies with physiological substrates and mechanism-based inhibitors support the proposed mechanism. Model studies have established that the proposed mechanism is plausible from a kinetic and thermodynamic standpoint; evidence from model systems suggests that each proposed step or sequence of steps would could occur in a kinetically competent fashion in the enzymatic mechanism.

These mechanistic studies have provided insights into how enzymes effect catalysis and control reactivity. One aspect of enzymatic catalysis that the ribonucleotide reductases illustrate well is the ability of enzymes to couple a thermodynamically unfavorable step to a subsequent thermodynamically favorable step, thus assisting in the cleavage of strong covalent bonds. In the case of the L. leichmannii reductase, the homolytic
cleavage of the carbon-cobalt bond of AdoCbl (uphill by ~ 30 kcal/mol) is coupled to the thermodynamically favorable formation of a thyl radical from 5'-dA• by virtue of both reactions occurring in a single concerted step. For both E. coli and L. leichmannii reductases, coupling of the abstraction of the 3' hydrogen by the thyl radical to the rapid and thermodynamically favorable dehydration of a 3'-nucleotide radical may be essential for allowing the hydrogen atom abstraction to occur and nucleotide reduction to proceed. The ribonucleotide reductases thus provide an example of how acceleration of a sequence of single elementary steps may be selected for in evolution, in addition to acceleration of each step individually.

The ribonucleotide reductases also illustrate the importance of protonation states in biological redox chemistry. Model systems suggest that generation of a thyl radical from a •Y in the E. coli reductase requires dynamic regulation of the protonation states of the protein residues involved in electron transfer. Model reaction and the reactions of mechanism-based inactivators also show that redox reactions between the substrate and the active site thiols of the enzyme are highly dependent on the protonation states of the substrate and of active site residues.

The necessity for control of active site residue protonation states also has implications for the design of mechanism-based inhibitors of ribonucleotide reductase and other redox enzymes. Many of the mechanism-based inhibitors of ribonucleotide reductases work in part by preventing the deprotonation of the cysteines that interact with the α face of the nucleotide, thus interfering with their ability to deliver reducing equivalents. These inhibitors show that the pKₐ of a leaving group can be as important to the mechanism of inactivation as its rate of expulsion.

Much remains to be learned about the mechanisms of ribonucleotide reductases, particularly with regard to spectroscopic characterization of intermediates in intersubunit electron transfer (for the E. coli enzyme) and of substrate-based radicals in the normal catalytic mechanism. Isotopic substitutions or other perturbations in conditions may allow these intermediates to exist long enough to be trapped and characterized. If so, spectroscopic techniques such as high field EPR, ENDOR, and ESEEM should furnish important information about the structures, electronic properties, and protonation states of these
intermediates. This information, in turn, will give further insight into how these fascinating enzymes control radical reactivity.
References


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Chapter 2:

Evidence for a Kinetically Competent Thiyl Radical Intermediate
Introduction

Over ten years ago, Stubbe and co-workers formulated a new hypothesis for the role of AdoCbl in the catalytic mechanism for the ribonucleoside triphosphate reductase from *Lactobacillus leichmannii*. They hypothesized that 5'-deoxyadenosyl radical, derived from homolytic cleavage of the carbon-cobalt bond of AdoCbl forms, reacts with a protein residue to form a protein radical that initiates nucleotide reduction (Ashley et al., 1986, Stubbe, 1989). The formation of a protein radical was proposed to be a general mechanistic feature of the ribonucleotidreductases (Stubbe, 1990). Site-directed mutagenesis on both the *E. coli* (Mao et al., 1992, Stubbe, 1990) and the *L. leichmannii* (Booker et al., 1994) reductases suggested that this putative protein radical is a thyl radical. Experiments in described in this chapter use rapid mixing and quenching techniques to provide evidence that a thyl radical is formed concomitantly with carbon-cobalt bond cleavage as a kinetically competent intermediate in catalysis by the *L. leichmannii* reductase.

Many of the experiments described in this chapter take advantage of the discovery of an unusual exchange reaction in which tritium from [5'-3H]-AdoCbl is transferred to the solvent (Beck et al., 1966, Hogenkamp et al., 1968). This process, which can take place in the absence of substrate, requires an allosteric effector and the reduced form of RTPR, and occurs with a rate constant ~20% that of the rate constant for nucleotide reduction (Booker, 1994). This reaction does not lead to inactivation of the enzyme or irreversible degradation of the cofactor, which occur in the absence of substrate on the timescale of minutes, much more slowly than the exchange reaction or turnover (Yamada et al., 1971). This tritium exchange was originally hypothesized to result from reversible hydride transfer between AdoCbl and the dithiol reductant. However, further studies, described below, demonstrated that one-electron chemistry, initiated through homolysis of the carbon-cobalt bond of AdoCbl, was responsible for this reaction. These studies paralleled developments that demonstrated that carbon-cobalt bond homolysis, rather than heterolysis, is responsible for the reactivity of AdoCbl in the AdoCbl-dependent rearrangement-catalyzing enzymes such as diol dehydrase and ethanolamine ammonia lyase (Abeles & Dolphin, 1976).
The exchange reaction was studied extensively in the early 1970s by rapid kinetic techniques. Tamao and Blakley (Tamao & Blakley, 1973) used stopped-flow UV-visible spectroscopy to examine the fate of cobalt in both the exchange and reduction reactions. Their experiments were carried out by rapidly mixing the contents of one syringe containing RTPR, reductant and allosteric effector (and/or substrate) with the contents of a second syringe containing AdoCbl and effector (and/or substrate) at 37°C. These studies revealed that cob(II)alamin, rather than cob(I)alamin, was formed, with a rate constant of 35 to 45 sec⁻¹. These studies were consistent with catalysis proceeding through carbon-cobalt bond homolysis and hydrogen atom transfer, rather than heterolysis and hydride transfer.

Both hydrogen atom transfer and hydride transfer mechanisms predict the formation of 5’-dA. However, Tamao and Blakley (Tamao & Blakley, 1973) found no 5’-dA formed above the level expected given the rate of the slow, kinetically non-competent degradation of AdoCbl catalyzed by the enzyme. Thus, while these experiments had established cob(II)alamin as a kinetically competent intermediate in both the exchange reaction and nucleotide reduction, they did not resolve the question of the fate of the 5’-deoxyadenosyl moiety of AdoCbl.

The proposal of homolytic cleavage of the carbon-cobalt bond, while strongly supported by the data, led to a difficulty in explaining the exchange reaction. In the hydride transfer mechanism, reduction of the cofactor by the dithiol is required to activate it for hydride transfer to the substrate. However, in a hydrogen atom transfer mechanism, there is no need for dithiol to interact directly with AdoCbl, since homolytic bond cleavage is sufficient to activate the cofactor for further reaction. There is thus no obvious link between AdoCbl and a source of exchangable hydrogens. Tamao and Blakley (Tamao & Blakley, 1973) postulated that tritium exchange results from a side reaction involving reversible hydrogen atom abstraction by 5’-dA• from the dithiol reductant.

In an effort to characterize further the role of AdoCbl in the early steps of catalysis, Hogenkamp and coworkers (Sando et al., 1975) carried out SF UV-vis studies on a number of AdoCbl analogues with substitutions at the 5’-deoxyadenosyl moiety, and determined the ability of these analogues to function as cofactors for the exchange reaction and nucleotide reduction. They observed that while RTPR was capable of catalyzing rapid, reversible
carbon-cobalt bond homolysis with some of these analogues, other analogues only underwent the slow, irreversible homolytic cleavage earlier reported by Yamada et al. (Yamada et al., 1971). Based on this observation, they hypothesized that stabilization of the 5'-deoxyadenosyl moiety was required for the rapid formation of cob(II)alamin. As one possible source of this stabilization, they proposed that 5'-dA • might cyclize to 5', 8-cycloadenosyl radical (cycloA •), and that cycloA • might be the radical that interacts with substrate.

Around the same time as cob(II)alamin was being identified as a kinetically competent intermediate by UV-vis spectroscopy, EPR spectroscopists were beginning to investigate cob(II)alamin and its interactions with RTPR. One question of interest was whether the environment of the enzyme's AdoCbl binding site perturbed the ligation or conformation of cob(II)alamin relative to cob(II)alamin free in solution. To investigate this question, EPR spectroscopy was used to characterize cob(II)alamin bound to the enzyme in the presence of 5'-deoxyadenosine (Hamilton et al., 1971). Cob(II)alamin bound to the enzyme was found to have an EPR spectrum similar to that of free cob(II)alamin (Bayston et al., 1970), with $g_{\perp} = 2.23, g_{||} = 2.0$, and a cobalt hyperfine splitting ($A_{||}$) of 110 gauss. These studies were not directly relevant to the kinetically competent cob(II)alamin formation described by Tamao and Blakley, however, since the EPR experiments involved adding cob(II)alamin and 5'-dA to resting RTPR, rather than trapping the intermediate. A species with $g \sim 2$, known as the "doublet" for its EPR lineshape, was trapped under the conditions of turnover by freezing reaction mixtures in liquid nitrogen (Hamilton et al., 1972). While this species was of interest as a possible substrate-based radical (see Chapter 5), it was not formed in a kinetically competent fashion, and thus could not be the intermediate observed by SF UV-vis spectroscopy.

Orme-Johnson et al. (Orme-Johnson et al., 1974) succeeded in characterizing the EPR spectrum of the kinetically competent cob(II)alamin-containing intermediate using rapid freeze quench (RFQ) techniques. They discovered that although this intermediate was present in the steady-state in concentrations that would be detectable using EPR (Tamao & Blakley, 1973), it could not be trapped by plunging samples into liquid nitrogen, explaining the previous observation of only the doublet signal under turnover conditions. They succeeding in trapping this intermediate by spraying samples into
isopentane at -140° C. The EPR spectrum of the intermediate was very broad (half-width at half-height ~200 G) and had \( g = 2.119 \) and resolved hyperfine couplings on the low-field edge of 54 G. Based on the large linewidth that the freeze-quenched intermediate exhibited and the spin concentration derived from the signal intensity, Orme-Johnson et al. suggested that this intermediate might be composed of 5\(^\prime\)-deoxyadenosyl radical (5\(^\prime\)-dA•) and cob(II)alamin, the expected products of homolysis of the carbon-cobalt bond of AdoCbl. However, they also observed that the use of [5\(^\prime\)-2H]- and [5\(^\prime\)-13C]-AdoCbl failed to alter the EPR spectrum of the intermediate (Orme-Johnson et al., 1974), as might have been expected if the hyperfine splittings arising from 5\(^\prime\)-dA• were observable in the spectrum.

An early effort to simulate the X-band EPR spectrum of the freeze-quenched intermediate (Coffman et al., 1976) suggested that this species was composed of two distinct cob(II)alamin species with distorted octahedral coordination. It was proposed that a backbone carbonyl or amide or a sidechain sulfhydryl group might furnish the axial ligand for cobalt in these species. Recognizing that the stoichiometry of the reaction required formation of a radical species other than cob(II)alamin, Orme-Johnson and co-workers suggested that 5\(^\prime\)-dA• might be present, but inhomogeneously broadened to the point of undetectibility. They also hypothesized that a sulfur-based radical, too broad to be observed, might underlie the observed spectrum.

Later studies with the AdoCbl analogue Coα-[α-(aden-9-yl)]-Coβ-adenosylcobamide (pseudocoenzyme B\(_{12}\)) were designed to test the hypothesis that the EPR spectrum of the intermediate was due to a distorted octahedral geometry of cobalt(II) (Blakley et al., 1979). In pseudocoenzyme B\(_{12}\), an adenine base substitutes for dimethylbenzimidazole, and does not coordinate to cobalt. It was thus hypothesized that if the axial ligation of cobalt(II) was a major determinant of the EPR lineshape, the use of this analogue would lead to a highly perturbed EPR spectrum. In fact, the spectrum of the freeze-quenched intermediate obtained using pseudocoenzyme B\(_{12}\) was identical to that observed using AdoCbl. However, it was also observed that the EPR spectrum of Coα-[α-(aden-9-yl)]-cob(II)amide bound to RTPR exhibited the superhyperfine coupling indicative of nitrogen ligation to cobalt, suggesting either that binding to the enzyme enforces ligation of the adenine base to cobalt or that a nitrogen-containing enzyme sidechain acts as a ligand. These
results, in addition to a re-evaluation of their earlier studies, led Orme-Johnson and co-workers to conclude that the freeze-quenched intermediate consisted of two coupled paramagnetic species, rather than a mixture of two distinct cob(II)alamin species.

Coffman and coworkers identified a potential spectroscopic model for the freeze-quenched intermediate (Ghanekar et al., 1981, Pezeshk & Coffman, 1985). They found that photolysis of polycrystalline AdoCbl under anaerobic conditions produced an EPR-active species with $g = 2.119$, similar to that observed in the enzymatic intermediate. They suggested that this unusual $g$ value was attributable to delocalization of spin density between cob(II)alamin ($g_\perp = 2.23$, $g_\parallel = 2.0$) and 5'-dA• (presumably having the $g = 2.0$ typical of organic radicals). They also reported observation of a $\Delta M = \pm 2$ or "half-field" transition, characteristic of a spin-spin interaction (Wertz & Bolton, 1986), and alluded to unpublished results indicating a similar half-field transition in the EPR spectrum of the enzymatic intermediate. Examining another signal, which was observed in the presence of oxygen and ascribed to superoxocobalamin interacting with an organic radical, they used computer simulations to model the spectrum and propose a distance of 8.7 Å between the two interacting spins (Pezeshk & Coffman, 1985), suggesting that the EPR spectrum of the enzymatic intermediate could be modeled by similar means. Pilbrow also speculated that the observed EPR spectrum could be accounted for by dipolar and exchange coupling between an organic radical and cob(II)alamin (Pilbrow, 1982). However, no spectral fits were reported. The composition of the intermediate remained unknown, and its EPR spectrum was still largely unexplained.

While spectroscopic studies were unable to determine the nature of the intermediate, mechanistic studies continued to provide new insights into what the role of the intermediate might be. Kinetic isotope effect studies using [3'-3H]-NTPs (Stubbe et al., 1981) and studies using 3' [3H] 2'-chloro-2'-deoxyuridine 5'-triphosphate (C1UTP) (Stubbe et al., 1983) indicated that the 3' hydrogen of the substrate was abstracted in the course of catalysis, but also replaced at the 3' position. These experiments were consistent with the intermediate radical species, possibly 5'-dA•, acting to abstract the 3' hydrogen of the substrate.

In 1986, however, studies of Ashley et al.(Ashley et al., 1986) led to a new hypothesis for the identity of the intermediate. The hypothesis that 5'-
dA acts directly to abstract the 3' hydrogen of substrate predicts that if a [3'-3H]-NTP is reduced under single turnover conditions, tritium will initially be incorporated into 5'-dA. When the putative 3' radical re-abstracts a hydrogen from 5'-dA, allowing the re-formation of AdoCbl, there will be at most a 1/3 chance that the tritium label will be replaced, as the three positions of the 5'-dA methyl group are equivalent. In fact, due to the kinetic isotope effect, tritium is likely to be re-abstracted much less than 1/3 of the time. The hypothesis that 5'-dA acts directly to abstract the 3' hydrogen of substrate thus predicts that in a single turnover of [3'-3H]-NTP, a substantial fraction of the tritium label will appear in re-isolated AdoCbl compared to product dNTP. In fact, the tritium initially in the substrate is found either in the product or in re-isolated substrate after a single turnover. This result requires the proposal that the function of AdoCbl is to generate a protein radical on RTPR from a monoprotic amino acid residue within the active site of the enzyme.

Site-directed mutagenesis studies have provided strong support for the involvement of a protein radical in both the L. leichmannii (Booker et al., 1994) and the E. coli reductases (Mao et al., 1992). In both cases, a cysteine residue is implicated as necessary for catalysis, suggesting that the protein radical is a thyl radical. Cysteine 439 of the R1 subunit appears to be essential for catalysis in the E. coli enzyme. Correcting for contaminating wild-type activity, the specific activity of the C439S mutant of the E. coli R1 subunit is <0.3% of the wild-type (the lower limit of detection of the assay used). In addition, C439S R1 acts as a competitive inhibitor of the wild-type R1's interaction with the R2 subunit. The recent three-dimensional structure of the R1 subunit (Eriksson et al., 1997, Uhlin & Eklund, 1994) bolsters the hypothesis that C439 forms the thyl radical that initiates catalysis by abstracting the 3' hydrogen of nucleotide substrates, showing this residue is in the substrate binding region, positioned to allow interaction with the 3' hydrogen.

A short sequence homology between the E. coli and L. leichmannii reductases was used to identify C408 of the L. leichmannii reductase as a likely homolog of C439 in the E. coli reductase. Site-directed mutagenesis experiments show that the C408S mutant is catalytically inactive with respect to both nucleotide reduction (<0.002% of wild-type) and the exchange reaction.
Scheme 2.1. The exchange reaction and nucleotide reduction.
(<0.006% of wild-type) (Booker et al., 1994). These data suggest that C408 forms a thiol radical and abstracts the 3' hydrogen from the substrate during catalysis.

These experiments support the mechanism in Scheme 2.1. In this mechanism, both the exchange reaction and nucleotide reduction begin with carbon-cobalt bond homolysis and formation of a thiol radical. The exchange of the 5' hydrogens of AdoCbl comes about on re-abstraction of a hydrogen from 5'-dA by the thiol radical, which puts this hydrogen (or tritium) in an exchangable position. If the thiol radical instead abstracts the 3' hydrogen of the substrate, nucleotide reduction is initiated. The mechanism in Scheme 2.1 suggests that the paramagnetic intermediate discovered by Orme-Johnson, Beinert, and Blakley consists of a thiol radical coupled to cob(II)alamin (the boxed intermediate in Scheme 2.1).

The experiments described in this chapter were designed to investigate the existence of the thiol radical-containing intermediate pictured at the center of Scheme 2.1. This intermediate consists of cob(II)alamin (which had already been identified (Tamao & Blakley, 1973)), 5'-dA, and the protein-based thiol radical. Rapid acid quench experiments using [5'-3H]-AdoCbl provided evidence for the intermediacy of 5'-dA in the exchange reaction. RFQ EPR experiments using [β-2H2]-cys-labeled RTPR provided evidence for the intermediacy of a thiol radical both in the exchange reaction and in nucleotide reduction.
Materials and Methods

Materials and General Methods

Ampicillin, protamine sulfate, ATP, dGTP, dimethylglutaric acid, and G-25 Sephadex were purchased from Sigma. Dithiothreitol was from Mallinkrodt. DE-52 anion-exchange resin was purchased from Whatman. Biogel HTP hydroxylapatite resin and Dowex AG 1-X2 were purchased from BioRad. \([3,3'^{-2}H_4]\)-cystine and \(D_2O\) were from Cambridge Isotope Laboratories. Ultrafiltration apparatus and membranes were purchased from Amicon. Sep-Paks were from Millipore. Reacti-Vials and \(N\)-methyl-\(N\)-(t-butyldimethyl-silyl)trifluoroacetamide were from Pierce. Scint-A, Soluene 350, and Hionic-Fluor were purchased from Packard.

\(E.\ cola\) thioredoxin (TR) and thioredoxin reductase (TRR) were purified as previously described (Lunn et al., 1984, Russel & Model, 1985). \(E.\ cola\) JM15 was a gift from John Kozarich (Merck Research Laboratories, Rahway, N. J.).

All operations involving AdoCbl were carried out under red light or in the dark.

Purification of Recombinant RTPR

The method described previously (Booker, 1994, Booker & Stubbe, 1993) was used with minor modifications to accommodate larger scale. Glycerol stocks of HB101/pSQUARE were streaked onto LB plates supplemented with ampicillin (50 \(\mu g/ml\)), and the plates were incubated at 37°C. A single colony was used to inoculate 5 ml of LB medium supplemented with ampicillin (50 \(\mu g/ml\)). A 1 ml aliquot of the saturated culture was used to inoculate 1.5 L of LB medium supplemented with ampicillin (50 \(\mu g/ml\)). The cultures were incubated at 37 °C with shaking to saturation (~14 h). The bacteria were pelleted by centrifugation at 9,000xG for 30 minutes, typically yielding ~20 g of cell paste. The pelleted bacteria were either used immediately or frozen with liquid nitrogen and stored at -80°C. All subsequent steps were performed at 4 °C.

The pelleted bacteria (~20 g) were resuspended in 100 ml of lysis buffer (200 mM potassium phosphate, 1mM EDTA, pH 7.2) and lysed by a single passage through a French pressure cell at 16,000 psi. \(\beta\)-Mercaptoethanol (\(\beta ME\)) (to 1 mM) and PMSF (to 0.1 mM) were added to the lysate. The lysate was centrifuged for 30 minutes at 16,000xG. Protamine sulfate (1% in buffer
A, 20 mM potassium phosphate, 1 mM EDTA, 1 mM βME, pH 7.2) was added to the supernatant over 15 minutes to a final concentration of 0.25%, after which the cloudy solution was stirred for 20 minutes. The solution was centrifuged at 9,000xG for 30 minutes. EDTA (to a final concentration of 5 mM) was added to the supernatant. Ammonium sulfate was added over 20 minutes to a final concentration of 243 g/L (40% saturation). The mixture was stirred for an additional 50 minutes, then centrifuged at 16,000xG for 30 minutes. The ammonium sulfate pellet was stable overnight at 4 °C.

The pellet was redissolved in a minimal amount (~10 ml) of buffer A and loaded onto a Sephadex G-25 column (1.6 x 100 cm) equilibrated in buffer A. The protein was eluted with buffer A. The protein was then loaded onto a DE-52 column (6x12 cm) equilibrated in buffer A. The column was washed with buffer B (100 mM potassium phosphate, 1 mM EDTA, 1 mM βME, pH 7.2) while collecting fractions (10 ml). When most of the initial peak of protein had eluted (as judged by the A280 of the fractions decreasing from a maximum of ~1.7 to ~0.5), the column was washed with buffer C (150 mM potassium phosphate, 1 mM EDTA, 1 mM βME, pH 7.2). The peak that eluted contained the majority of the RTPR. After elution of this peak, the remaining protein was eluted with buffer D (500 mM potassium phosphate, 1 mM EDTA, 1 mM βME, pH 7.2). The second peak (RTPR-containing fractions) was exchanged by Amicon ultrafiltration (100 fold net dilution of salt, PM30 membrane) into buffer E (10 mM potassium phosphate, 1 mM EDTA, 1 mM βME, pH 6.8). The protein was then loaded onto a hydroxylapatite (Biocel HTP) column (6x12 cm) equilibrated in buffer E. The column was washed with 500 ml of buffer E, and the protein was eluted by running a 500 x 500 ml gradient of buffer E to buffer F (200 mM potassium phosphate, 1 mM EDTA, 1 mM βME, pH 6.8). Fractions were collected, and protein-containing fractions (eluting from approximately 40 to 80 mM, as judged by A280) were pooled and concentrated by Amicon ultrafiltration (PM30 membrane) to approximately 20 ml. The protein was dialyzed against 4 l of buffer G (0.1 M sodium citrate, 1 mM EDTA, 1 mM DTT, 0.025% NaN3, 20 % glycerol, pH 5.6) for nine hours, after which it was divided into 500 μl aliquots, placed in microcentrifuge tubes, and quick-frozen with liquid nitrogen. The protein was stored at -80 °C. The specific activity of RTPR was 1.4-1.5 μmol dATP formed per minute per mg of enzyme, assaying spectrophotometrically using the consumption of NADPH (Booker, 1994).
Pre-Reduction of RTPR

Pre-reduction of RTPR was carried out by incubating the enzyme (300 nmol) with 30 mM DTT in sodium dimethylglutarate buffer (100 mM, pH 7.3) for 30 min at 37 °C. The mixture was loaded onto a Sephadex G-25 (1.5 X 8 cm) equilibrated in in sodium dimethylglutarate buffer (5 mM, pH 7.3). RTPR eluted in the column buffer. Fractions containing RTPR were pooled and concentrated in a Centricon 30 device.

Deoxygenation of Solutions for SF-UV vis and Rapid Freeze Quench Experiments

The materials (0.5-2 ml) were placed in a septum-sealed 10 ml round-bottom flask equipped with a stirrer. If 100 µl or less was to be used, a septum-sealed Eppendorf tube was used, and the stirrer was omitted. The container was purged with argon (blown over the solution) for 20 min while stirring at 0 °C. All materials were transferred via gas-tight Hamilton syringe.

Formation of Cob(II)alamin by RTPR

An Applied Photophysics DX.17MV spectrophotometer was used for the SF spectroscopy. Loading syringes and sample lines were filled with 50 mM dithionite 12 h prior to the actual experiments. They were then flushed with 20 ml of 0.2 M sodium dimethylglutarate, pH 7.3, which had been deoxygenated by bubbling argon through it for 2-3 h. Reaction mixtures were transferred to the loading syringes via gas-tight syringe. Loading syringes, the windows of the drive unit, and exposed lines were covered in foil after addition of the reaction mixtures. Argon was bubbled through the bath that controlled the temperature of the sample holding unit for 3 h preceding data acquisition and throughout the course of the experiment. In later experiments, it was shown that samples that were not anaerobic gave identical rates and amounts of cob(II)alamin formation within the first 1 s of reaction.

Pre-reduced RTPR (100 µM), 20 µM TR, 1 µM TRR, 2 mM NADPH, and 1 mM dGTP in 200 mM sodium dimethylglutarate, pH 7.3 were placed in one syringe and mixed with an equal volume from a second syringe of the same reaction buffer containing 100 µM AdoCbl and 1 mM dGTP. The formation of cob(II)alamin was measured by monitoring change in A525 at 37°C.
Preparation of 5'δ,8-Cycloadenosine

5'δ,8-Cycloadenosine was prepared by a modification of the method of Brady and Barker (Brady & Barker, 1961). AdoCbl (2 μmoles, as a 200 μM solution in H₂O) was degassed by three cycles of freeze/pump/thaw. The solution was photolyzed using a 90W lamp for 3 h, with the lamp was placed approximately 5 cm from the flask. The reaction mixture was loaded onto C18 Sep-Paks (2 ml of reaction mixture/Sep-Pak), and 5'δ,8-cycloadenosine was eluted with 20% methanol (10 ml/Sep-Pak). The partially purified 5'δ,8-cycloadenosine was evaporated (to a final volume of 9 ml) purified by reversed phase-C18 HPLC (Econsil C18 10U, 4.6x250 mm), using an isocratic elution with 20% MeOH. 5', 8-cycloadenosine eluted at 9 min.

Synthesis of [5'-3H]-AdoCbl

N6-Benzoyl-2',3'-O-isopropylidene adenosine (Ad) was prepared as reported (Jenkins et al., 1976). This was converted to N6-benzoyl-2',3'-O-isopropylidene Ad 5'-aldehyde via a Moffatt oxidation (Ranganathan et al., 1973). The aldehyde hydrate was reduced with Na3BH4 (Dupont NEN, 500 μCi, 13.8 μCi/μmol) (Gleason & Hogenkamp, 1971), and the benzoyl protecting group was removed using the method of Gaudemer et al. (Gaudemer et al., 1981).

Deprotection of [5'-3H]-2',3'-O-isopropylidene Ad (160 mg) was achieved by dissolving it in 9:1 (v/v) aqueous trifluoroacetic acid (10 mL) and stirring for 3.5 h. The solvent was then removed in vacuo leaving an oil, which was re-dissolved in 7:3 (v/v) methanol/H₂O. The solution was neutralized with 10 mL Dowex AG 1-X2 (50-100 mesh, hydroxide form). The resin was washed with 1.1 L of 7:3 methanol/H₂O until all the Ad had been removed as evaluated by A260. The combined washings were pooled and concentrated in vacuo to give a white powder. The yield of [5'-3H]-Ad was 94% (specific activity: 2x10^8 cpm/μmol).

[5'-3H]-5'-Chloro-5'-dA was prepared from [5'-3H]-Ad by reaction with thionyl chloride (Robins et al., 1991). [5'-3H]-AdoCbl was prepared from [5'-3H]-5'-chloro-5'-dA by reduction of hydroxocobalamin to cob(I)alamin, followed by nucleophilic displacement of the 5'-chloride (Gaudemer et al., 1981). Partial purification of [5'-3H]-AdoCbl was carried out by chromatography on Dowex 50W X2. AdoCbl-containing fractions (as judged
by UV-visible spectroscopy) were pooled, extracted into phenol, back-extracted into water, concentrated to 1 – 3.5 mM and stored in foil-wrapped containers, at -20 °C (Gleason & Hogenkamp, 1971). The yield of [5'-3H]-AdoCbl (specific activity 3.3x10^8 cpm/μmol) was 55 mg (5% from N6-benzoylated 2', 3'-isopropylidene adenosine). The apparent increase in specific activity from [5'-3H]-Ad to [5'-3H]-AdoCbl is probably due to non-radioactive impurities in [5'-3H]-Ad.

**Fate of the 5'-Deoxyadenosyl Moiety of [5'-3H]-AdoCbl**

A Kin-Tek model RQF-3 rapid quench apparatus was used. To allow reproducible loading in dim light, the sample loops were loaded using Luer-tip gas-tight syringes that had been calibrated so that the displacement required to fill each sample loop was marked on the barrel of the syringe.

Pre-reduced RTPR (100 μM), 20 μM TR, 1 μM TRR, 2.6 mM NADPH, 1 mM dGTP and 100 mM sodium dimethylglutarate, pH 7.3 were placed in one syringe and rapidly mixed at 37°C with an equal volume from a second syringe containing [5'-3H]-AdoCbl (100 μM, 2x10^7 cpm/μmol), and 1 mM dGTP in the reaction buffer. After the specified time, the reaction was quenched with 2 percent perchloric acid (60 to 220 μl), collected in tubes containing 5'-dA (55 nmol) and 5', 8-cycloadenosine (15 nmol). Immediately after quenching, samples were neutralized with equal volumes of 0.4 M KOH and 0.5 M sodium dimethylglutarate, pH 7.3 (50 to 200 μl each). Neutralized samples were immediately quick-frozen in liquid nitrogen and stored on dry ice. The neutralized reaction mixtures were stable at -20 °C for up to a week. A zero time point was generated by mixing the contents of Loop B with water.

For analysis, each sample was thawed and loaded onto a C18 Sep-Pak. 3H2O was eluted with H2O (3 ml) and analyzed by scintillation counting. 5'-dA and 5', 8-cycloadenosine were eluted with 50% CH3OH/50% H2O (10 ml) and the solvent removed by lyophilization. Each lyophilized mixture was dissolved in 450 μl of H2O and analyzed by reversed phase-C18 HPLC (Econosil C18 10U, 4.6x250 mm), with a linear gradient. Solvent A was H2O, and solvent B was MeOH: 0 to 5 min, 0 to 20% B; 5 to 24 min, 20% B; 24 to 34 min, 20% to 100% B; 34 to 40 min, 100% B. The flow rate was 1 ml/min, the elution profile was monitored by A260, and 1 ml fractions were collected. Adenine, 5', 8-cycloadenosine, and 5'-dA eluted at 12, 19 and 21 minutes, respectively. UV-vis spectroscopy (ε260 =13400 M^-1 cm^-1, ε264 = 15100 M^-1
cm⁻¹, and ε₂₆₀ = 15200 M⁻¹ cm⁻¹, respectively) was used to measure the recovery, and radioactivity was analyzed by scintillation counting (8.5 ml Scint-A per milliliter of eluate).

The protein pellets were washed with 2 portions (100 µl each) of water and re-pelleted in an Eppendorf microcentrifuge. The pellets were suspended in 100 µl of water, and an alkaline solubilizing agent (Soluene-350, 1 ml) was added to each tube. The tubes were sealed and incubated at 50°C for 3 hours. The samples were then mixed with scintillation fluid (Hionic Fluor, 10 ml) and counted.

*Preparation of [5'-²H₂]-AdoCbl*

This material was prepared by a modification of the procedure of Hogenkamp *et al.* (Hogenkamp *et al.*, 1968). The following components were combined in a final volume of 1 ml and lyophilized: 100 mM sodium dimethylglutarate (pH 7.3), 2 mM dGTP, 20 µM TR, 1.9 mM NADPH, and 24 nmol RTPR. The residue was dissolved in 1 ml D₂O (99%), and TRR and AdoCbl (final concentrations of 1 µM and 100 µM) were added to start the reaction, which was incubated at 37 °C for 15 min. The solution was then loaded onto a C18 Sep-Pak, washed with water, and [5'-²H₂]-AdoCbl was eluted with 100 % methanol. The AdoCbl was then further purified by C18 RP-HPLC using a 10 min isocratic elution with H₂O, followed by a 30 min linear gradient to 100% MeOH (flow rate=1.0 ml/min). The appropriate fractions were pooled and the solvent removed *in vacuo*.

[5'-²H₂]-AdoCbl and unlabeled AdoCbl were characterized by electrospray mass spectrometry. The most abundant ion, [M + 2H]²⁺, for AdoCbl, has a m/z of 789.95, in good agreement with the calculated value of 790.33. [5'-²H₂]-AdoCbl had an m/z of 790.93. The shift in m/z of one unit corresponds to a change of two mass units, since the charge of the ion is +2. A shoulder in the spectrum of the deuterated sample was observed at m/z 790.4 (a shift of one mass unit from the unlabeled material), accounting for ~10% of the total abundance. This shoulder was assigned as the monodeuterated compound. These results show that the deuterated sample consists of 90% dideuterated AdoCbl and 10% monodeuterated AdoCbl, with an overall isotopic incorporation of 95%.
Purification of $[\beta^2H\text{-cysteine}]-\text{RTPR}$: Expression in JM105

The method of Hibler, et al. was used (Hibler et al., 1989). M9 medium was supplemented as follows: FeCl$_3\cdot$6H$_2$O, 33 g/L; CuSO$_4\cdot$6H$_2$O, 0.16 g/L; CoCl$_2\cdot$6H$_2$O, 0.18 g/L; Na$_2$EDTA·2H$_2$O, 38 g/L; Zn(OAC)$_2$, 0.18 g/L; thiamine, 0.05 g/L; biotin, 0.05 g/L; amino acids (Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val, Trp), 0.1 g/L of each, added as an autoclaved suspension with the exception of Trp, which was sterile filtered; ampicillin (50 μg/ml).

A 5 ml saturated culture of JM105/pSQUIRE in LB medium supplemented with ampicillin (50 μg/ml) was used to inoculate a 25 ml of the supplemented M9 medium described above. These cultures were grown to saturation with shaking at 37 °C, and the entire 25 ml culture was used to inoculate 500 ml of supplemented M9 medium. This culture was incubated with shaking at 37° C to 1 O.D., after which [3,3'-2H$_4$]-cystine (400 mg in 4 ml of 0.2 M HCl) was added to the medium. After 30 minutes, IPTG was added to 1 mM. The cultures were grown to saturation (~2 O.D.) Purification was carried out as described by Booker and Stubbe (Booker & Stubbe, 1993) with the exception that the hydroxylapatite chromatography step was omitted.

Purification of $[\beta^2H\text{-cysteine}]-\text{RTPR}$: Expression in the Cysteine Auxotroph JM15

JM15 was made competent by the calcium chloride method described by Sambrook, et al. (Sambrook et al., 1989). Plasmid pSQUIRE was isolated from HB101/pSQUIRE using a Qiagen plasmid isolation kit. Transformation of JM15 with pSQUIRE was carried out according to a modification of the procedure in Sambrook, et al. (Sambrook et al., 1989). The frozen competent JM15 cells were thawed and placed on ice for 30 min. 10 ng of plasmid pSQUIRE was added to the competent cells, and the cells were incubated on ice for 30 min. The cells were incubated at 42 °C for 90 s and placed on ice. LB (800 μl) was added, and the cells were incubated at 37 °C (water bath) for 45 min. LB plates supplemented with ampicillin (50 μg/ml) were spread with aliquots of the cells (50-600 μl), and incubated at 37°C.

JM15 was grown according to the procedure of Cheng, et al. (Cheng et al., 1995). The medium contained salts, amino acids, and nucleic acids as follows: Na$_2$HPO$_4$ (7 g/L); K$_2$HPO$_4$ (3 g/L); NH$_4$Cl (1 g/L); NaCl (1 g/L); NaOH (0.5 g/L); Ala (0.5 g/L); Asp (0.4 g); Asn (0.4 g/L); Gln (0.4 g/L); Glu (0.74 g/L);
Gly (0.55 g/L); His (0.15 g/L); Ile (0.23 g/L); Arg (0.4 g/L); Leu (0.23 g/L); Lys-HCl (0.42 g/L); Met (0.25 g/L); Phe (0.23 g/L); Pro (0.1 g/L); Ser (2.1 g/L); Thr (0.23 g/L); Tyr (0.17 g/L); Val (0.23 g/L); adenine (0.5 g); guanosine (0.65 g); thymine (0.2 g); uracil (0.5 g); and cytosine (0.2 g). The medium containing the above was autoclaved. After autoclaving, the following were added as solutions in deionized water through 0.22 micron filters: glucose (5 g.); MgCl₂ (0.21 g); CaCl₂ (0.014 g); Trp (0.1 g); thiamine (0.1 g); nicotinic acid (0.1 g). Before inoculation, [3,3'-²H₄]-cystine (0.12 mg/ml) was added as a suspension in 0.2 N HCl.

To ensure that JM15, rather than a contaminating strain, had been transformed, JM15/pSQUIRE was used to inoculate both 5 ml of the above medium containing both cysteine and ampicillin and 5 ml of the above medium lacking cysteine. As expected, JM15/pSQUIRE grew in the presence of ampicillin, and failed to grow in the absence of cysteine, thus verifying that the transformed strain both contained the plasmid and exhibited cysteine auxotrophy.

A 1 ml aliquot of a saturated JM15/pSQUIRE culture was used to inoculate 50 ml of the medium described above (containing unlabeled cysteine). When the 50 ml culture had reached 1.5 O.D., 1 ml was used to inoculate a 500 ml of medium containing the labeled cystine. IPTG was added to 1 mM when the culture reached 1 O.D. The cells were harvested when the culture reached saturation (~12 h), quick-frozen, and stored at -80 °C. Four 500 ml cultures yielded 3.8 g of cells. Purification was carried out as described by Booker and Stubbe (Booker & Stubbe, 1993) with the exception that the hydroxylapatite chromatography step was omitted.

**Determination of the Isotopic Incorporation of [β-²H₂-cys]RTPR**

Because cysteine residues are recovered in poor yield from acid hydrolysis of proteins, RTPR was labeled with iodoacetamide before hydrolysis. [β-²H₂-cys]-RTPR (0.09 mg, 1 nmol) was reduced with DTT (5 mg, 32 μmol) in 8 M guanidine hydrochloride in 0.3 M Tris-HCl, 5 mM EDTA, pH 8.0 (1 ml) at 37°C for 2 h. Iodoacetamide (74 mg, 400 μmol) was added, and the mixture was incubated in the dark at room temperature for 30 min. βME (30 μl) was added to quench the iodoacetamide, and the reaction mixture was dialyzed against NH₄HCO₃ (1 L) overnight.
The labeled protein was lyophilized and submitted to the MIT Biopolymers Laboratory for acid hydrolysis. The hydrolysis was carried out in 6 N HCl, 0.1% phenol at 150°C for 1 h, after which the hydrolyzed protein was lyophilized.

The GC-MS analysis of the hydrolysis was carried out by the procedure of Mawhinney, et al. (Mawhinney et al., 1986). The lyophilized hydrolysate was taken up in EtOH (25 µl), and transferred to a dry Reacti-Vial (Pierce), which was sealed with a Teflon-silicone cap. The hydrolysate was dried by adding benzene (200 µl) to the vial and blowing nitrogen over the solution to evaporate the solvent. DMF (5 µl) and N-methyl-N-(t-butyldimethylsilyl)trifluoroacetamide (25 µl) were added to the vial via syringe. The reaction mixture was incubated in a heating block at 70 °C for 20 min, then at room temperature for 90 min.

Aliquots (1-2 µl) of the derivatized amino acid mixture were injected onto a GC-MS (Hewlett-Packard 5890 Series 2 GC with 5971 Series MS detector) equipped with an HP-1 column. Mass spectral monitoring was begun 10 min after injection so that the solvent and silylating reagent peaks would not be admitted into the mass spectrometer. The derivatized amino acids were eluted with the following program: 100 °C for 2 min after injection, followed by a linear gradient from 100 °C to 280 °C over 20 min. Authentic S-carboxymethylcysteine (Sigma) was used to prepare tBDMS-S-carboxymethyl-cysteine (tBDMS-SCM-cys) and determine its GC retention time (24.5 min with the gradient used). For detection of S-carboxymethylcysteine, mass spectral detection was carried out in the single ion monitoring mode.

RFQ EPR: Evidence for Thiyl Radical Formation

An Update Instruments System 1000 was used. X-band EPR tubes were from Wilmad. Q-band EPR tubes and the corresponding funnels were as described by Burdi et al. (Burdi et al., 1996).

RTPR (525 µM) and 50 µM TR, 3 µM TRR, 1.7 mM NADPH, 1 mM dGTP in 100 mM sodium dimethylglutarate, pH 7.3 were placed in one syringe and mixed (using a ram velocity ram velocity of 1 cm/sec) with an equal volume from a second syringe of the same reaction buffer containing 400 µM AdoCbl and 1 mM dGTP in the reaction buffer, followed by quenching in isopentane (-140°C). In experiments using deuterated protein,
[β-²H₂-cysteine]-RTPR (250 μM) was mixed with AdoCbl (300 μM), with other reaction conditions identical to those used for unlabeled RTPR. In experiments using deuterated cofactor, RTPR (250 μM) was mixed with [5'-²H₂]-AdoCbl (300 μM), with other reaction conditions identical to those used for unlabeled RTPR. For experiments under turnover conditions, reaction and quenching conditions were identical to those described for unlabeled AdoCbl and RTPR, except that ATP (1 mM) was present in both syringes, and the reaction was quenched at 20 ms (the time at which cob(II)alamin formation was maximal, as judged by SF UV-vis spectroscopy, see Chapter 5).

The X-band EPR spectra were recorded on a Bruker ESP-300. The cooling system consisted of the quartz sample holder and temperature controller from a Bruker VT 1000 system connected to a transfer line delivering N₂ (at 25 l/min) through a copper heat exchanger maintained at 77 K with liquid N₂. The samples were maintained at 100 K. For recording at temperatures below 100 K, an Oxford Instruments ESR 900 continuous flow liquid helium cryostat was used. The EPR spectra were recorded with spectrometer frequency 9.41 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 4 gauss; time constant, 1.3 s; and scan time, 671 s.

The spin concentration of the samples was quantitated by comparing the signal intensity of the RFQ EPR samples to a CuSO₄ standard (Ballou & Palmer, 1970, Malmström et al., 1970). In these experiments, isopentane is packed into the tubes along with frozen sample, resulting in variable amounts of dilution of spin concentration (Ballou & Palmer, 1970). Based on packing of CuSO₄, a dilution factor, or "packing factor," of 0.7 was used in the calculation of spin concentration for comparison with SF UV-vis spectroscopic results.

Temperature dependences for signal intensity were measured both for the cob(II)alamin-(5'-dA)-RTPR complex (Hamilton et al., 1971) and the intermediate reported by Orme-Johnson, et al. (Orme-Johnson et al., 1974). The temperature was allowed to equilibrate for 10 min before recording spectra. The cob(II)alamin sample was prepared by thawing a rapid freeze-quench sample under argon, incubating the tube under argon at 37 °C for 3 h, and re-freezing the reaction mixture. Power saturation curves were also recorded at temperatures ranging from 5 to 20 K.
Continuous-wave Q-band EPR spectra were recorded by Jean-Paul Willems in the laboratory of Prof. Brian Hoffman at Northwestern University on a modified Varian E109 EPR spectrometer equipped with an E110 35 GHz microwave bridge in the dispersion mode. A 100 kHz field modulation was used under conditions of rapid passage (Werst et al., 1991). The spectra were recorded with spectrometer frequency 34.93 GHz; temperature 2 K; modulation frequency 100 kHz; modulation amplitude 2 G; time constant 64 ms; scan time 240 s.

EPR spectra in parallel mode were recorded with the assistance of Dr. Ralph Weber of Bruker Instruments using a Bruker DM9304 resonator. The samples were the same as those used for perpendicular mode EPR. The spectra were recorded with spectrometer frequency 9.463 GHz; temperature 10 K; modulation frequency 100 kHz; modulation amplitude 4 G; time constant 327 ms; scan time 335 s.

Reversibility of Formation of the Paramagnetic Intermediate: Effect of Thawing and Freezing

A freeze-quench sample quenched at 175 ms under exchange reaction condition was septum-sealed and purged with argon while still frozen. The tube was kept under passive argon and plunged quickly into a beaker of ice water (1 l). The septum seal was blown off, and quickly replaced. An aliquot of the sample was removed and assayed for RTPR activity. The EPR tube was then dried and slowly immersed in liquid nitrogen to re-freeze the contents. An EPR spectrum was recorded at 100 K as described above. The sample was then thawed as described above, incubated at 37 °C for 3 h, and re-frozen. The EPR spectrum was again recorded, and the temperature dependence of the signal intensity was measured. The sample was observed to be orange, with a small layer of pink material at the top.
Results

Fate of the 5'-Deoxyadenosyl Moiety of [5'-3H]-AdoCbl

The mechanism in Scheme 2.1 predicts that AdoCbl reacts with RTPR to form cob(II)alamin and 5'-dA, with the oxidation of C408 to a thyl radical. The predictions this mechanism makes about the fate of AdoCbl can tested with SF UV-vis spectroscopy to monitor cob(II)alamin formation, rapid chemical quench with acid to monitor the formation of 5'-dA or 5'-dA•, and RFQ EPR spectroscopy to monitor the cob(II)alamin and thyl radical formation. We have repeated the SF UV-vis experiments of Tamao and Blakley, using the TR, TRR and NADPH reducing system in place of the dihydrolipoate used in their experiments, and obtained a similar rate constant for cob(II)alamin formation by SF (42 s⁻¹) (Fig. 2.1).

In a parallel set of experiments, the fate of the axial ligand was established with [5'-3H]-AdoCbl in the second syringe and a third syringe containing 2 percent perchloric acid to rapidly quench the reaction. Less than 0.01 equivalent of tritium co-eluted with cycloA in any sample, and the precipitated protein pellets contained <0.004 equivalent of tritium each. Only 5'-dA and H₂O were detected as tritium-labeled species, and the rate constant for their formation ([5'-3H]-dA + ³H₂O) was identical to that observed for formation of cob(II)alamin (Fig. 2.1). Stoichiometric isolation of 5'-dA relative to cob(II)alamin supports the mechanism in Scheme 2.1 and provides direct evidence for the fate of the adenosyl moiety of AdoCbl during the RTPR-catalyzed reaction.

An apparent first-order rate constant for tritium washout of ~0.3 eq. of tritium/eq. of protein s⁻¹ can be calculated from the time course in Fig. 2.1. This is comparable to the value of 0.16 s⁻¹ determined for tritium washout in the steady state (Booker, 1994).

 Determination of the isotopic incorporation of [3-²H₂-cys]-RTPR isolated from JM105/pSQUIRE

Because JM105/pSQUIRE is not a cysteine auxotroph, incorporation of deuterated cysteine into RTPR was not guaranteed to be complete in this expression system. In order to interpret the results of RFQ EPR experiments using [β-²H₂-cys]-RTPR isolated from JM105/pSQUIRE, it was thus necessary to measure the incorporation of deuterated cysteine into the protein. As
Fig. 2.1. Formation of cob(II)alamin and 5'-dA by RTPR. RTPR (100 µM), TR/TRR/NADPH, and dGTP (1 mM) were placed in one syringe and mixed with an equal volume from a second syringe of the same reaction buffer containing 100 µM AdoCbl (or [5'-3H]-AdoCbl for rapid acid quench experiments) and 1 mM dGTP. The dotted trace shows the equivalents of cob(II)alamin formed, calculated directly from the SF absorbance trace using the difference in extinction coefficients between AdoCbl and cob(II)alamin (4800 M⁻¹ cm⁻¹). The data is the average of three trials. The solid line is the fit to a single exponential. The circles represent equivalents of 5'-dA formed, and the triangles represent equivalents of ³H₂O as measured subsequent to rapid chemical quench. The average of 2–3 replicate experiments is shown. A linear fit is shown to the ³H₂O data.
previously reported by Mawhinney et al. (Mawhinney et al., 1986), the GC-MS of tBDMS-SCM-cys prepared from a hydrolysate of unlabeled RTPR showed an M-57 ion at m/z=464 (resulting from loss of the t-butyl moiety) and an M-159 ion at m/z=362 (resulting from loss of the tBDMS ester moiety, including the carbonyl group), both of which included C3 of cysteine and its two hydrogens. Thus, the MS of tBDMS-SCM-cys from a total hydrolysate of \([\beta-2H_2\text{-cys}]\)-RTPR (prepared from growth of JM105/pSQUIRE in minimal media supplemented with 3,3'-2H_4-cystine) was predicted to exhibit a shift of two mass units in both the M-57 ion and the M-159 ion.

The observed MS (Fig. 2.2) shows that the most abundant ion in the M-57 family is at m/z 466, rather than 464. However, the presence of the ions at m/z=464 and 465 indicate that non-deuterated and/or monodeuterated material are also present. Using the calculated isotopic abundances for the M-57 ion, the abundance of the unlabeled ion was determined, and subtracted from the total abundance to give the abundance of the di-deuterated species. Since the abundance of the ion at m/z=465 was, to within experimental error, that predicted for the m/z=465 peak arising from an unlabeled M-57 ion due to natural abundance of heavier isotopes, it was determined that the monodeuterated species was not present in significant amounts. Based on the analysis of the M-57 ion, the didodeuterated species composed 60±7% of the tBDMS-SCM-cys isolated. The M-159 ion at m/z=362 gave similar results. As an internal control, the isotopic incorporation of the M-R ion (the fragment arising from loss of the amino acid side chain), with m/z=302 as the most abundant ion, was determined. As expected, the family of MS peaks arising from this ion was for unlabeled and deuterated tBDMS-SCM-cys, as this ion has lost the deuterium-containing side chain moiety. Finally, no differences in mass spectra relative to the unlabeled amino acids were observed for any of the other tBDMS-derivatized amino acids prepared from the \([\beta-2H_2\text{-cys}]\)-RTPR hydrolysate, indicating that no scrambling of the deuterium label had occurred. These results indicate that the cysteine residues, and only the cysteine residues, of RTPR incorporated the deuterium label, albeit only at a level of 60%.
Fig. 2.2. Mass spectra of silylated S-carboxymethylcysteine, showing the m/z = 464 peak. Iodoacetamide-labeled RTPR was subjected to acid hydrolysis, and the tBDMS derivatives of the amino acids in the hydrolysate were prepared by reaction with N-methyl-N-(t-butyldimethylsilyl)trifluoroacetamide. Main figure: Hydrolysate of [3-²H₂-cys]-RTPR isolated from JM105/pSQUIRE. Inset: Hydrolysate of unlabeled RTPR.
Abundance

Scan 297 (24.543 min): [BBB1]LICHTRJ.D

466

464

468

469

436

506

450

500

m/z ---> 456 458 460 462 464 466 468 470 472 474 476 478
RFQ EPR: Evidence for Thyl Radical Formation

The model in Scheme 2.1 predicts that rapid quenching of the reaction in liquid isopentane at -140°C could trap cob(II)alam in and a 5'-dA•, a thyl radical, or both. Cob(II)alam is EPR active (S, the electron spin, is 1/2), and the hyperfine interaction with the cobalt nucleus (I, the nuclear spin, is 7/2) is evident in its spectrum (Hamilton et al., 1971). The appearance of the actual EPR spectrum of the intermediate is difficult to predict because of the possibility of coupling of the cob(II)alam unpaired spin to one or more radical species via spin exchange interactions, dipolar interactions or both. In order to investigate whether 5'-dA• is one of the species in the trapped intermediate, [5'-2H]-AdoCbl was used in freeze quench experiments. If 5'-dA• were a component in the intermediate, [5'-2H]-AdoCbl would be expected to produce narrowed features in the EPR spectrum because of the difference in gyromagnetic ratios between 1H and 2H, which result in a difference in hyperfine interactions (Wertz & Bolton, 1986). As previously observed by Orme-Johnson and co-workers under slightly different conditions (Orme-Johnson et al., 1974), the substitution of [5'-2H]-AdoCbl does not give rise to any significant change in the EPR spectrum of the intermediate (Fig. 2.3). Thus, a 5'-dA• does not appear to be a component of the observed EPR signal.

Alternatively, the model in Scheme 2.1 predicts that cob(II)alam could be interacting with a thyl radical rather than a 5'-dA•. To test this hypothesis, RTPR was grown under conditions that allow incorporation of β-[2H]-labeled cysteine. If a thyl radical is a component of the intermediate, this substitution would narrow the features of the EPR signal as described above for the [5'-2H]-AdoCbl experiments (Fig. 2.3). The spectrum of the intermediate formed on mixing [β-2H-cys]-RTPR with AdoCbl in the presence of allosteric effector is shown in Fig. 2.4. The sharpening of the features associated with the cobalt hyperfine structure relative to Fig. 2.4 (the spectrum observed with unlabeled RTPR) is readily apparent. The effect of 2H-labeling of cysteine on the cobalt hyperfine features defines unambiguously that a cysteine is adjacent to and interacting with cob(II)alam. These results, in conjunction with the SF and acid quench experiments described above, support the proposal (Scheme 2.1) that the function of the AdoCbl is to generate 5'-dA, cob(II)alam, and a thyl radical.
Fig. 2.3. Effect of isotopic substitution of AdoCbl on EPR spectra of freeze-quenched reaction mixtures. Reaction conditions for the top spectrum: unlabeled RTPR (525 μM) and 50 μM TR, 3 μM TRR, 1.5 mM NADPH, 1 mM dGTP in 100 mM sodium dimethylglutarate, pH 7.3 were mixed with an equal volume of 400 μM AdoCbl and 1 mM dGTP in the reaction buffer, and the reactions were quenched in isopentane (-140 °C) at 175 ms. Reaction conditions for the bottom spectrum were the same, with the exception that 250 μM RTPR and 300 μM [5′-2H2]-AdoCbl were used. The EPR spectra were recorded at 100 K with spectrometer frequency 9.41 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 4 gauss; time constant, 1.3 s; and scan time, 671 s. In each case, 10 scans were recorded.
Fig. 2.4. Effect of deuteration of the cysteines of RTPR on the EPR signal of the intermediate. (Top) The EPR spectrum that resulted when RTPR (525 µM) and 50 µM TR, 3 µM TRR, 1.7 mM NADPH, 1 mM dGTP in 100 mM sodium dimethylglutarate, pH 7.3 were mixed with an equal volume of 400 µM AdoCbl and 1 mM dGTP in the reaction buffer, and the reactions were quenched in isopentane (-140 °C) at 175 ms. (Bottom) The spectrum observed in the presence of [β-²⁴H₂-cysteine]-RTPR (250 µM) and AdoCbl (300 µM). This labeled RTPR was prepared using E. coli JM105 containing plasmid pSQUIRE The EPR spectra were recorded at 100 K with spectrometer frequency 9.41 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 4 gauss; time constant, 1.3 s; and scan time, 671 s. In each case, 10 scans were recorded. The feature at g = 2 (*) was observed in variable amounts in both unlabeled and isotopically labeled samples. While thawing of the sample resulted in disappearance of the intermediate species, there was a g = 2 signal that remained after thawing and re-freezing of the sample, suggesting that this feature is unrelated to the spectrum of the intermediate.
Reversibility of Formation of the Paramagnetic Intermediate: Effect of Thawing and Freezing

RFQ methods could conceivably result in detection of a species that is an artifact of the quenching process. In fact, it has been suggested that the spectral narrowing observed on incorporation of [β-2H]-cysteine (Fig. 2.4) could also indicate a thiolate ligated to cob(II)alamin. Several experiments have shown this not to be the case. First, if this hypothesis were correct, an additional EPR-active species should be detected, because an amino acid residue would have to be oxidized in order to reduce a thiol radical to a thiolate. No such species was observed. Second, if the signal was due to a kinetically competent intermediate, then warming the sample to 0 °C under anaerobic conditions should result in reformation of the carbon-cobalt bond, since the equilibrium between cob(II)alamin and AdoCbl on the enzyme lies toward AdoCbl at this temperature (Tamao & Blakley, 1973). If the observed signal is the result of a quenching artifact, however, cob(II)alamin, which is stable under anaerobic conditions, should still be present and readily detected by EPR spectroscopy. When this experiment was performed the spectrum revealed no cob(II)alamin.

Spin Quantitation of the Intermediate and Temperature Dependence of EPR Signal Intensity

The proposed mechanism also predicts that the intermediate observed by EPR spectroscopy should be produced with the same rate constant as cob(II)alamin observed by SF and should contain two spins per equivalent of cob(II)alamin. The half-time for formation of the EPR signal under exchange conditions is 10 to 20 ms (Fig. 2.5), consistent with the rate constant of 49 s⁻¹ (t₁/₂ = 14 ms) observed by SF UV-vis spectroscopy under identical conditions, and in accord with the results of Orme-Johnson, et al., who measured identical rate constants for intermediate formation using SF UV-vis and RFQ EPR methods (Orme-Johnson et al., 1974). The SF experiments also show that 48 μM cob(II)alamin (0.18 equivalents per equivalent of RTPR) is formed at 175 ms (i.e., in the steady state). The amount of spin formed at 175 ms has been quantitated by comparing the RFQ EPR samples to a CuSO₄ standard at 100 K (Ballou & Palmer, 1970, Malmström et al., 1970). If the paramagnetic intermediate is assumed to have S=1/2, the average spin concentration in the steady state is 80±20 μM, consistent with the formation
Fig. 2.5. Kinetics of formation of paramagnetic intermediate as judged by RFQ EPR spectroscopy. Reaction conditions were as described in Fig. 2.4. Triangles: equivalents of spin/equivalent of RTPR, calculated assuming $S = 1/2$ and a packing factor of 0.7. Solid line: time course expected based on kinetics of cob(II)alamin formation measured by SF UV-vis spectroscopy.
of two equivalents of spin per equivalent of cob(II)alamin, as was reported by Orme-Johnson et al. (Orme-Johnson et al., 1974).

The spin quantitation is also consistent with the hypothesis that the intermediate species comprises two spins that are coupled strongly enough to be described as a triplet. If the paramagnetic intermediate were assumed to be a triplet, the spin concentration would be calculated to be $30 \pm 8 \, \mu M$, in reasonable agreement with the value of $48 \, \mu M$ predicted from stopped-flow studies (one equivalent of a triplet intermediate per equivalent of cob(II)alamin). The spin state of the intermediate is relevant to the issue of whether this intermediate contains one or two paramagnetic species. If the intermediate exhibits behavior characteristic of a triplet state, there must be two paramagnetic centers present.

The temperature dependence of the EPR signal intensity can be used to measure the exchange coupling between two unpaired spins (Wertz & Bolton, 1986). To investigate the spin state of the intermediate, the temperature dependence of its EPR signal intensity was determined by recording spectra from 4 to 99 K, with the use of a non-saturating power at each temperature, and with the intensities being normalized for power as necessary. As a control, a similar temperature dependence was determined for the cob(II)alamin-(5'-dA)-RTRP complex (Hamilton et al., 1971). From 4 K to 99 K, the EPR signal intensity of this complex follows the Curie-Weiss law behavior expected for a single unpaired spin (Wertz & Bolton, 1986) (Fig. 2.6). In contrast, the signal intensity of the intermediate generated from quenching deviates from Curie-Weiss law behavior below 10 K (Fig. 2.6); the intensity at 5.4 K is $53 \pm 6$ percent of the intensity predicted by the Curie-Weiss law. These data are consistent with a model in which the intermediate is composed of two exchange-coupled spins, with a singlet ground state and a thermally accessible triplet excited state. At low temperatures, the triplet state is depopulated, and the EPR signal intensity decreases (Wertz & Bolton, 1986). An approximate treatment of the temperature dependence of the EPR signal intensity (Wertz & Bolton, 1986), neglecting the effects of singlet-triplet mixing, predicts that the signal intensity will be proportional to

$$\frac{3}{T} \exp \left( \frac{-Jh}{kT} \right) \quad \text{Eq. 2.1}$$
Fig. 2.6. Temperature dependence of signal intensity for the paramagnetic intermediate and cob(II)alamin bound to RTPR. The paramagnetic intermediate was freeze-quenched as described in Fig. 2.4. The cob(II)alamin sample was prepared by thawing a rapid freeze-quench sample under argon, incubating the tube under argon at 37 °C for 3 h, and re-freezing the reaction mixture. Spectra were recorded at non-saturating power at each temperature, with the intensities being normalized for power as necessary. The data for cob(II)alamin bound to RTPR is fit to a line. The data for the paramagnetic intermediate is fit to Eq. 2.1.
where $T$ is the temperature (Kelvin), $J$ is the exchange coupling, $h$ is Planck's constant, and $k$ is Boltzmann's constant (Wertz & Bolton, 1986). Fitting the data in Fig. 2.6 to Eq. 2.1 gives a $J$ coupling of $2.8 \pm 0.6$ cm$^{-1}$.

**Characterization of the Paramagnetic Intermediate by Parallel Mode EPR**

The observation of a $\Delta M = \pm 2$ or "half-field" transition is considered to be diagnostic of spin-spin interaction (Wertz & Bolton, 1986). Observation of such a transition in the freeze-quenched intermediate would thus support the hypothesis that this intermediate consists of two paramagnetic species, rather than one. Reports that such a transition had been observed in the RTPR system have been alluded to in the literature (Ghanekar et al., 1981, Pilbrow, 1982), but never published. Since this transition is semi-forbidden when the magnetic field vector of the incident microwave is perpendicular to the applied field ("perpendicular mode") (Wertz & Bolton, 1986), but allowed when the microwave magnetic field vector is parallel to the applied field ("parallel mode"), a microwave cavity that allows recording in parallel mode is often used to detect half-field lines. Using the cavity available at Bruker Instruments, we detected an absorption at $g \sim 4$ (Fig. 2.7). However, oxygen, being a triplet, also a half-field transition at $g \sim 4$, which was detected in a sample of oxygen dissolved in toluene (Fig. 2.7). Since the observed half-field line was fairly isotropic, it was interpreted as resulting from oxygen in the isopentane, although differences in lineshape are evident.

**Characterization of the Paramagnetic Intermediate by Q-band EPR**

Simulation of the EPR spectra provide another means of assigning the identity of the paramagnetic intermediate. In order to distinguish between a thyl radical coupled to cob(II)alamin and other possibilities for the identity of the paramagnetic intermediate, it was necessary to simulate EPR spectra at two frequencies: X-band and Q-band (Gerfen et al., 1996). Simulating spectra recorded at two frequencies is useful because it distinguishes between spectral broadening due to $g$ anisotropy and broadening due to exchange and dipolar coupling. While $g$ values are proportional to the microwave frequency, exchange and dipolar couplings are independent of microwave frequency (Wertz & Bolton, 1986).

Reaction mixtures were therefore quenched into Q-band tubes, and spectra recorded at Q-band (35 GHz) (Fig. 2.8). Simulations of the spectra at
Fig. 2.7. Parallel mode EPR spectra using a Bruker DM9304 resonator. Top: O₂ dissolved in toluene. Bottom: Freeze-quenched reaction mixture as described in Fig. 2.4. The spectra were recorded with spectrometer frequency 9.463 GHz; temperature 10 K; modulation frequency 100 kHz; modulation amplitude 4 G; time constant 327 ms; scan time 335 s.
Fig 2.8. Q-band EPR spectrum. Experimental data of enzyme with [$\beta$-$^1$H$_2$]-cysteine-labeled RTPR. The reaction conditions are identical to those previously described in Fig. 2.4. Spectrometer frequency 34.93 GHz; temperature 2 K; modulation frequency 100 kHz; modulation amplitude 2 G; time constant 64 ms; scan time 240 s; the displayed spectrum is a pseudomodulation (Hyde, 1992; van der Donk, 1995) of the sum of 16 saturated dispersion spectra. The starred feature is a contaminant present in variable amounts in the freeze-quenched samples.
both X-band and Q-band support the assignment of the paramagnetic intermediate as an enzyme-based thiyl radical coupled to cob(II)alamin (Gerfen et al., 1996).

**Paramagnetic Intermediate Formed During Turnover**

Orme-Johnson et al. (Orme-Johnson et al., 1974) showed that the EPR spectrum of the paramagnetic intermediate observed during turnover is similar to the EPR spectrum observed in the absence of substrate. It was therefore expected that this intermediate comprised a thiyl radical and cob(II)alamin both during turnover and in the absence of substrate. To test this hypothesis, RFQ EPR experiments were carried out with [β-2H2-cys]-RTPR under turnover conditions (Fig. 2.9). In this case, the [β-2H2-cys]-RTPR was purified from the cysteine auxotroph JM15/pSQUIRE, so that the incorporation of deuterated cysteine was expected to be complete. Samples were quenched at 20 ms, as this was the time when cob(II)alamin formation was found to be maximal (see Chapter 4).

As reported by Orme-Johnson et al., the spectrum of the paramagnetic intermediate trapped during turnover is similar to the spectrum observed in the absence of substrate. The most obvious difference between the spectra is an increased absorbance at g ~2 in the spectrum of the sample obtained by freeze-quenching of the turnover reaction. A feature at g ~2 is also observed in variable amounts when the exchange reaction is freeze-quenched. This feature appears to increase in amplitude with time over a period of months after quenching. However, in the case of the turnover reaction, the feature at g ~2 appears to have an amplitude proportional to the overall spin concentration. This observation suggests that, when the turnover reaction is freeze-quenched, the feature at g ~2 is either part of the spectrum of the thiyl radical-cob(II)alamin spin system or represents some species formed concurrently with the thiyl radical-cob(II)alamin intermediate. Nonetheless, the appearance of the spectrum is consistent with very similar intermediates being trapped during turnover and in the absence of substrate.

As expected, deuteration of the cysteines of RTPR has a pronounced effect on the breadth of the hyperfine features in the intermediate trapped
Fig. 2.9. Effect of deuteration of the cysteines of RTPR on the EPR signal of the intermediate generated in the presence of substrate. Reaction and quenching conditions were identical to those described in Fig. 2.4 except that ATP (1 mM) was present in both syringes, and the reaction was quenched at 20 ms. Top: Spectrum observed when unlabeled RTPR was used. Bottom: Spectrum observed in the presence of [β-²H₂-cysteine]RTPR. In this experiment, [β-²H₂-cys]-RTPR was purified from the cysteine auxotroph JM15/pSQUIRE. The EPR spectra were recorded at 6 K with spectrometer frequency 9.47 GHz; microwave power, 1 mW; modulation frequency, 100 kHz; modulation amplitude, 4 gauss; time constant, 1.3 s; and scan time, 671 s. In each case, eight scans were recorded.
during turnover (Fig. 2.9). The effect appears to be more pronounced that that observed in the absence of substrate, probably because of the higher incorporation of deuterium made possible by expression in the auxotroph. This experiment indicates that the kinetically competent paramagnetic intermediate formed during turnover includes an enzyme-based thiyl radical coupled to cob(II)alamin.
Discussion

Fate of the 5′-Deoxyadenosyl Moiety of [5′-3H]-AdoCbl

The mechanism in Scheme 2.1 predicts that 5′-dA should be formed as a kinetically competent intermediate in both the exchange reaction and nucleotide reduction, regardless of whether carbon-cobalt bond homolysis and thyl radical formation are stepwise or concerted. Tamao and Blakley (Tamao & Blakley, 1973), in an effort to test a similar mechanism, attempted to trap this species by quenching a reaction mixture with perchloric acid. However, they found no 5′-dA formed above the level expected given the rate of the slow, kinetically non-competent degradation of AdoCbl catalyzed by the enzyme. Thus, while evidence for catalysis by RTPR of slow, irreversible formation of 5′-dA from AdoCbl had previously been obtained, there was no direct evidence for the proposed fate of the axial ligand of AdoCbl during the exchange reaction or turnover. Obtaining evidence for or against the role of 5′-dA as a kinetically competent intermediate is thus required to evaluate the mechanism in Scheme 2.1.

When RTPR is mixed with AdoCbl in the presence of an allosteric effector, a reasonably large amount of cob(II)alamin (0.2-0.3 eq.) is formed in the steady state (Tamao & Blakley, 1973). It is therefore surprising that 5′-dA could not be isolated in the earlier experiments. One possibility that is consistent with the mechanism in Scheme 2.1 is that adding perchloric acid by hand does not give fast enough mixing and quenching to trap 5′-dA as an intermediate. This would suggest that the use of a rapid mixing apparatus would allow trapping of 5′-dA as a kinetically competent intermediate. Another possibility, proposed by Hogenkamp and coworkers (Sando et al., 1975), is that the 5′-dA• could cyclize to give the 5′,8-cycloadenosyl radical. This hypothesis predicts that cycloA would be formed as a kinetically competent intermediate. A third hypothesis postulates reaction of 5′-deoxyadenosyl radical with a disulfide bond on the enzyme, forming a thioether and a thyl radical. This hypothesis predicts that the 5′-deoxyadenosyl moiety would be covalently linked to the enzyme.
In order to distinguish among these hypotheses, rapid acid quench experiments were carried out. The observation that 5'-dA is formed at approximately the same rate as cob(II)alamin (measured in SF UV-vis experiments) (Fig 2.1) supports the hypothesis that 5'-dA is a kinetically competent intermediate in the exchange reaction, thus supporting the mechanism in Scheme 2.1. This observation also suggests that quenching the reaction using a rapid mixing device is required to observe this intermediate. An inability to trap 5'-dA using hand-mixing is consistent with carbon-cobalt bond homolysis being readily reversible. If denaturation of the protein is slow compared to re-formation of the carbon-cobalt bond, and acidic pH (<6.5) favors AdoCbl over cob(II)alamin, as suggested by SF UV-vis studies of Tamao and Blakley (Tamao & Blakley, 1973), hand-mixing of a reaction mixture with perchloric acid could lead to carbon-cobalt bond re-formation occurring before denaturation of the protein, thus preventing the trapping of 5'-dA.

This experiment does not distinguish between trapping of 5'-dA and 5'-'dA•. If 5'-dA• were present, it would be likely to abstract a hydrogen from the protein or perchloric acid during quenching, thus appearing as 5'-dA. It might be possible to distinguish between 5'-dA and 5'-'dA• by preparing the quench solution in D2O or [3H]-H2O. The extent of deuterium or tritium labeling of the 5'-dA isolated from the quenched reaction mixture would reflect the extent to which any 5'-dA• abstracted deuterium (or tritium) from the quench solution. 5'-dA that was present in the reaction mixture before addition of the quench solution would not be labeled.

A small fraction of the tritium in the reaction mixture was observed to wash out to the solvent. By fitting the kinetics of tritiated water washout to a line, a rate of 0.2-0.3 eq. of tritiated water s⁻¹ was measured for tritium washout. This is approximately the same as the steady-state rate of tritium washout (0.16 s⁻¹) measured by Squire Booker (Booker, 1994). The observation that tritium washout occurs at approximately the same rate as in the steady state supports the idea that this process is on the catalytic pathway. If thyl radical formation and washout occurred as a slow side reaction, as suggested by Tamao and Blakley (Tamao & Blakley, 1973) (Scheme 2.2), the kinetics of
Scheme 2.2. The exchange reaction hypothesized to be off the normal nucleotide reduction pathway.
tritium washout might exhibit a lag phase, as the $5'$-dA• intermediate which partitioned between immediate re-formation of the carbon-cobalt bond and the washout reaction would need to build up to its steady-state concentration before the washout reaction reached its maximum rate. In contrast, if thyl radical formation accompanies each carbon-cobalt bond homolysis (Scheme 2.1), washout will occur without a lag phase during the approach to equilibrium observed by SF UV-vis and rapid acid quench experiments. This result also suggests that $5'$-dA•, if it exists as a discrete intermediate, has a short lifetime. If $5'$-dA• were a long-lived intermediate, tritium washout, which would presumably proceed through this intermediate, would exhibit a lag time corresponding to the formation of $5'$-dA•.

Finally, since C408 has been shown to be essential for the exchange reaction (Booker et al., 1994), these results provide further indirect support for the hypothesis that a thyl radical is the X• proposed to function as the hydrogen abstracting species in both tritium washout and nucleotide reduction (Ashley et al., 1986). The simultaneous observation of cob(II)alamin, $5'$-dA, and tritium washout suggests that carbon-cobalt bond cleavage, X• formation, and the re-formation of XH and the carbon-cobalt bond are all happening on the millisecond timescale. Since C408 is required for tritium washout, these results complement steady state experiments suggesting that C408 acts as X• (Booker et al., 1994). SF UV-vis experiments described in the next chapter indicate that C408 is required for efficient carbon-cobalt bond homolysis.

**RFQ EPR: Evidence for Thyl Radical Formation**

Given the biochemical evidence that C408 serves as the X• in the exchange reaction and nucleotide reduction, it was hypothesized that the paramagnetic intermediate trapped by freeze-quenching consisted of cob(II)alamin coupled to the C408 thyl radical. This hypothesis predicted that deuteration of the β-position of the cysteines of RTPR would perturb the EPR spectrum of the intermediate due to the interaction of the β-hydrogens with the thyl radical/cob(II)alamin radical pair.
As shown in Fig. 2.4, the spectrum of [β-2H₂-cys]-RTTPR exhibits narrowing in the cobalt hyperfine features throughout the spectrum. The effect of ²H-labeling of cysteine on the cobalt hyperfine features defines unambiguously that a cysteine is adjacent to and interacting with cob(II)alamin. This result is particularly striking considering that ²H (Fig. 2.3) or ¹³C labeling of the 5' position of AdoCbl fails to perturb the EPR spectrum of the intermediate (Orme-Johnson et al., 1974). These results thus indicate that if the paramagnetic intermediate represents a pair of radical species, it is a thyl radical, rather than 5'-dA•, that is interacting with cob(II)alamin. However, the isotopic labeling studies do not define unambiguously that the observed paramagnetic intermediate consists of two interacting paramagnetic species.

Reversibility of Formation of the Paramagnetic Intermediate: Effect of Thawing and Freezing

Investigating the reversibility of formation of the paramagnetic intermediate provides an avenue toward determining whether it comprises one or two paramagnetic species. Carbon-cobalt bond homolysis leads to the formation of two paramagnetic species. In order for carbon-cobalt bond re-formation to occur reversibly, there must be two paramagnetic species present throughout the reaction. If one of the paramagnetic species is reduced to a diamagnetic species as an artifact of freeze-quenching, the reaction will no longer be reversible.

The experiments of Tamao and Blakley indicate that the rapid carbon-cobalt bond homolysis is reversible on cooling to 5 °C or below (Tamao & Blakley, 1973). If the freeze-quenched samples contain the reversibly-formed intermediate characterized in solution, thawing of the samples in an ice/water bath should lead to re-formation of the carbon-cobalt bond, thus providing evidence that the paramagnetic intermediate trapped by freeze quenching consisted of two paramagnetic species. If, however, the freeze-quenched samples contain a single paramagnetic species due to artifactual reduction of a second species, thawing of the samples will not lead to the re-formation of the carbon-cobalt bond, and cob(II)alamin will be observed.
EPR spectroscopy on thawed and re-frozen samples reveals no cob(II)alamin, indicating that the carbon-cobalt bond is found to be re-formed on thawing of the sample at 0° C. This result supports the hypothesis that the paramagnetic intermediate consists of two paramagnetic species.

_Spin Quantitation of the Intermediate and Temperature Dependence of EPR Signal Intensity_

Spin quantitation of the freeze-quenched samples provides a more direct method of evaluating whether the intermediate comprises two paramagnetic species. The hypothesis that the paramagnetic intermediate consists of a thyl radical interacting with cob(II)alamin predicts that the spin concentration of the intermediate as measured by the EPR signal intensity should be twice the concentration of cob(II)alamin as measured by SF UV-vis spectroscopy. The measured spin concentration is 80±20 μM (assuming S = 1/2 for the two paramagnetic species), and the measured cob(II)alamin concentration under the same conditions is 48 μM, consistent with the hypothesis that the paramagnetic intermediate consists of two interacting paramagnetic species. However, the spin quantitation is subject to large errors due to the variable and unknown amount of isopentane packed into the sample tubes along with the reaction mixture. While packing of a standard of known concentration allows an estimate of the packing factor, or fraction of the sample occupied (Ballou & Palmer, 1970), this method is not ideally suited for precise determination of spin concentrations (see the large scatter in the spin concentration data in Fig. 2.5.)

However, the EPR signal intensity depends not only on the spin concentration but also on the spin state of the paramagnetic species. The signal intensity increases directly with S(S+1), where S is the spin multiplicity of the paramagnetic species (Wertz & Bolton, 1986). Since the paramagnetic intermediate trapped by freeze-quenching is proposed to consist of two interacting paramagnetic species, it is possible that the two species are coupled tightly enough to be viewed as a triplet state. If this is the case, the spin concentration calculated for S = 1/2 does not accurately reflect the true spin concentration. It is thus necessary to
evaluate the spin state of the intermediate in order to perform the appropriate spin quantitation. More importantly, evidence for triplet character of the paramagnetic intermediate would constitute evidence for the existence of two coupled paramagnetic species.

Analysis of the temperature dependence of the EPR signal intensity can be an effective way to investigate the spin state of a paramagnetic entity. In a sample consisting of isolated unpaired spins, the EPR signal intensity should vary inversely with temperature (the Curie-Weiss law) (Wertz & Bolton, 1986). Exchange coupling will alter this temperature dependence. Exchange coupling breaks the degeneracy between singlet and triplet states. Depending on the sign of the exchange coupling (ferromagnetic or antiferromagnetic coupling), either the singlet or the triplet state will lie lower in energy. Since the singlet state represents paired spins, it is EPR-silent, while the triplet state will be EPR active (Smith & Pilbrow, 1974). Decreasing the temperature will increase the population of the ground state: if the ground state is the singlet state, this will cause a reduction in EPR signal intensity, while if the ground state is the triplet state, it will cause an increase in EPR signal intensity. If the energy difference between the singlet and triplet states (\(Jh\), where \(J\) is the exchange coupling) is \(\sim kT\), both singlet and triplet states will be populated to a significant extent. Regardless of the sign of the coupling, the EPR signal intensity should deviate from Curie-Weiss law behavior, with the signal intensity, neglecting singlet-triplet mixing, described by Eq. 2.1 (Wertz & Bolton, 1986) (see Results section).

The observed temperature dependence of signal intensity for cob(II)alamin bound to RTPR follows Curie-Weiss law behavior (intensity directly proportional to \(1/T\)) (Fig. 2.6), consistent with cob(II)alamin being a single \(S = 1/2\) species. In contrast, the paramagnetic intermediate exhibits pronounced deviation from Curie-Weiss law behavior, and the temperature dependence of its signal intensity is well fit to Eq. 2.1. While the fit of this data to a straight line is acceptable, the intercept of the best fit line is significantly greater than zero. A non-zero intercept is not physically reasonable, because regardless of the spin multiplicity, the signal intensity will approach
zero in the limit of infinite temperature. Fitting the data to Eq. 2.1 was thus considered to be more reasonable.

The observation that the observed signal intensities for the paramagnetic intermediate are lower than predicted by the Curie-Weiss law at low temperatures indicates that the singlet state is the ground state and the triplet state is the thermally excited state. As the temperature decreases, the thermally excited, EPR-active triplet state becomes depopulated, making the signal intensity less than predicted by the Curie-Weiss law. The fit of the data to the simplified model of a thermally accessible triplet state gives a value of 2.8±0.6 cm\(^{-1}\) for J. This is much lower than values typically observed for exchange coupling in paramagnetic systems in which two paramagnetic centers have a direct covalent linkage. For example, the diferric iron center of the E. coli R2 protein, in which the iron is connected by an oxo bridge, has a |J| of 94±7 cm\(^{-1}\) (Hirsh et al., 1992). As will be discussed later, however, this value for the exchange coupling is consistent with a thiol radical participating in through-space orbital overlap with cob(II)alamin.

This first-order estimate for J can be used to obtain an estimate of the spin concentration assuming a triplet state, answering the question of whether amount of triplet state paramagnetic intermediate measured by EPR is consistent with the amount of cob(II)alamin measured by SF UV-vis spectroscopy. With a J coupling of 2.8 cm\(^{-1}\), the Boltzmann distribution (Eisenberg & Crothers, 1979) predicts that the triplet state should be 75% populated at 100 K. Since the singlet state is EPR silent, one would expect a 36 μM spin concentration in the steady state (75% of 48 μM cob(II)alamin), in good agreement with the value of 31±5 μM calculated from the observed signal intensity using S = 1. The temperature dependence of the EPR signal intensity thus provides a strong argument that the paramagnetic intermediate consists of two interacting paramagnetic centers, rather than a single paramagnetic center.

**Characterization of the Paramagnetic Intermediate by Parallel Mode EPR**

In an effort to obtain independent evidence for the hypothesis that the paramagnetic intermediate consists of two interacting centers,
spectroscopic experiments were carried out to look for $\Delta M = \pm 2$ or "half-field" transition. Reports that such a transition had been observed in the RTPR system have been alluded to in the literature (Ghanekar et al., 1981, Pilbrow, 1982), but never published. Investigations of half-field transitions in the parallel $B_1$ mode (Bleaney, 1951, Eaton et al., 1983) might allow estimation of the electron-electron dipolar interaction parameters $D$ (the dipolar coupling) and $E$ (the zero-field splitting). These parameters, especially the dipolar coupling, are relevant to structural modeling of a pair of interacting paramagnetic centers.

Unfortunately, it appears that dissolved oxygen (which, being a triplet, also has a half-field transition) in the sample was present at a high enough concentration to obscure any half-field transition arising from the paramagnetic intermediate. While the half-field transition observed in the freeze-quenched sample (Fig. 2.7) exhibits anisotropy that might optimistically be interpreted as a manifestation of the $g$ anisotropy of the thyl radical/cob(II)alamin system, the feature is fairly isotropic overall and is likely to arise mostly from dissolved oxygen in the sample. Degassing the isopentane used for freeze-quenching, perhaps by bubbling nitrogen through it immediately before use, might allow a better chance for observation of a half-field transition. However, the parameters derived from simulations of the EPR spectrum of the intermediate (Gerfen et al., 1996) suggest the half-field transition would be difficult to observe even in the absence of contaminating oxygen, as the maximum intensity of this transition is expected to be two orders of magnitude less than that of the main transition (Professor Gary Gerfen, personal communication).

*Characterization of the Paramagnetic Intermediate by Q-band EPR*

One of the most rigorous tests for the identity of a paramagnetic species is simulation of the observed EPR spectrum at multiple frequencies. By constraining $g$-values and hyperfine coupling constants to those appropriate for both a thyl radical and cob(II)alamin within a model that includes exchange and dipolar couplings between the unpaired spins, and seeing how well this model simulates the observed data, it is possible to evaluate whether the hypothesized composition of the intermediate is consistent with the EPR
data. Professor Gary Gerfen wrote a computer program to calculate EPR spectra of a pair of interacting paramagnetic centers, with dipolar couplings, zero-field splitting, exchange coupling, and Euler angles as adjustable parameters (Gerfen et al., 1996). By extensive variation of the adjustable parameters, Prof. Gerfen was able to simulate the observed EPR data at both X-band (Fig. 2.10) and Q-band (Fig. 2.11). The parameters used for the simulation are shown in Table 2.1.

These simulations reproduce the general features of the experimentally acquired X-band EPR spectrum, which include the "effective" g-value of 2.12, the spectral width and intensities, and the scaling of the cobalt hyperfine splitting by approximately a factor of two (from ~110 G in cob(II)alamin (Hamilton et al., 1971) to ~50 G in the intermediate for A33) (Gerfen et al., 1996). Furthermore, simulated replacement of the thiy radical β-proton with a deuteron (I=1, g_{nD}/g_{nH} = 0.154) produces the experimentally observed line-narrowing (compare Fig. 2.10b and 2.10d). The simulations thus provide strong evidence that the paramagnetic intermediate consists of an enzyme-based thiy radical coupled to cob(II)alamin.

Prof. Gerfen also used simulations to evaluate two alternative models for the composition of the paramagnetic intermediate: a single paramagnetic species (presumably arising from as an artifact of freeze-quenching) and cob(II)alamin coupled to a carbon-centered radical (the putative 5'-dA• or, conceivably, a carbon-centered amino acid radical). The simulation of the 9 GHz spectrum assuming the single electron spin 1/2 model (compare Fig. 2.12a and Fig. 2.12b) was calculated using parameters previously presented by Pilbrow (Pilbrow, 1982). Although the fit appears reasonable, the g-values, hyperfine coupling constants and principal axes orientations required to simulate the spectrum are unprecedented for cob(II)alamin and cob(II)inamide coordination complexes (Bayston et al., 1970, Hamilton et al., 1971, Jörin et al., 1979, Pilbrow, 1982, Schrauzer & Lee, 1970). Moreover, the simulation fails to reproduce the intensities at the spectral edges. A simulation of the Q-band spectrum (compare Fig. 2.13a and Fig. 2.13b) using these same parameters completely fails to reproduce the overall spectral width and lineshape. Thus, as Pilbrow previously concluded based on X-band data (Pilbrow, 1982), a single electron spin model is inadequate to describe the observed experimental data.
Fig. 2.10. X-band EPR spectra and simulations of the paramagnetic intermediate in the presence of [β-1H₂]-cysteine-labeled RTPR (A and B) and [β-2H₂]-cysteine-labeled RTPR (C and D). A and C: Experimental spectra obtained as in Fig. 2.4. Spectrometer frequency 9.41 GHz; temperature 100 K; microwave power 10 mW; modulation frequency 100 kHz; modulation amplitude 0.4 mT; time constant 1.3 s; scan time 671 s; each experimental spectrum is the sum of ten scans. The starred feature is a contaminant present in variable amounts in the freeze-quenched samples. B and D: Simulations using parameters in Table I and a gaussian line broadening of 3.0 mT. B: 100% proton simulation; D: 60:40 sum of deuteron:proton simulations.
Fig. 2.11. Q-band EPR spectrum and simulation of the paramagnetic intermediate. Experimental conditions were as in Fig. 2.4. A: Experimental data of enzyme with [β-¹H₂]-cysteine-labeled RTPR. Spectrometer frequency 34.93 GHz; temperature 2 K; modulation frequency 100 kHz; modulation amplitude 2 G; time constant 64 ms; scan time 240 s; the displayed spectrum is a pseudomodulation (Hyde, 1992; van der Donk, 1994) of the sum of 16 saturated dispersion spectra. The starred feature is a contaminant present in variable amounts in the freeze-quenched samples. B: Simulation using parameters in Table I. The calculated spectrum was convolved with a saturated dispersion lineshape function and pseudomodulated in a manner analogous to the experimental spectrum. The overall width of the convolved lineshape function was approximately 5.2 mT.
Fig. 2.12. X-band EPR spectrum of the paramagnetic intermediate and simulation for a single electron spin 1/2 species. Experimental conditions for the reaction and quenching were as in Fig. 2.4. A. Identical to the spectrum in Fig. 2.10. B: Simulation assuming a single electron spin 1/2 species. The g-values (1.995, 2.198, 2.140) and cobalt hyperfine splitting constants (47.1 G, 53.5 G, 7.5 G) were taken from a similar figure published previously by Pilbrow (Pilbrow, 1982).
Fig. 2.13. Q-band EPR spectrum of the paramagnetic intermediate and simulation for a single electron spin 1/2 species. Experimental conditions for the reaction and quenching are as in Fig. 2.4. A and B: Identical to the spectrum and simulation, respectively, in Fig. 2.11. C: Simulation assuming a single electron spin 1/2 species. The g-values (1.995, 2.198, 2.140) and cobalt hyperfine splitting constants (47.1 G, 53.5 G, 7.5 G) were taken from a similar figure published previously by Pilbrow (Pilbrow, 1982).
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\textsuperscript{a}A in MHz; $J_{\text{ex}}$, $D$ and $E$ in $10^{-4}$ cm\textsuperscript{-1}. Euler angles, in degrees, are defined as in Goldstein (Herbert Goldstein, Classical Mechanics, Addison Wesley, Reading MA, 1967). \textsuperscript{b}Relates each hyperfine interaction principal axis system to the respective $g$-principal axis system. \textsuperscript{c}Hyperfine coupling constants (MHz) for cobalt nucleus. \textsuperscript{d}Hyperfine coupling constant (MHz) for $\beta$-methylene proton. Simulations of deuterated species used this value multiplied by 0.1535. \textsuperscript{e}The approximately isotropic nature of the hyperfine interaction of the $\beta$-methylene proton renders the simulation insensitive to these angles. Values used were arbitrarily set to those reported for cysteiny1 radical. \textsuperscript{f}Relates dipole coupling principal axes to cobalt $g$-principal axes. \textsuperscript{g}Relates thiyl radical $g$ principal axis system to cobalt $g$ principal axis system.

Table 2.1. Parameters used for X- and Q-band simulations of the paramagnetic intermediate.

Although isotopic labeling studies suggested that the intermediate is probably not composed primarily of 5'-dA• and cob(II)alamin, Prof. Gerfen modeled in nearly isotropic $g$-values typical of a 5'-dA• (Orme-Johnson et al., 1974) or amino acid radicals ($g$-values in the range from 2.000 to 2.010) to investigate the hypothesis that the intermediate might include a carbon-centered radical. Use of these isotropic $g$-values rather than the anisotropic $g$ matrix of the thiyl radical (Table 2.1) failed to achieve reasonable fits to the experimental data, lending further support for a thiyl radical as the major component of this intermediate.

Prof. Gerfen's simulations also offer insight into the nature of the interaction between the thiyl radical and cob(II)alamin. As indicated in Table 2.1, adequate modeling of the data at both X-band and Q-band requires $D$ values that lie in the range of 1$x10^{-2}$ to 2$x10^{-2}$ cm\textsuperscript{-1} and $|J| > 0.45$ cm\textsuperscript{-1} (a value of $\approx 4.17$ cm\textsuperscript{-1} was used in the simulations shown in Figs. 2.10 and 2.11). The exchange coupling indicates an interaction of the molecular orbitals containing the unpaired spins. The calculated range for $J$ is in agreement with the estimate of $J = 2.8 \pm 0.6$ cm\textsuperscript{-1} obtained from the temperature dependence of the EPR signal intensity. The EPR lineshape and the EPR
signal intensity thus represent independent lines of evidence pointing toward the identification of the intermediate as a coupled spin system.

The limiting values which can be set for both D and $J_{\text{ex}}$ provide structural information regarding the \textit{cob(II)alamin-thyl} radical pair. Prof. Gerfen has calculated that if the interaction giving rise to D is exclusively assigned to a dipolar coupling between point dipoles, this range provides a distance of approximately 5 to 7 Å between paramagnetic centers (Gerfen et al., 1996). However, since this interaction may also contain contributions from "pseudo-dipolar" terms arising from anisotropic exchange and spin orbit coupling (Bleaney & Bowers, 1952, Smith & Pilbrow, 1974), and since electron delocalization may render the point dipole assumption inaccurate (Belford et al., 1969), this distance determination must be considered an estimate (Gerfen et al., 1996). The value of $|J| > 0.45 \text{ cm}^{-1}$ derived from the simulations is consistent with this estimate. The magnitude of J tends to decrease with increasing distance between the species because exchange coupling between unpaired electrons may arise from direct orbital overlap or be mediated via the orbitals of intervening atoms (Eaton & Eaton, 1989, Forbes & Bhagnt, 1993, Rajca, 1994, Smith & Pilbrow, 1974). The complexity of the interaction prevents the unique inference of structure from values of $J_{\text{ex}}$. However, in well-characterized systems with $|J_{\text{ex}}| \geq 0.45 \text{ cm}^{-1}$, distances between unpaired electrons are generally less than 8 Å unless the intervening bonding network is highly conjugated (Eaton & Eaton, 1988, Eaton & Eaton, 1989, Matsumoto et al., 1992, Smith & Pilbrow, 1974). Based on these previous studies, an upper limit of 8 Å for the \textit{cob(II)alamin-thyl} radical can be assigned. This value is consistent with the distance estimates based on the value of the dipolar coupling parameter D and provides further evidence for the proximity of the two radical species in the enzyme.

The simulations (Figs. 2.10 and 2.11) require a large number of adjustable parameters (Table 2.1), and there may exist discrepancies between the assumed and actual g and A values for both \textit{cob(II)alamin} and thyl radical. Thus, it is clear that a unique fit to the experimental data is not possible at this stage. However, these simulations have provided the framework for experiments that will lead to refinement of these parameters. Fitting EPR spectra obtained at other EPR frequencies (Eaton et al., 1980) (4, 95, 140 and 250 GHz) may prove to be an effective method for the refinement of Hamiltonian parameters and the structural information these parameters
provide. Spectra recorded at lower frequencies should accentuate the effects of the electron-electron dipolar and electron-nuclear hyperfine interactions, while spectra obtained at higher frequencies will be more sensitive to g-values and will provide more precise limits on the value of J. Nonetheless, the success of the simulations shown in Figs. 2.10 and 2.11 provides compelling evidence that the kinetically competent intermediate species in the RTPR-catalyzed exchange and reduction reactions consists of a thyl radical interacting with cob(II)alamin.

Paramagnetic Intermediate Formed During Turnover

Our hypothesis (Scheme 2.1) predicts that the exchange reaction and the turnover reaction share a common intermediate: a thyl radical, cob(II)alamin, and 5'-dA. The work of Tamao and Blakley (Tamao & Blakley, 1973) and our own observations have demonstrated that, on mixing of RTPR and AdoCbl in the presence of dGTP, ATP, and a reducing system, cob(II)alamin is formed and rapidly reaches a maximum value ($t_{1/2} < 3$ ms using our conditions), then more slowly declines to a steady-state level. Our mechanism explains this kinetic behavior as rapid formation of the paramagnetic intermediate (comprising thyl radical, cob(II)alamin, and 5'-dA), followed by slower re-formation of the carbon-cobalt bond following nucleotide reduction. Thus, freeze-quenching a reaction mixture under turnover conditions should allow EPR spectroscopic observation of a paramagnetic intermediate similar to that observed on quenching reaction mixtures under exchange reaction conditions.

RFQ EPR experiments using [$\beta^{2}$H$_2$-cys]-RTPR support the hypothesis that the paramagnetic intermediate formed under turnover conditions consists of a thyl radical coupled to cob(II)alamin. As shown in Fig. 2.9, the intermediate trapped under turnover conditions using [$\beta^{2}$H$_2$-cys]-RTPR exhibits pronounced narrowing in the cobalt hyperfine features throughout the spectrum, just as observed in the absence of substrate (Fig. 2.4). In both cases, this narrowing is the result an interaction between the $\beta$-hydrogens of a cysteine residue with the thyl radical/cob(II)alamin spin system.

The observation of a thyl radical under turnover conditions shows that this paramagnetic intermediate is likely to be crucial for catalysis. It also demonstrates the utility of the exchange reaction conditions for observation of this important intermediate under conditions that lead to kinetics that are
simpler than those of the turnover reaction itself. The further study of the exchange reaction that chemical and spectroscopic characterization of intermediates makes possible is can thus provide insight not only into this chemically unusual reaction but also into the mechanism of ribonucleotide reduction.
References


Bleaney, B. (1951) Phil. Mag. 42, 441.


Chapter 3:

Evidence that Carbon-Cobalt Bond Homolysis and Thiyl Radical Formation are Concerted
Introduction

The ribonucleoside triphosphate reductase (RTPR) of Lactobacillus leichmannii catalyzes the adenosylcobalamin (AdoCbl)-dependent reduction of nucleoside triphosphates to deoxyribonucleoside triphosphates (dNTP). The reducing equivalents are provided by a pair of cysteine residues (C419 and C119) in the enzyme's active site (Booker et al., 1994, Lin et al., 1987). Reduction of the resulting cystine requires a second pair of cysteine residues (C731 and C736). These C-terminal cysteines can accept reducing equivalents from small organic dithiols or the thioredoxin (TR)/thioredoxin reductase (TRR)/NADPH reducing system to effect multiple turnovers (Vitols et al., 1967b). Ribonucleotide reduction also requires dNTPs as allosteric effectors with one binding site (Chen et al., 1974), controlling both substrate specificity and turnover rate. The fact that dNTPs are both products and allosteric effectors has made the steady-state (Vitols et al., 1967a) and transient state (Tamao & Blakley, 1973) kinetic analysis of this system complex.

However, in addition to nucleotide reduction, RTPR also catalyzes an unusual reaction in which the 5' hydrogens of AdoCbl exchange with the solvent (Scheme 2.1, Chapter 2) (Beck et al., 1966, Hogenkamp et al., 1968). This reaction proceeds in the absence of nucleotide substrates, simplifying its kinetics relative to those observed for nucleotide reduction. We have recently proposed that this exchange reaction is an attractive model system for studying early events in catalysis (Booker et al., 1994). As shown in Scheme 2.1 (Chapter 2), these reactions are proposed to share a common intermediate: a protein-based thyl radical residing on C408. The model postulates that the function of AdoCbl is to generate, in a concerted or stepwise fashion, this thyl radical, 5'-deoxyadenosine (5'-dA), and cob(II)alamin. Generation of the thyl radical in the absence of substrate can lead to exchange of tritium from [5'-3H]-AdoCbl by re-abstraction of hydrogen from 5'-dA in the course of reversal of carbon-cobalt bond homolysis. Alternatively, in the presence of substrate, the thyl radical initiates the nucleotide reduction process by 3'-hydrogen atom abstraction. After a complex set of transformations, hydrogen atom abstraction from the thiol group by a deoxynucleotide-based radical regenerates the thyl radical.

Several experiments have provided evidence that the exchange reaction and nucleotide reduction share common intermediates. First, the
exchange of tritium from $[5^\prime-3^\text{H}]-\text{AdoCbl}$ occurs with $k_{\text{obs}}$ of 0.3 s$^{-1}$. The rate constant for hydrogen exchange can be estimated to be $\sim6$ s$^{-1}$ by making the reasonable assumption of a selection effect of 10 on this process (due to the isotope effect on carbon-tritium bond cleavage) and a statistical correction of 2 (for the three hydrogens of the 5$'$-dA intermediate in this process). This rate constant is similar to that determined for nucleotide reduction (2 s$^{-1}$) (Booker, 1994), making it likely that the exchange reaction is a mechanistically informative process.

Second, transient-state kinetic experiments have provided direct evidence that the exchange reaction and nucleotide reduction share common intermediates that are kinetically competent in their respective transformations (Licht et al., 1996, Orme-Johnson et al., 1974, Tamao & Blakley, 1973). In the exchange reaction, both cob(II)alamin, monitored by stopped-flow (SF) UV-vis spectroscopy, and 5$'$-dA, monitored by rapid acid quench methods, are produced with observed rate constants of $\sim40$ s$^{-1}$ (Licht et al., 1996, Tamao & Blakley, 1973). Furthermore, rapid freeze quench (RFQ) EPR experiments (Licht et al., 1996, Orme-Johnson et al., 1974) under identical conditions reveal an unusual paramagnetic species generated on the same time scale. A repetition of the RFQ EPR experiments using $[\beta-2^\text{H}_2]$-cysteine labeled RTPR and simulation of the resulting spectra (Gerfen et al., 1996) indicate that the paramagnetic intermediate is a thiyl radical interacting with cob(II)alamin via exchange coupling and dipolar interactions.

Similar SF and RFQ experiments in the presence of substrate show that cob(II)alamin is formed with a $k_{\text{obs}}>200$ s$^{-1}$. The observed paramagnetic species is almost identical to that observed in the absence of substrate. These studies thus suggest that information on the role of AdoCbl in thiyl radical formation, obtained by a detailed examination of the exchange reaction, is relevant to nucleotide reduction.

In this chapter, requirements for the exchange reaction have been identified and quantified. The effects of deuteration of the solvent and the cofactor ($[5^\prime-2^\text{H}_2]-\text{AdoCbl}$) on the pre-steady-state kinetics of cob(II)alamin formation and on the amounts of cob(II)alamin formed have been determined. These studies provide additional support for the model in Scheme 2.1 (Chapter 2), and, in particular, for the importance of a thiyl radical in catalysis. The simplest interpretation of the reported isotope effect analysis is that this thiyl radical is generated by AdoCbl in a concerted fashion.
Materials and Methods

General methods.

Nucleotides, nucleosides, and NADPH were obtained from Sigma. RTPR and mutant RTPRs were purified as reported (Booker & Stubbe, 1993). Wild-type (wt) RTPR has a specific activity of 1.4 – 1.5 µmol min⁻¹ mg⁻¹ using ATP as substrate. Pre-reduced and pre-oxidized RTPR were prepared as previously described (Booker, 1994). TR and TRR were purified as previously described (Lunn et al., 1984, Russel & Model, 1985). HPLC analyses were carried out on a Beckman Model 334 system. UV-visible spectroscopy was performed on a Cary 3 or Hewlett-Packard 8452A. Scint-A was obtained from Packard. Centricon-30 microconcentrators were obtained from Millipore. SF UV-vis experiments were carried out using an Applied Photophysics DX.17MV spectrophotometer. RFQ EPR experiments were carried out using an Update Instruments System 1000.

Synthesis of [5'-3H]-AdoCbl.

Synthesis and purification of [5'-3H]-AdoCbl were as described in Chapter 2.

Preparation of [5'-2H₂]-AdoCbl.

Synthesis and purification of [5'-2H]-AdoCbl were as described in Chapter 2.

Deoxygenation of Solutions for Kinetic Experiments.

The materials (0.5-2 ml) were placed in a septum-sealed 10 mL round-bottom flask equipped with a stirrer. If 100 µL or less was to be used, a septum-sealed Eppendorf tube was used, and the stirrer was omitted. The container was purged with argon (blown over the solution) for 20 min while stirring at 0 °C. All materials were transferred via a gas-tight Hamilton syringe. For SF experiments, the syringes and sample lines were filled with 50 mM dithionite 12 h prior to the actual experiments. They were then flushed with 20 mL of 0.2 M sodium dimethylglutarate, pH 7.3, which had been deoxygenated by bubbling argon through it for 2-3 h. Reaction mixtures were transferred to the loading syringes via gas-tight syringe. Loading syringes, the windows of the drive unit, and exposed lines were covered in foil after
addition of the reaction mixtures. Argon was bubbled through the bath that controlled the temperature of the sample holding unit for 3 h preceding data acquisition and throughout the course of the experiment.

*Ability of Cysteine-to-Serine Mutants of RTPR to Catalyze Steady-State and Pre-Steady State Cob(II)alamin Formation.*

The reaction mixture included in a final volume of 600 µL: 200 mM HEPES (pH 7.5), 50-60 µM AdoCbl, 0.12 mM TR, 1 µM TRR, 1 mM NADPH, and 70-75 µM C119S or C419S RTPR. This mixture was deoxygenated, and a 450 µL aliquot of the reaction mixture was transferred via a gas-tight syringe to a septum-sealed cuvette which had been purged with argon. The mixture was equilibrated at 37 °C. The UV-vis spectrum was recorded. To initiate the reaction, a degassed solution of dGTP was added to a final concentration of 5 mM. Spectra were then recorded every 15 min for 75 min.

For SF studies under anaerobic conditions, 70 µM C419S RTPR, 20 µM TR, 1 µM TRR, 2 mM NADPH, and 1 mM dGTP in 200 mM sodium dimethylglutarate, pH 7.3 were mixed with an equal volume of the same reaction buffer containing 100 µM AdoCbl and 2 mM dGTP, and the formation of cob(II)alamin was measured by monitoring change of A525 at 37 °C.

*Kinetic and Equilibrium Isotope Effects on Cob(II)alamin Formation Using [5'-1H2]- and [5'-2H2]-AdoCbl in H2O and D2O.*

To prepare 0.5 M dimethylglutarate buffer (pD 7.3), the acid was dissolved in D2O and titrated to pH 6.9 using 5 N NaOD. For studies in D2O, non-protein components of the reaction mixture were lyophilized and re-dissolved in D2O. RTPR (20-30 mg, ~200 µL) was exchanged into D2O by dilution into 2 mL of deuterated 5 mM sodium dimethylglutarate (pD 7.3), and concentrating using a Centricon 30 apparatus. TR and TRR were exchanged the same way (~50 µL of protein solution in ~2 mL of deuterated buffer), except a Centricon 3 device was used. The total amount of H2O introduced by this exchange procedure was calculated to be <5%. Reaction mixtures were deoxygenated by purging with D2O-saturated argon.

RTPR (80-100 µM), 20 µM TR, 1 µM TRR, 2 mM NADPH, and 1 mM dGTP in 200 mM sodium dimethylglutarate, pH 7.3 (or pD 7.3) were mixed with an equal volume of the same reaction buffer containing 60-100 µM
AdoCbl (or [5'-2H2]-AdoCbl) and 1 mM dGTP, and the formation of cob(II)alamin was measured by monitoring change of A525 at 37 °C. Experiments with labeled and unlabeled coenzyme were carried out on the same day with the same batch of RTPR-containing reaction mixture.

To ensure that isotope effects were not due to the difference in pK_a between H_2O and D_2O, SF UV-vis experiments were also carried out at pH 6.9, the pH expected to be equivalent to a pD of 7.3 (Schowen & Schowen, 1982).

**Kinetic Analysis.**

Fits to single or double exponentials were carried out using the least-squares fitting program included in the Applied Photophysics operating software or with Kaleidagraph. For a single exponential, traces were fit to the equation \( A_t = A_\infty + C \times \exp(-kt) \), where \( A_t \) is the absorbance at time \( t \), \( A_\infty \) is the final absorbance, \( C \) is the total absorbance change, and \( k \) is the observed rate constant; \( A_\infty \), \( C \), and \( k \) are the adjustable parameters. For a double exponential, the equation used was \( A_t = A_\infty + C_1 \times \exp(-k_1t) + C_2 \times \exp(-k_2t) \), where \( C_1 \) and \( C_2 \) are absorbance changes and \( k_1 \) and \( k_2 \) are rate constants.

Kinetic simulations were carried out using HopKINSIM 1.3, a Macintosh version of KINSIM (Barshop et al., 1983). Simulations had as starting parameters the experimentally used concentrations of AdoCbl and RTPR, so small differences in concentration between the different isotopic experiments compared are accounted for. The greatest difference in [AdoCbl] is between experiments A and B in Table 3.1, representing ~10% of the total reactant concentration. Although a discrepancy of this size may affect the observed rates and amounts to a small extent (~10% or less) their effect is included in the simulations. A successful model will predict both the large effects arising from isotopic substitution and any smaller effects arising from differences in concentrations.

Global analysis was carried out using the program Dynafit.220 (Kuzmic, 1996). Values for rate constants not involving transfer of deuterium were obtained by global analysis of the kinetics of cob(II)alamin formation as a function of [AdoCbl] (see Chapter 4). The rapid equilibrium assumption for AdoCbl binding was accomodated by fixing the association rate constant at 10^8 M\(^{-1}\) s\(^{-1}\) (assumed to be the diffusion-controlled limit), and allowing the dissociation rate constant to vary to obtain the equilibrium constant. To accommodate the rapid equilibrium assumption for thyl radical formation in
the stepwise mechanism, the rate constant for thiol radical formation was set at 1000 s\(^{-1}\) (~100-fold greater than the rate constants for carbon-cobalt bond cleavage and re-formation), and the rate constant for re-formation of thiol was allowed to vary to obtain the equilibrium constant. Exchange of the thiol with solvent was written as a second order reaction of the enzyme with solvent (H\textsubscript{2}O or D\textsubscript{2}O), and the concentration of H\textsubscript{2}O or D\textsubscript{2}O was set at 55 M. Offsets of absorbance traces (i.e., the absorbance at time zero, which is subject to uncertainty due to the dead time of the instrument) and enzyme concentrations were allowed to vary to within 10% of their input values.


Results

Ability of Mutant RTPRs to Catalyze C-Co Bond Homolysis.

The two simplest mechanisms to account for the observed exchange reaction involve AdoCbl-mediated thyl radical formation in a concerted or a stepwise process (Scheme 2.1) (Licht et al., 1996). Mutagenesis studies have suggested that C408 is the source of this thyl radical. If thyl radical formation occurs by a stepwise mechanism, mutation of C408 to serine would not be expected to prevent carbon-cobalt bond cleavage from occurring transiently. For a concerted formation of thyl radical and cob(II)alamin, however, the difference in homolytic bond dissociation energies between O-H (119 kcal/mol) and S-H (88-91 kcal/mol) (Benson, 1978, Griller & Martinho Simoes, 1990, McMillen & Golden, 1982) might be expected to prevent such a reaction. C408S RTPR has therefore been examined by presteady state SF UV/vis spectroscopy to determine its ability to catalyze transient formation of cob(II)alamin. The results of these experiments are shown in Fig. 3.1. Monitoring at 525 nm (or from 465 to 535 nm) reveals no apparent absorbance change up to 200 ms, indicating that <0.01 equivalent of cob(II)alamin (the lower limit of detection) is generated. Thus, C408S RTPR is either unable to catalyze transient carbon-cobalt bond cleavage, or if this cleavage occurs, re-formation of the carbon-cobalt bond occurs within the dead time of the instrument (< 5 ms).

Similar presteady state as well as steady state experiments have been carried out with the C419S and C119S RTPRs. In the steady state, C119S gives no detectable change at 525 nm, while C419S gives rise to 0.36 equivalents of cob(II)alamin after 1 h at 37 °C. This slow reaction is most likely analogous to the slow wt RTPR-mediated breakdown of AdoCbl shown by Yamada et al. to occur with a rate constant of $3 \times 10^{-4}$ s⁻¹ ($t_{1/2} = 38$ min) (Yamada et al., 1971). Consistent with this interpretation is the observation that when C419S is mixed with AdoCbl in the presence of dGTP in the presteady state, <0.02 equivalents of cob(II)alamin are formed. Inability to detect cob(II)alamin in the presteady state with either of these mutants is consistent with their slow exchange rates and suggest that these exchange rates result from an inability to cleave the carbon-cobalt bond efficiently.
Fig. 3.1. Effect of the C408S mutation on carbon-cobalt bond cleavage catalyzed by RTPR. A. C408S RTPR (70 μM), 20 μM TR, 1 μM TRR, 2 mM NADPH, and 1 mM dGTP in 200 mM sodium dimethylglutarate, pH 7.3 were mixed with an equal volume of the same reaction buffer containing 109 μM AdoCbl and 1 mM dGTP, and the formation of cob(II)alamin was measured by monitoring change of $A_{525}$ at 37 °C. The wt experiment was the same, with the exception that 100 μM wt RTPR was used. The solid traces show the equivalents of cob(II)alamin formed, calculated directly from the SF absorbance trace using the difference in extinction coefficients between AdoCbl and cob(II)alamin (4800 M$^{-1}$ cm$^{-1}$).
Kinetic and Equilibrium Isotope Effects on Carbon-Cobalt Bond Homolysis.

Kinetic and equilibrium isotope effects comparing rates and amounts of cob(II)alamin formed from [5'-1H2]-AdoCbl in H2O with [5'-2H2]-AdoCbl in H2O and D2O have been carried out in an effort to distinguish between the concerted and stepwise models shown in Scheme 2.1 (Eq. 3.1a and 3.1b).

Eq. 3.1a

Eq. 3.1b

First, the rate of cob(II)alamin formation was measured with [5'-2H2]-AdoCbl in H2O and compared to the results with unlabeled AdoCbl. Tamao and Blakley have previously reported a similar experiment using dihydrolipoate as reductant and monitoring cob(II)alamin for only 200 ms (Tamao & Blakley, 1973). Fits of their data to a single exponential allowed them to report a kH/kD of 1.4. We have made a similar measurement using TR/TRR/NADPH as reductant, but have monitored the reaction for 5 s. The results are shown in Fig. 3.2. The data in the all-protonated case fit well to a single exponential, while that with [5'-2H2]-AdoCbl fit well to two exponentials (22±2 s⁻¹ and 1.3±0.2 s⁻¹). Comparison of the kobs in the first case to k1 in the second case gives an apparent kH/kD=1.7±0.2 (Table 3.1), similar to the number reported by Tamao and Blakely. However, what is strikingly evident in Fig. 3.2, and has not been reported by previous workers, is that at
Fig. 3.2. Kinetic isotope effect using [5′-2H_2]-AdoCbl as cofactor. A. A transient maximum in cob(II)alamin formation. Reaction conditions were as described in Fig. 3.1, except 120 μM wt RTPR and 100 μM unlabeled AdoCbl or 80 μM [5′-2H_2]-AdoCbl were used. The fit to a double exponential is superimposed (solid line) on the data for [5′-2H_2]-AdoCbl. Residuals of the fit are shown beneath the graph. At longer times, a slower phase of cob(II)alamin formation becomes apparent, as is described in the text.
~200 ms in the case of [5'-2H₂]-AdoCbl, 35% more cob(II)alamin is observed than in the case of unlabeled AdoCbl. Cob(II)alamin then declines slowly (~1 s⁻¹) from this maximum to give a concentration of cob(II)alamin identical to that observed in the all-protonated case. This slow decline is most reasonably associated with exchange of the deuterium from AdoCbl with solvent. Its rate constant can be compared with the rate constant for tritium washout from [5'-3H]-AdoCbl of 0.3 s⁻¹, measured in either the presteady state or steady state (Booker et al., 1994, Licht et al., 1996).

To obtain further insight into the mechanism of the exchange reaction, the SF UV-vis experiments were next carried out in D₂O with AdoCbl and D₂O with [5'-2H₂]-AdoCbl and compared with the all protonated case. The results of all of these experiments are summarized in Table 3.1. In D₂O with AdoCbl, the absorbance trace can be fit to two exponentials: one with a rate constant of 24±2 s⁻¹ accounting for 70% of the absorbance change and a second with a rate constant of 1.0±0.1 s⁻¹ accounting for the remainder of the absorbance change (Fig. 3.3). Comparison of the rate constant for the first phase with that in H₂O gives an apparent solvent isotope effect of 1.6±0.2. The rate constants and amounts of cob(II)alamin formed were the same whether the all-protonated experiment was carried out at pH 7.3 or pH 6.9 (the pH equivalent to pD 7.3), thus ruling out the possibility that the pKₐ difference between D₂O and H₂O is responsible for the observed effects (Schowen & Schowen, 1982).

When the reaction is carried out with [5'-2H₂]-AdoCbl in D₂O, the resulting trace is also best fit to two exponentials (Fig. 3.3) giving k₉obs of 15±1 s⁻¹ and 0.4±0.03 s⁻¹. Comparison of the faster rate constant with that measured for AdoCbl in H₂O gives an apparent combined solvent and cofactor isotope effect of 2.7±0.3 (Table 3.1). The implications of these results in terms of the two mechanisms under consideration will be discussed subsequently.

In addition to the differences in rate of cob(II)alamin formation, D₂O also causes a dramatic perturbation of the amounts of cob(II)alamin formed relative to the amount in the all-protonated case (Fig. 3.3). In the all-protonated case from 100 ms to 5 s, 0.2 equivalents of cob(II)alamin are present. In the D₂O case with [5'-2H₂]-AdoCbl, the formation of cob(II)alamin
Fig. 3.3. Equilibrium isotope effect observed with D$_2$O, [5'-$^2$H$_2$-AdoCbl]. A. Kinetic traces. Reaction conditions were as described in Fig. 3.1, except RTPR was 80 μM and AdoCbl (or [5'-$^2$H$_2$-AdoCbl]) was 60 μM. The trace for the D$_2$O, [5'-$^2$H$_2$-AdoCbl] experiment was fit to a double exponential. B. Residuals for the D$_2$O, [5'-$^2$H$_2$-AdoCbl] experiment.
Table 3.1. Comparison of experimental results with simulations

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<td>0.21</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>3 30</td>
<td>1.0</td>
<td>0.20</td>
<td>0.22</td>
</tr>
<tr>
<td>D. [²H₂]-AdoCbl, D₂O</td>
<td>1 15±1</td>
<td>0.5±0.1</td>
<td>0.4±0.03</td>
<td>0.04±0.01</td>
</tr>
<tr>
<td></td>
<td>2 27</td>
<td>-</td>
<td>0.29</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3 12</td>
<td>-</td>
<td>0.36</td>
<td>-</td>
</tr>
</tbody>
</table>

1 = experimental data, 2 = simulation of stepwise mechanism, 3 = simulation of concerted mechanism. Amplitudes are expressed as equivalents of cob(II)alamin. Final concentrations after mixing were: A. 60 µM RTPR, 50 µM AdoCbl, H₂O. Similar k₁ (38 s⁻¹) and amplitude (0.19 eq.) were observed and simulated with 40 µM RTPR, 30 µM AdoCbl. B. 60 µM RTPR, 40 µM [⁵⁻²H₂]-AdoCbl. C. 60 µM RTPR, 45 µM AdoCbl, D₂O. D. 40 µM RTPR, 30 µM [⁵⁻²H₂]-AdoCbl, D₂O.

is best fit by two exponentials with amplitude factors of 0.4 and 0.04, respectively. The small amplitude factor associated with the second phase suggests that it results from our inability experimentally to make this system 100% deuterated.¹

Finally, in the D₂O case with [⁵⁻¹H₂]-AdoCbl, the formation of cob(II)alamin is best fit by two exponentials with amplitude factors of 0.3 and 0.1 respectively (data not shown). The second phase can be associated with the wash-in of deuterium into the cofactor. By 5 s, 0.36 equivalents of cob(II)amin are present. This number is identical (within experimental error)

¹ The [⁵⁻²H₂]-AdoCbl used was prepared by RTPR-catalyzed wash-in of deuterium from D₂O. The incorporation was ~95% by atom, compared to ~98% by atom for D₂O.
to that observed in the all-deuterium labeled case and substantially different from the all-protonated case.

A number of possible mechanisms can account for the shift in equilibrium in favor of cob(II)alamin in the totally deuterium labeled system. The favored model is that this perturbation is associated with the influence that the low fractionation factor of sulfhydryl groups (Lienhard & Jencks, 1966, Schowen & Schowen, 1982) has on the thyl radical/thiol equilibrium. Fractionation factors of 0.4-0.6 of thiol groups indicate that transfer of deuterium from -SD to a group with fractionation factor ~1 (e.g. water or a carbon center) is thermodynamically favorable (Cleland, 1987). Thus, according to our working model for the exchange mechanism in Scheme 2.1 (Chapter 2), deuteration of C408 should destabilize the thiol state of the enzyme relative to the state that contains the thyl radical and cob(II)alamin, in which the deuterium is transferred to the methyl group of 5'-dA. Cob(II)alamin formation would therefore be more thermodynamically favorable in D₂O than in H₂O.

Rapid, Reversible Binding of AdoCbl to RTPR.

The association and dissociation rates of substrate and cofactor binding to enzymes are often observed to be rapid compared to subsequent steps (Booker et al., 1994, Fersht, 1985, Johnson, 1992). When this is the case, kinetic modeling is simplified considerably. As a preliminary step toward the kinetic modeling required to distinguish between concerted and stepwise mechanisms (Eqs. 3.1a and 3.1b), we examined the kinetics of cob(II)alamin formation at low concentrations of AdoCbl and RTPR. If the association and dissociation rates are fast compared to the rate of carbon-cobalt bond cleavage, cob(II)alamin formation will be observed immediately after mixing, with no lag phase. If the association and dissociation rates are slower than or

---

2 The fractionation factor is defined as \((D/H)_{XL}/(D/H)_{L2O}\), where \((D/H)_{XL}\) and \((D/H)_{L2O}\) are the ratios of deuterium to hydrogen at equilibrium at the group of interest and the solvent, respectively.

3 In fact, a true equilibrium between these species can never be obtained, for as outlined above, RTPR catalyzes a slow breakdown of AdoCbl (\(t_{1/2} = 38\) min). This rate is sufficiently slow, however, relative to the conditions examined, that a pseudo-equilibrium between these species can be measured. All subsequent sections refer to this pseudo-equilibrium as an equilibrium.
comparable to the rate of carbon-cobalt bond cleavage, cob(II)alamin formation will exhibit a lag phase. This lag phase will be more pronounced at low substrate concentrations, since the RTPR-AdoCbl association reaction is second-order (Johnson, 1992).

Evidence to support the validity of the rapid equilibrium assumption was initially provided by the studies of Tamao and Blakley (Tamao & Blakley, 1973). They showed that rapid mixing of RTPR (~10 μM), reductant, and dGTP with variable concentrations of AdoCbl (25 to 150 μM) and dGTP resulted in the formation of cob(II)alamin, with apparent first order rate constants in the range of 35-45 s⁻¹. At all concentrations of AdoCbl tested, the time dependence followed a single exponential. We have reproduced and extended these results. As shown in Fig. 3.4, at 12 μM AdoCbl and 3 μM RTPR, the data are well fit to a single exponential. Careful examination of the reaction time course did not reveal any lag phase in the reaction kinetics at this or other low substrate concentrations examined. The t₁/₂ for approach to equilibrium for the binding reaction must therefore be small compared to the dead time of the instrument (3-5 ms), suggesting that the kobs for binding is >>200 s⁻¹ (compared to kobs of ~40 s⁻¹ for cob(II)alamin formation). This data is thus consistent with the rapid equilibrium assumption.

The observation of monoexponential kinetics exhibiting no lag or burst phase over a range of [AdoCbl]s also has consequences for distinguishing between concerted and stepwise reactions. The stepwise mechanism, which has two steps, can give rise to monoexponential kinetics only in certain special cases, the most probable of which is slow cob(II)alamin formation followed by rapid thyl radical formation. The constraints that monoexponential kinetics put on the mechanistic possibilities will be examined in detail in the Discussion.

Kinetic Modeling Using Experimental Data to Distinguish Between a Stepwise and a Concerted Mechanism.

Numerical simulations of the kinetics of cob(II)alamin formation for both a concerted and a stepwise mechanism have been carried out in order to

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4 The [AdoCbl] dependence of the kinetics of cob(II)alamin formation has been investigated in detail (Chapter 4).
Fig. 3.4. Cob(II)alamin formation is fit well by a single exponential even at low [AdoCbl]. Reaction conditions were as described in Fig. 3.1, except RTPR was 6 μM and AdoCbl was 24 μM. A. Cob(II)alamin formation, fit to a single exponential (solid line). B. Residuals for the fit in A.
determine whether the experimental data enumerated above can make a distinction between the two. The model used for the concerted mechanism, which explicitly takes into account equilibration of the isotopomers of AdoCbl and 5'-dA, is shown in Fig. 3.5. It assumes: 1. that binding of AdoCbl is well approximated as a rapid equilibrium and that deuterium in the solvent or cofactor has no effect on the $K_d$ for this process; 2. that the allosteric effector, dGTP, is bound throughout the catalytic cycle; and 3. that rotation of the methyl group of 5'-dA is very rapid so that stereo-isotopomers are functionally indistinguishable. The last assumption requires the application of the appropriate statistical factors to the abstraction of hydrogen from mixed isotopomers of 5'-dA (e.g., a statistical factor of 1/2 on abstraction of hydrogen from CHD$_2$Ad). A fourth and final assumption is that the exchange of the C408 thiol proton with solvent occurs only from the free form of the enzyme (as would be the case if AdoCbl binding blocked solvent access to C408).

An analogous stepwise mechanism is shown in Fig. 3.6. In addition to the four assumptions mentioned above, two additional assumptions have been made. One is that abstraction of hydrogen from the C408 thiol and the corresponding reverse reaction are fast compared to carbon-cobalt bond homolysis and reformation. This assumption is justified in the discussion, where other possibilities for the stepwise mechanism have been considered and eliminated based on experimental considerations. The other additional assumption is that there is no secondary isotope effect on carbon-cobalt bond homolysis. As described in the Discussion, chemical precedent suggests that these secondary isotope effects will have a negligible effect on the observed kinetics (Abeles & Dolphin, 1976). Finally, the thiol radical/thiol equilibrium constants for mixed isotopomers ($K_8$ and $K_{12}$, $K_9$ and $K_{13}$) are increased ($K_8$ and $K_{12}$) or decreased ($K_9$ and $K_{13}$) by a factor of 2 relative to $K_{16}$. This statistical factor is required to account for the fact that the thyl radical can react to form two different products when mixed isotopomers are present, while it can form only one product when only one isotope is present.

The purpose of these simulations is to reproduce the unusual isotope effects observed experimentally in an effort to make a distinction between the two mechanistic hypotheses. Insufficient experimental data is available at present to obtain microscopic rate constants that uniquely fit the experimental time courses for cob(II)alamin formation. The latter goal can only be achieved through more extensive studies on the [AdoCbl] dependence of the
Fig. 3.5. Concerted mechanism for the exchange reaction, treating isotopomers explicitly. Although this diagram depicts linear sequences of reactions, in the kinetic simulations, equilibria were not treated as sequential. a. Binding equilibria. b. Carbon-cobalt bond cleavage and thyl radical formation reactions with mixed isotopomers (i.e., containing both H and D). c. Carbon-cobalt bond cleavage and thyl radical formation reactions with H only. d. Carbon-cobalt bond cleavage and thyl radical formation reactions with D only. e. Solvent exchange.
Fig. 3.6. Stepwise mechanism for the exchange reaction, treating isotopomers explicitly. As in Fig. 3.5, linear sequences of reactions are not meant to imply an obligate order for isotope exchange.  

a. Binding equilibria.  
b. Carbon-cobalt bond cleavage and thiyl radical formation reactions with mixed isotopomers (i.e., containing both H and D).  
c. Carbon-cobalt bond cleavage and thiyl radical formation reactions with H only.  
d. Carbon-cobalt bond cleavage and thiyl radical formation reactions with D only.  
e. Solvent exchange.
observed isotope effects on cob(II)alamin formation and analysis of the isotopomers of 5'-dA using rapid acid quench methods.

The starting point for the simulations involved obtaining reasonable values for rate constants in the stepwise and concerted reactions with unlabeled AdoCbl in H$_2$O by reproducing the observed $A_{525nm}$ vs. time traces. The microscopic rate constants used are also consistent with the dependence of the rate of cob(II)alamin formation on AdoCbl concentration, which sets additional constraints on the possible rate constants.

For the concerted mechanism, three additional constraints are required to successfully model the experimental data with rate constants and isotope effects that are chemically reasonable. First, only primary isotope effects less than 7-fold have been considered. This is the upper limit for classical deuterium primary isotope effects (Cleland et al., 1977, Westheimer, 1961). Second, since the ratio of the isotope effect on carbon-cobalt bond homolysis/thiyl radical formation and the isotope effect on carbon-cobalt bond re-formation/thiol reformation should be equal to the thiol fractionation factor, this ratio has been constrained to be 0.4-0.6, in keeping with the range of literature values (Schowen & Schowen, 1982). Third, the rate constant of proton exchange for the C408 thiol with bulk solvent ($k_{+13}$ or $k_{-13}$ in Fig. 3.5) has been constrained to be $\leq 5$ s$^{-1}$. In varying this rate constant from 2 s$^{-1}$ to 500 s$^{-1}$, the best fits, particularly for the case with unlabeled AdoCbl and D$_2$O, were observed with values $\leq 5$ s$^{-1}$. This constraint is reasonable given the experimentally measured rate constant of 0.3 s$^{-1}$ for tritium washout observed in presteady state and steady state experiments (Booker et al., 1994, Licht et al., 1996). The intrinsic rate of proton exchange between the C408 thiol can be calculated to be $\sim 10^3$ s$^{-1}$ based on a pKa of 8.5 (Jencks, 1969). The difference of 200-fold between the proposed exchange rate and the intrinsic exchange rate would require that the cysteine be shielded from the solvent in the free form of the enzyme. This shielding effect has substantial precedent in exchange rates of amide protons in folded proteins, which vary by a factor of 10$^4$ (Englander & Kallenbach, 1984).

The best fit to the experimental data in the four sets of experiments described above (unlabeled AdoCbl and H$_2$O, [5'-2H$_2$]-AdoCbl and H$_2$O, unlabeled AdoCbl and D$_2$O, and [5'-2H$_2$]-AdoCbl and D$_2$O) has been obtained with the values in Table 3.2 for the concerted mechanism. Computing isotope effects from these rate constants gives an isotope effect of 1.7 on
Table 3.2. Rate constants employed in simulation of the concerted mechanism.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>K₁</td>
<td>4000 M⁻¹</td>
<td>k₉⁺</td>
</tr>
<tr>
<td>K₂</td>
<td>4000 M⁻¹</td>
<td>k₉</td>
</tr>
<tr>
<td>K₃</td>
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</tr>
<tr>
<td>K₄</td>
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<td>k₁₀⁻</td>
</tr>
<tr>
<td>K₅</td>
<td>4000 M⁻¹</td>
<td>k₁₁⁺</td>
</tr>
<tr>
<td>K₆</td>
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</tr>
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<td>k₇⁻</td>
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<tr>
<td>k₈⁺</td>
<td>4.3 s⁻¹</td>
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<td>k₈⁻</td>
<td>32.4 s⁻¹</td>
<td>k₁₃⁻</td>
</tr>
</tbody>
</table>

See Fig. 3.5 for definitions of rate and equilibrium constants. For simulation of reactions in H₂O, k₁₃⁺ was 0 s⁻¹, and k₁₃⁻ was 5 s⁻¹.

carbon-cobalt bond homolysis/thiyl radical formation (k₉⁺₁₁/k₉⁺₁₂ in Fig. 3.5) and an isotope effect of 4.2 on carbon-cobalt bond re-formation/thiol reformation (k₁₁⁻/k₁₂⁻). The magnitude of these isotope effects is consistent with moderate primary isotope effects, indicating that violating the constraint that isotope effects should be ≤7 is not required to obtain a reasonable fit. These calculated rate constants also give an equilibrium isotope effect of 2.5 (fractionation factor for the thiol of 0.4), which is the highest value within the range tested (2.0–2.5).

The best fit to the data for the stepwise mechanism was obtained with the parameters in Table 3.3, which also correspond to an equilibrium isotope effect of 2.5. Here, the equilibrium isotope effect is the ratio of K₁₈/K₁₆ (Fig. 3.6).

The isotope effects on rates and amounts of cob(II)alamin formation predicted by the simulations of the stepwise and concerted mechanisms, in comparison with the experimental data, are shown in Figs. 3.7 and 3.8 and summarized in Table 3.4. Both the stepwise and concerted mechanisms qualitatively predict a transient maximum of cob(II)alamin in the experiment with [5⁺²H₂]-AdoCbl in H₂O (Fig. 3.7) and an increase in the equilibrium
Fig. 3.7. Comparison of stepwise and concerted simulations with experimental data for [5'-2H2]-AdoCbl in H2O. Eq. cob(II)[D]/eq. cob(II)[H]∞ is the ratio of the amount of cob(II)alamin formed with [5'-2H2]-AdoCbl at time t and the amount formed at equilibrium in the all-protonated experiment. The solid bar indicates the experimental error in the maximum eq. cob(II)[D]/eq. cob(II)[H]∞. Both stepwise and concerted mechanism qualitatively reproduce the transient maximum observed in cob(II)alamin formation. However, as shown in Table 3.1, the rate constant for the initial formation of cob(II)alamin (κ1 in Table 3.1) is better fit by the concerted mechanism than the stepwise mechanism.
eq. cob(II)/final eq. cob(II)

- - - concerted
- - - stepwise
- - - exp. data
Fig. 3.8. Comparison of stepwise and concerted simulations with experimental data for [5′-2H₂-AdoCbl] in D₂O. Eq. cob(II)[D,D]/eq. cob(II)[H,H]∞ is the ratio of the amount of cob(II)alamin formed with both [5′-2H₂]-AdoCbl and D₂O and the amount formed in the all-protonated experiment at equilibrium. The solid bar indicates the experimental error in the maximum eq. cob(II)[D,D]/eq. cob(II)[H,H]∞. The concerted mechanism accounts for the increase in cob(II)alamin formation in D₂O vs. H₂O to within experimental error, while the stepwise mechanism does not. In addition, as shown in Table 3.1, the concerted mechanism also predicts the kinetic isotope effect observed, while the stepwise mechanism does not.
concentration of cob(II)alamin in D₂O vs. H₂O (Fig. 3.8). However, the stepwise mechanism fails to account for both the observed kinetic isotope effects and the magnitude of the equilibrium solvent effect (Fig. 3.8). The concerted mechanism accounts for all observed kinetic isotope effects within experimental error (<1.5 standard deviations). In addition, it more faithfully reproduces the isotope effect on equilibrium amounts of cob(II)alamin.

Table 3.3. Rate constants employed in simulation of the stepwise mechanism.

<table>
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<tr>
<th>K₁</th>
<th>4000 M⁻¹</th>
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</tr>
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<tr>
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<td>k⁻₁₄</td>
<td>55 s⁻¹</td>
</tr>
<tr>
<td>K₅</td>
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<td>k⁺₁₅</td>
<td>55 s⁻¹</td>
</tr>
<tr>
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</tr>
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<td>K₁₆</td>
<td>1</td>
</tr>
<tr>
<td>k⁻₇</td>
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<td>k⁺₁₇</td>
<td>55 s⁻¹</td>
</tr>
<tr>
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<td>k⁻₁₇</td>
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<tr>
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<td>k⁺₁₉</td>
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<tr>
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<tr>
<td>k⁻₁₁</td>
<td>55 s⁻¹</td>
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</table>

See Fig. 3.6 for definitions of rate and equilibrium constants. For simulation of reactions in H₂O, k⁺₁₉ was 0 s⁻¹, and k⁻₁₉ was 5 s⁻¹.

Rate constants and amplitudes predicted by the two mechanisms for representative sets of conditions are shown in Table 3.1. For the concerted mechanism, most of the rate constants and amplitudes fit to within 30% of the observed values. Less well fit are k₂ for the experiment with [5′⁻²H₂]-AdoCbl in H₂O and the relative amplitudes for the experiment with unlabeled AdoCbl in D₂O (although the total equilibrium amount of cob(II)alamin formed agrees well with the experimental data). Clearly, more detailed kinetic studies are required to determine the actual isotope effects on microscopic steps. However, the kinetic simulations suggest that a simple
Table 3.4. Summary of observed and predicted isotope effects

<table>
<thead>
<tr>
<th></th>
<th>$H_{k_1}/D_{k_1}$, AdoCbl$^a$</th>
<th>$H_{k_1}/D_{k_1}$, Solvent$^b$</th>
<th>$H_{k_1}/D_{k_1}$, AdoCbl + Solvent$^c$</th>
<th>Overshoot$^d$</th>
<th>Cob(II)$_D$/Cob(II)$_H$ $^e$</th>
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</thead>
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<td>exp. data</td>
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<td>1.6±0.2</td>
<td>2.7±0.3</td>
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<td>2.1±0.4</td>
</tr>
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<td>1.5</td>
<td>1.2</td>
<td>1.45</td>
</tr>
<tr>
<td>concerted</td>
<td>1.5</td>
<td>1.4</td>
<td>3.1</td>
<td>1.6</td>
<td>1.8</td>
</tr>
</tbody>
</table>

a. $H_{k_1}/D_{k_1}$, AdoCbl is the rate constant for cob(II)alamin formation observed in the all-protonated case divided by the rate constant of the first kinetic phase observed with [5'-2H]-AdoCbl at comparable enzyme and cofactor concentration; i.e., the ratio of $k_1$, A and $k_1$, B in Table 3.1.

b. $H_{k_1}/D_{k_1}$, Solvent is the rate constant for cob(II)alamin formation observed in the all-protonated case divided by the rate constant of the first kinetic phase observed with D$_2$O at comparable enzyme and cofactor concentration; i.e., the ratio of $k_1$, A and $k_1$, C in Table 3.1.

c. $H_{k_1}/D_{k_1}$, AdoCbl + Solvent is the rate constant for cob(II)alamin formation observed in the all-protonated case divided by the rate constant of the first kinetic phase observed with [5'-2H]-AdoCbl and D$_2$O at comparable enzyme and cofactor concentration; i.e., the ratio of ; i.e., the ratio of $k_1$, A and $k_1$, D in Table 3.1, except the rate constant and amplitude for A are those observed with 40 µM RTPR, 30 µM AdoCbl (38 s$^{-1}$, 0.19 eq. cob(II)alamin.

d. Overshoot is the maximum [cob(II)alamin] observed with [5'-2H$_2$]-AdoCbl divided by the maximum [cob(II)alamin] observed in the all-protonated case at comparable enzyme and cofactor concentrations; this is approximately equal to the ratio of Amp. 1, B and Amp. 1, A in Table 3.1.

e. Cob(II)$_D$/Cob(II)$_H$ is the maximum [cob(II)alamin] formed in the all-deuterated case divided by the maximum [cob(II)alamin] formed in the protonated case at the same enzyme and cofactor concentrations; i.e., the ratio of Amp. 1 + Amp. 2, D and Amp. 1, A in Table 3.1, except the rate constant and amplitude for A are those observed with 40 µM RTPR, 30 µM AdoCbl (38 s$^{-1}$, 0.19 eq. cob(II)alamin.

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concerted mechanism can account for a variety of isotope effects, both on rates and amounts of cob(II)alamin formed, while a stepwise mechanism cannot.

**Global analysis of isotope effect data to obtain best fit rate and equilibrium constants.**

The concerted model used for simulations provided a reasonable fit for the observed isotope effects, but did not provide fits that overlaid the experimental kinetic traces. This is probably due in part to the difficulty of finding a global best fit by iterative simulation when each new set of parameters is entered by hand. Another reason for the difficulty in getting a global best fit from the simulations is that most of the rate constants in this model are constrained in one way or another based on chemical considerations (e.g. statistical factors, fractionation factors, etc.). It was therefore of interest to use a computer program to determine the rate constants that provide a global best fit to all of the isotope effect data. The results could then be compared with the predictions of the simplified concerted mechanism (with the constraints described above) to see to what extent the global best fit rate constants support the assumptions made from chemical considerations.

Global analysis of kinetic data allows non-linear least squares regression analysis of an entire set of data simultaneously to find the parameters that best fit all the data (Beechem, 1992). In this case, global analysis of the isotope effect data was carried out using the program DynaFit 2.2 (Kuzmic, 1996). First, best fit rate constants for the experiments with unlabeled cofactor and H₂O were determined by global fitting of stopped-flow traces obtained over a range of [AdoCbl] (10-500 ÌΜ). This method allowed direct determination of values for K₁, k₁₁₁, and k₁₁ (Figs. 3.5 and 3.9). All rate constants involving transfer of deuterium were allowed to vary as adjustable parameters. Sets of data representing all four sets of isotopic conditions (AdoCbl with H₂O, [5'-2H₂]-AdoCbl with H₂O, AdoCbl with D₂O, [5'-2H₂]-AdoCbl with D₂O) were fit simultaneously. The fits to the data for the concerted mechanism are shown in Fig. 3.9, and the best fit parameters are in Table 3.5. As illustrated in Fig. 3.9, the concerted mechanism provides a reasonable fit to all the data.

The same procedure was used for fitting the same data to the stepwise mechanism (Fig. 3.10). For reasons described in the Discussion, thyl radical
Fig. 3.9. Global best fit of the concerted mechanism to presteady state isotope effect data. The fits are shown as dashed lines. (A): Unlabeled AdoCbl, H$_2$O (experimental conditions as in Fig. 3.2). (B): [5'-2H$_2$]-AdoCbl, H$_2$O (experimental conditions as in Fig. 3.2). (C): AdoCbl, D$_2$O (experimental conditions as in Fig. 3, except 60 μM AdoCbl was used in place of [5'-2H$_2$]-AdoCbl). (D): [5'-2H$_2$]-AdoCbl, D$_2$O (experimental conditions as in Fig. 3). Each tick mark represents 0.2 eq. of cob(II)alamin. Traces are shown offset from each other for clarity. The residuals are derived from the complete global analysis, which involved simultaneous fitting of seven traces.
Fig. 3.10. Global best fit of the stepwise mechanism to presteady state isotope effect data. The fits are shown as dashed lines. (A): Unlabeled AdoCbl, H₂O (experimental conditions as in Fig. 3.2). (B): [5'-2H₂]-AdoCbl, H₂O (experimental conditions as in Fig. 3.2). (C): AdoCbl, D₂O (experimental conditions as in Fig. 3, except 60 µM AdoCbl was used in place of [5'-2H₂]-AdoCbl). (D): [5'-2H₂]-AdoCbl, D₂O (experimental conditions as in Fig. 3). Each tick mark represents 0.2 eq. of cob(II)alamin. Traces are shown offset from each other for clarity. The residuals are derived from the complete global analysis, which involved simultaneous fitting of seven traces.
Table 3.5. Best fit rate constants for the concerted mechanism from global analysis

<table>
<thead>
<tr>
<th>$K_1$</th>
<th>4000 M$^{-1}$</th>
<th>$k_{+9}$</th>
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<tr>
<td>$K_2$</td>
<td>4000 M$^{-1}$</td>
<td>$k_{9}$</td>
<td>13 s$^{-1}$</td>
</tr>
<tr>
<td>$K_3$</td>
<td>4000 M$^{-1}$</td>
<td>$k_{+10}$</td>
<td>~1 s$^{-1}$</td>
</tr>
<tr>
<td>$K_4$</td>
<td>4000 M$^{-1}$</td>
<td>$k_{-10}$</td>
<td>42 s$^{-1}$</td>
</tr>
<tr>
<td>$K_5$</td>
<td>4000 M$^{-1}$</td>
<td>$k_{+11}$</td>
<td>60 s$^{-1}$</td>
</tr>
<tr>
<td>$K_6$</td>
<td>4000 M$^{-1}$</td>
<td>$k_{-11}$</td>
<td>28 s$^{-1}$</td>
</tr>
<tr>
<td>$k_{+7}$</td>
<td>60 s$^{-1}$</td>
<td>$k_{+12}$</td>
<td>42 s$^{-1}$</td>
</tr>
<tr>
<td>$k_{-7}$</td>
<td>~7 s$^{-1}$</td>
<td>$k_{12}$</td>
<td>6 s$^{-1}$</td>
</tr>
<tr>
<td>$k_{+8}$</td>
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</tr>
<tr>
<td>$k_{-8}$</td>
<td>42 s$^{-1}$</td>
<td>$k_{-13}$</td>
<td>0 s$^{-1}$</td>
</tr>
</tbody>
</table>

Rate constants are as defined in Fig. 3.5. For simulation of reactions in H$_2$O, $k_{+19}$ was 0 s$^{-1}$, and $k_{-19}$ was 5 s$^{-1}$.

formation was assumed to be much faster than carbon-cobalt bond homolysis. Therefore, thyl radical formation was modeled as a rapid equilibrium. As described above, best fit rate and equilibrium constants for the experiments with unlabeled cofactor and H$_2$O were determined by global fitting of stopped-flow traces obtained over a range of [AdoCbl] (10-500 μM), $^6$ determining values for the rate and equilibrium constants $K_1$, $k_{+15}$, $k_{-15}$, and $K_{16}$ (Fig. 3.6). With these rate constants fixed, $K_8$, $K_9$, $K_{12}$, $K_{13}$, and $K_{18}$, the only parameters involving transfer of deuterium, were allowed to vary. The fits are shown in Fig. 3.10, and the best fit parameters are summarized in Table 3.6. Where the fit does not define the parameter to within a factor of 2 (i.e., error >100%), no value is shown. The stepwise mechanism does not allow global fitting of all the isotope effect data. While none of the fits to the data are as good as for the concerted mechanism, the fit fails most obviously for the experiments with unlabeled cofactor and D$_2$O (Fig. 3.10c).

EPR Evidence for the Nature of Paramagnetic Intermediates.

EPR spectroscopy may also be useful in distinguishing between a stepwise and concerted mechanism. In a stepwise mechanism for example,
Table 3.6. Best fit rate constants for the stepwise mechanism from global analysis.

<table>
<thead>
<tr>
<th></th>
<th>5000 M⁻¹</th>
<th></th>
<th>63 s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₁</td>
<td></td>
<td>K₁₂</td>
<td></td>
</tr>
<tr>
<td>K₂</td>
<td>5000 M⁻¹</td>
<td>K₁₃</td>
<td></td>
</tr>
<tr>
<td>K₃</td>
<td>5000 M⁻¹</td>
<td>K₊₁₄</td>
<td>63 s⁻¹</td>
</tr>
<tr>
<td>K₄</td>
<td>5000 M⁻¹</td>
<td>K₋₁₄</td>
<td>55 s⁻¹</td>
</tr>
<tr>
<td>K₅</td>
<td>5000 M⁻¹</td>
<td>K₊₁₅</td>
<td>55 s⁻¹</td>
</tr>
<tr>
<td>K₆</td>
<td>5000 M⁻¹</td>
<td>K₋₁₅</td>
<td>63 s⁻¹</td>
</tr>
<tr>
<td>K₇</td>
<td>55 s⁻¹</td>
<td>K₁₆</td>
<td>1.3</td>
</tr>
<tr>
<td>K₈</td>
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<td>K₊₁₇</td>
<td>55 s⁻¹</td>
</tr>
<tr>
<td>K₉</td>
<td>~10</td>
<td>K₋₁₇</td>
<td>63 s⁻¹</td>
</tr>
<tr>
<td>K₁₀</td>
<td>~0.2 s⁻¹</td>
<td>K₁₈</td>
<td>8</td>
</tr>
<tr>
<td>K₊₁₀</td>
<td>63 s⁻¹</td>
<td>K₊₁₉</td>
<td>~10 s⁻¹</td>
</tr>
<tr>
<td>K₋₁₀</td>
<td>55 s⁻¹</td>
<td>K₋₁₉</td>
<td>0 s⁻¹</td>
</tr>
</tbody>
</table>

Rate constants are as defined in Fig. 3.6. The standard errors on K₁₂ and K₁₃ (used as a qualitative measure of uncertainty (Kuzmic, 1996) ) are >200%, indicating that these parameters are not meaningfully determined in this analysis. For simulation of reactions in H₂O, k₊₁₉ was 0 s⁻¹, and k₋₁₉ was ~10 s⁻¹.

5'-dA• could be detectable as an intermediate if the rate of thiol radical formation was sufficiently slow to allow its accumulation. RFQ-EPR experiments with [5'-²H₂]-AdoCbl were therefore carried out to investigate this possibility. In agreement with previous studies of Orme-Johnson, et al. (Orme-Johnson et al., 1974), no perturbation of the EPR spectrum quenched at 28 ms (compare top and bottom spectra in Fig. 2.3, Chapter 2) is apparent. Simulations of these spectra using a carbon-centered radical in place of a thiol radical (as recently observed in the case of several AdoCbl dependent enzymes that catalyze rearrangements) (Beatrix et al., 1995, Keep et al., 1993, Michel et al., 1992, Padmakumar & Banerjee, 1995, Zelder et al., 1994, Zhao et al., 1994) indicate that if the 5'-dA• were 10 % of the observed paramagnetic species, it
should have been detected. These results contrast with those from RFQ experiments carried out with RTPR in which all cysteines have been [β–2H₂]-labeled (compare top and bottom spectra in Fig. 2.4, Chapter 2), where a marked sharpening of hyperfine features associated with cob(II)alamin is detectable (Licht et al., 1996). These experiments are thus consistent with the hypothesis that 5′-dA• is not a required intermediate. The possibility of hydrogen atom transfer from C408 to a putative 5′-dA• during the freeze quenching process, however, cannot be excluded.
Discussion

*Importance of C408 in the Exchange Reaction.*

Our model for the exchange reaction (Scheme 2.1, Chapter 2) postulates a unique role for C408. Our detailed studies of this process using a variety of reductants and site-directed mutants (Booker, 1994) support this proposal and eliminate previous proposals that invoked the requirement for and direct involvement of the reductant (Hogenkamp et al., 1968, Ong et al., 1992). Furthermore, these studies suggest that carbon-cobalt bond homolysis and formation of the thyl radical are subtly dependent on the protein conformation, which in turn is influenced by active site cysteine conformation and/or allosteric binding of reductant. The rate constants and requirements for the exchange process, suggest, as we originally proposed (Licht et al., 1996), that it is an excellent model for the early stages in the nucleotide reduction process. Hence the detailed analysis of this simplified system was undertaken.

*Equilibrium Solvent and Cofactor Isotope Effects as Evidence for a Thyl Radical in the Exchange Reaction.*

Further evidence that supports the role of the thyl radical in the exchange process comes from the observation that D₂O perturbs the amount of cob(II)alamin formed at equilibrium (Fig. 3.3) by a factor of ~2 relative to H₂O. The simplest explanation attributes this phenomenon to the fractionation factor of 0.4 to 0.6 associated with thiols (Jencks, 1969, Schowen & Schowen, 1982).

The hypothesis that this equilibrium effect arises from deuteration of C408 is also consistent with the observation of a transient maximum in cob(II)alamin formation when [5′-²H₂]-AdoCbl is studied in similar experiments (Fig. 3.2). If C408 is relatively inaccessible to solvent, more than one cycle of carbon-cobalt bond cleavage/thyl radical formation and carbon-cobalt bond re-formation/thiol regeneration might occur before the hydrogen (deuterium) of the C408 thiol exchanges with the solvent. The deuterium would thus fractionate between AdoCbl and the C408 thiol. This process would initially favor formation of the thyl radical and cob(II)alamin over re-formation of the carbon-cobalt bond.
These effects suggest that the residue that interacts directly with AdoCbl is a cysteine, consistent with the EPR data obtained by the RFQ method and isotopically [β-²H]cysteine labeled RTPR (Chapter 2) (Licht et al., 1996). Furthermore, qualitatively similar equilibrium isotope effects are observed in the presence of substrate, suggesting that a thyl radical is also an intermediate in nucleotide reduction, again in accord with freeze quench EPR results. Obtaining evidence for a thyl radical intermediate from a continuous method carried out at a physiological temperature strongly supports the idea that this species is a true catalytic intermediate and not an artifact of the freeze-quenching method.

*Mechanism of Thiyl radical Formation: Concerted vs Stepwise.*

The issue of whether carbon-cobalt bond cleavage and thyl radical formation occur in a concerted or stepwise fashion is relevant to explaining how the enzyme can accelerate carbon-cobalt bond homolysis by the factor of $10^{10}$ required for the exchange reaction. The concerted mechanism is appealing because it helps explain how RTPR catalyzes two reactions expected to be thermodynamically unfavorable. The homolytic bond dissociation energy of AdoCbl is reported to be ~30 kcal/mol in solution (Finke & Hay, 1984; Halpern et al., 1984), which would make carbon-cobalt bond very endergonic in a stepwise mechanism. However, coupling this step to C-H bond formation and S-H bond cleavage would be expected to reduce the enthalpy cost by ~10 kcal/mol, since since the homolytic bond dissociation energy of S-H is 88-91 kcal/mole, while that of C-H of a CH₃ is ~ 100 kcal/mole (Benson, 1976; Griller & Martinho Simoes, 1990, McMillen & Golden, 1982). Similarly, coupling the thyl radical's unfavorable re-abstraction of hydrogen from the methyl group of 5'-dA to carbon-cobalt bond re-formation would be expected to result in a net negative enthalpy change. This coupling is not possible in the stepwise mechanisms.

Two approaches have been taken to address the question of whether the reaction proceeds in a concerted or stepwise fashion (Eq. 3.1a and 3.1b). The first approach examines the ability of C408S RTPR to catalyze homolysis of the carbon-cobalt bond. The second makes an attempt to quantitatively explain the observed isotope effects in terms of microscopic rate constants using reasonable chemical and biochemical assumptions based on what is known about this system.
To organize the different possibilities for the kinetics of the stepwise mechanism, three cases have been considered: case A, in which carbon-cobalt bond cleavage is much faster than abstraction of hydrogen from C408; case B, in which the abstraction of hydrogen from C408 is much faster than carbon-cobalt bond cleavage; and case C, in which the two steps occur with comparable rates.

*The Inability of C408S RTPR to Catalyze Co-C Bond Cleavage is Consistent with a Concerted Mechanism.*

The observation that making hydrogen atom abstraction energetically unfavorable (O-H vs S-H, 120 vs 88-91 kcal/mole) also makes carbon-cobalt bond cleavage unfavorable suggests that these two reactions take place in a single step. Alternatively, this phenotype is consistent with a stepwise mechanism in which the first step (carbon-cobalt bond cleavage) is thermodynamically unfavorable, while the second step (thiyl radical formation) is thermodynamically more favorable, and drives the reaction toward products. The phenotype of the C408S mutant is thus consistent with either the concerted mechanism or the stepwise mechanism, case B (Fig. 3.1). This result argues against cases A and C of the stepwise mechanism, as these cases would require thiyl radical formation to be thermodynamically much more favorable than carbon-cobalt bond homolysis, but also slower (case A) or not significantly faster (case C) than that step. However, model systems suggest that the activation barrier for thiyl radical formation is not large. Hydrogen abstraction from thiols by carbon-centered radicals occurs at diffusion-controlled rates (~10^8 M^{-1} s^{-1}) in model systems (Akhlaq et al., 1987), and hydrogen abstraction from 2-propanol by penicillamine thiyl radical occurs with a rate constant of ~10^3-10^4 M^{-1} s^{-1} (Schöneich et al., 1989). Thus, with even modest effective concentrations of ~1 M for 5'-dA• and thiyl radical at the active site, the rate of approach to equilibrium would be at ~10^4 s^{-1}, three orders of magnitude larger than the observed rate constant. It is thus difficult to rationalize why the approach to equilibrium for a thermodynamically favorable hydrogen atom abstraction step in the stepwise mechanism could be slower than or comparable in rate to carbon-cobalt bond cleavage, unless a slow protein conformational change were required for this step to occur. However, if a conformational change were rate-limiting in thiyl radical formation, it would be difficult to explain the magnitude
observed of the isotope effects (Table 3.4), as there would be only a secondary isotope effect on carbon-cobalt bond homolysis and no isotope effect on thiy1 radical formation.

*Presteady State Kinetic and Spectroscopic Experiments Argue Against Stepwise Mechanisms A and C.*

Cob(II)alamin formation in the presteady state is observed to proceed without an observable lag phase with a time course that can be fit well to a single exponential (Fig. 3.4). As described in the results section, this observation indicates that binding of AdoCbl to RTPR is rapid compared to cob(II)alamin formation. It also argues against cases A and C of the stepwise mechanism. The stepwise mechanism, case A, would predict biphasic kinetics in which a burst is observed, followed by a slower increase in cob(II)alamin formation. Case C would also predict biexponential behavior, unless the rate constants for the two steps happened to be equal or very close to equal. Under conditions designed to maximize detection of this phenomenon (i.e., low [AdoCbl] and [RTPR]), only a single exponential is required to fit the data (Fig. 3.4) (see Chapter 4).

EPR evidence provides another argument against the hypotheses of a stepwise reaction with approximately equal barriers (case C) or with thiy1 radical formation much slower than carbon-cobalt bond homolysis (case A). In both of these cases, 5'-dA• should be observed in the steady-state. However, RFQ EPR experiments carried out using [5'-2H2]-AdoCbl provide no evidence for formation of 5'-dA• as a discrete intermediate (Fig. 3.11) (Orme-Johnson et al., 1974; Licht et al., 1996). Given the experimental evidence and the chemical precedents, the most plausible possibility for the stepwise mechanism is thus case B, in which hydrogen atom abstraction is much faster than cobalt-carbon bond homolysis and reformation.

*Solvent and Cofactor Kinetic Isotope Effects on Cob(II)alamin Make Distinct Predictions about Concerted and Stepwise Mechanisms.*

In order to distinguish the concerted reaction from the stepwise mechanism, case B, kinetic isotope effects have been measured with [5'-2H2]-AdoCbl, D2O, and both [5'-2H2]-AdoCbl and D2O. The two mechanisms predict different isotope effects on microscopic steps, which in turn predict different observed kinetic and equilibrium isotope effects.
The stepwise mechanism, case B (Fig. 3.6), predicts that only secondary kinetic isotope effects would be observed on the carbon-cobalt bond cleavage reaction itself, but that an isotope effect of up to 2.5 might be observed on the equilibrium between 5'-dA• and the thiol radical (Schowen & Schowen, 1982). It is thus likely that equilibrium effects on K8, K9, K12, K13 (Fig. 3.6) would have the largest effect on the observed rate and equilibrium constants. The upper limit for a secondary isotope effect is ~1.25 per deuterium (Cleland, 1987). The carbon-cobalt bond homolysis reaction involves a change in hybridization from sp³ to sp², and thus would be expected to exhibit a normal secondary effect on this process. Reformation of the carbon-cobalt bond involves rehybridization from sp³ to sp² and would be expected to exhibit an inverse effect. These two steps in concert would thus be expected to reduce the observed rate of approach to equilibrium only modestly. In addition, these secondary isotope effects would produce an equilibrium isotope effect favoring AdoCbl over cob(II)alamin and 5'-dA•, the opposite of the experimental observation. The possibility that this isotope effect is due to a magnetic isotope effect (Harkins & Grissom, 1995), which would reflect the efficiency of cage escape vs. geminate recombination, has been considered and discarded due to the expected small magnitude of its contribution. Thus, effects on K8, K9, K12, and K13 (Fig. 3.6) seem the most likely to be the source of observed isotope effects for case B. The deuterium initially present in AdoCbl should increase K8, 1/K9, K12, and 1/K13 due to the low fractionation factor of the thiol group, as discussed above. Depending on what the Kd for AdoCbl is, this could lead to an equilibrium isotope effect of up to 2.5 on this step; the greater the extent to which the binding equilibrium is saturated, the more fully the maximum equilibrium isotope effect will be expressed.

The concerted mechanism, in contrast, predicts that primary isotope effects can affect all the bond-breaking steps. When deuterium is present on the thiol of C408, this mechanism predicts a primary kinetic isotope effect on k_{+11} and the other rate constants describing carbon-cobalt bond homolysis/thiol radical formation (Fig. 3.5). When deuterium is present at the 5' position of 5'-dA, this mechanism predicts a primary kinetic isotope effect on k_{-11} and the other rate constants describing carbon-cobalt bond reformation/thiol radical re-formation (Fig. 3.5). This mechanism also predicts an equilibrium isotope effect (K_{11}/K_{12}, Fig. 3.5) of 2.0–2.5 due to the low fractionation factor of the thiol group (Schowen & Schowen, 1982).
These considerations, in addition to the other constraints and assumptions mentioned in the Results section, were used to narrow the range of rate constants used for numerical simulations of the kinetics of cob(II)alamin formation. In order to make quantitative predictions about how isotope effects on individual steps will affect the observed rate constants, kinetic simulations of the stepwise and concerted mechanisms were carried out using the program HopKINSIM. The differences in the kinetics predicted by these simulations have been shown, as outlined below, to allow discrimination between the two mechanisms.

**Numerical Simulations Allow Discrimination Between the Concerted and Stepwise Mechanisms.**

Initially, kinetic simulations were used to examine how the different isotope effects expected for the concerted and stepwise mechanism would be expected to affect the observed rates and amounts of cob(II)alamin formation. In order to simplify kinetic modeling, AdoCbl binding was approximated as a rapid equilibrium for both stepwise and concerted mechanisms, as justified above. Parameters were varied within the constraints of statistical factors and plausible isotope effects to obtain rate constants that provided qualitative fits to the data. The rate constants and amplitudes of cob(II)alamin formation predicted by the two mechanisms are shown in Figs. 3.7 and 3.8 for two of the isotopic experiments performed. Ratios of kinetic parameters obtained from simulations of pairs of isotopic experiments were compared to the ratios of kinetic parameters actually observed in those experiments (Table 3.1, primary data, and Table 3.4, summary).

The results in Table 3.4 show that the stepwise mechanism predicts kinetic isotope effects that are systematically lower than those observed experimentally in all three isotopic substitution experiments. The stepwise mechanism also predicts a smaller equilibrium isotope effect on cob(II)alamin formation than is experimentally observed. In contrast, the concerted mechanism (Table 3.4) predicts kinetic isotope effects that agree with those observed within experimental error (≤1.5 standard deviations) and also accounts for the observed equilibrium isotope effect.

The success of the concerted mechanism in predicting the observed isotope effects can be explained qualitatively in terms of the statistical factors and isotope effects on individual steps initially used as constraints in the
simulations. When [5'-2H2]-AdoCbl and D2O are present (Fig. 3.8), the primary isotope effects on carbon-cobalt bond homolysis (k\textsubscript{+11}/k\textsubscript{+12} = 1.7 in the best fit HopKINSIM simulation) and reformation (k\textsubscript{-11}/k\textsubscript{-12} = 4.2 in the best fit HopKINSIM simulation) produce an observed kinetic isotope effect of ~3. The equilibrium isotope effect on thyl radical formation (~2.5, based on the HopKINSIM simulations) manifests itself in the observed difference between amounts of cob(II)alamin formed in H2O and D2O (~2). In the case when [5'-2H2]-AdoCbl and H2O are present (Fig. 3.7), the transient maximum of cob(II)alamin formation comes about because initially, carbon-cobalt bond homolysis/thyl radical formation requires hydrogen atom abstraction, while the carbon-cobalt bond reformation/thiol formation reaction requires deuterium abstraction 2/3 of the time. This situation favors cob(II)alamin formation. In addition, as discussed above, the equilibrium isotope effect also contributes when the C408 thiol is transiently deuterated. However, as deuterium washes out to solvent, the system approaches the equilibrium observed in the absence of any isotopic label. When D2O and unlabeled AdoCbl are present, the same kinetic and equilibrium isotope effects produce the observed biphasic kinetics, with the steady-state amount of cob(II)alamin the same as when [5'-2H2]-AdoCbl and D2O are present.

The stepwise mechanism gives a qualitatively similar result for the case when [5'-2H2]-AdoCbl and D2O are present: that is, kinetic and equilibrium isotope effects on cob(II)alamin formation. However, the predicted effects are significantly lower than the observed effects (Table 3.4, 1.5 vs the experimentally observed 2.7). This is because the rate of carbon-cobalt bond cleavage and the final amount of cob(II)alamin formed depend on three steps, one isotope-sensitive (the equilibrium between thyl radical and thiol, steps 8, 9, 12, 13, 16, and 18, Fig. 3.6) and two relatively isotope-insensitive (carbon-cobalt bond cleavage and reformation, steps 7, 10, 11, 14, 15, and 17, and binding of AdoCbl to RTPR, steps 1—6, Fig. 3.6). The presence of the isotope-insensitive steps has the effect of decreasing the predicted kinetic and equilibrium isotope effects. For the same reason, the kinetic isotope effects predicted by the stepwise mechanism are also smaller than the observed isotope effects (Table 3.4) when [5'-2H2]-AdoCbl and H2O or unlabeled AdoCbl and D2O are present.
Global Analysis of Isotope Effect Data to Obtain Best Fit Rate and Equilibrium Constants

The numerical simulations described above demonstrate that the isotope effect data can be qualitatively modeled using a relatively small number of chemically reasonable assumptions about statistical factors and magnitudes of kinetic and equilibrium isotope effects in the concerted mechanism. However, while this analysis showed that these constraints were consistent with the mechanism, it did not address whether rate constants accomodating these constraints were actually required to fit the data, or whether other sets of kinetic parameters might also fit the data, even if these parameters were less consistent with chemical precedent. The question of whether other acceptable sets of kinetic parameters exist is particularly important for the stepwise mechanism, for which a set of rate constants that both satisfied the initial assumptions and fit the data was not found. Global kinetic analysis with a concerted mechanism was used to determine whether the rate constants that provided the best fit to all the data were consistent with the constraints placed on the rate constants in the original simulations. Global analysis with a stepwise mechanism was used to determine whether there was a set of rate constants that fit the data, or whether no satisfactory fit to all the kinetic data could be obtained with this mechanism.

The parameters derived from the global fitting of the data to the concerted mechanism provide a reasonable fit to the data (Fig. 3.9). The best defined rate constants (i.e., those subject to the lowest uncertainty) are those for the all-deuterated conditions. These represent a kinetic isotope effect \((k_{+11}/k_{+12})\) of 1.4 for carbon-cobalt bond homolysis/thiyl radical formation and 5 for carbon-cobalt bond re-formation/thiol regeneration \((k_{-11}/k_{-12})\). The ratio of these isotope effects is the best fit fractionation factor, which, at 0.3, is in reasonable agreement with the values of 0.4-0.6 measured in non-enzymatic systems (Schowen & Schowen, 1982) and used as a constraint in the previous modeling.

According to the concerted model, the rate constants for hydrogen abstractions involving mixed isotopomers \((k_{+7}, k_{+8}, k_{+9}, k_{+10}\) Fig. 3.5) should be calculable by applying the appropriate statistical factors to the rate constants for the all-protonated case and the all-deuterated case. These statistical factors were used as a constraint in the original simulations. The rate constants \(k_{-11},\)
k.9, and k.7 should be in the proportion 3:2:1, as should the rate constants k.12, k.8, and k.10. The global best fit values for these rate constants (Table 3.5) are subject to large uncertainties. However, they are in agreement with the predicted values to within the experimental uncertainties. The best fit rate constants derived from global analysis are thus in general agreement with the constraints put on these rate constants based on chemical considerations, although the uncertainties are too large to say that the data require a model that includes the assumed statistical factors.

Global analysis using the concerted mechanism also supports a pseudo first-order rate constant for deuterium washout of ~5 s\(^{-1}\) that the more constrained simulations suggested would be necessary to fit the data. Previous studies suggest that, at least under turnover conditions, C408 does not exchange rapidly with bulk solvent. Ashley, Harris, and Stubbe found that <0.3% of the tritium from [3^-3H]-ATP exchanged with the solvent. Since the rate constant for a single turnover is ~50 s\(^{-1}\) (S. Licht and J. Stubbe, unpublished results), the rate constant for exchange of C408 with bulk solvent must be much less than this number under these conditions. C408 might also be shielded from solvent in the free form of the enzyme.

The major shortcoming of the simplified concerted model presented here is that it fails to take into account the small differences in rate and equilibrium constants that are likely to arise from carrying reactions out in D\(_2\)O in comparison to H\(_2\)O. However, even without taking these effects into account, the model has too many parameters for all of them to be determined with low uncertainties. To refine this model further, it will be necessary to measure isotopic incorporation into 5'-dA and/or AdoCbl as a function of time. This could be achieved by rapid acid quench experiments, with mass spectrometric characterization of isotopic incorporation in 5'-dA and AdoCbl.

The stepwise mechanism, in contrast, fails to provide an acceptable global fit to the data (Fig. 3.10). The fit is reasonably good for the all deuterated experiment, somewhat worse for the experiment with [5'-2H\(_2\)]-AdoCbl in H\(_2\)O, and very much in discord with the experiment with AdoCbl in D\(_2\)O. Allowing other parameters to vary (e.g., allowing for secondary isotope effects by varying k\(_{\pm 7}\), k\(_{\pm 10}\), k\(_{\pm 11}\), k\(_{\pm 14}\), and k\(_{\pm 17}\), Fig. 3.6) did not improve the fits.

The isotope effect experiments thus support the concerted mechanism over the stepwise mechanism. These experiments do not rule out 5'-dA•
existing as a very high energy metastable intermediate rather than as a true transition state. However, the experiments described in this work argue that the potential energy surface describing the reaction is dominated by a single barrier.

**Significance of a Concerted Mechanism For Carbon-Cobalt Bond Homolysis.**

A variety of kinetic experiments now support concerted formation of a thiy radical in the exchange reaction. A concerted process would help to explain how the proposed mechanism for exchange could be accounted for thermodynamically. With such a mechanism, thermodynamically unfavorable steps such as carbon-cobalt bond homolysis and re-abstraction of hydrogen from 5'-dA by a thiy radical are coupled to energetically favorable steps such as carbon-hydrogen bond formation and carbon-cobalt bond re-formation. The concerted mechanism may thus help explain both rate acceleration by the enzyme and the remarkable perturbation of the thermodynamics of carbon-cobalt bond cleavage at the enzyme's active site. In addition, the unusual equilibrium isotope effect can be interpreted in the context of the concerted mechanism as arising from a low fractionation factor of the C408 thiol, thus corroborating the EPR evidence favoring a thiy radical as a reaction intermediate.

With the establishment of a minimal chemical mechanism for the exchange reaction, further kinetic and thermodynamic analysis of this reaction becomes possible. The concerted mechanism argued for in this work forms the basis of a study of the thermodynamics and activation parameters of this reaction (Chapter 4).
References


Chapter 4:

Kinetics and Thermodynamics of Carbon-Cobalt Bond Homolysis
and Thiyl Radical Formation
Introduction

One of the central mechanistic questions in the biochemistry of adenosylcobalamin (AdoCbl) is how enzymes activate the carbon-cobalt bond of the coenzyme for homolytic bond cleavage. Studies from the Halpern and Finke laboratories determined that the homolytic bond dissociation energy of the carbon-cobalt bond is ~30 kcal/mol in aqueous solution (Finke & Hay, 1984, Halpern et al., 1984), and that the rate constant for this process is $10^{-9}$ s$^{-1}$ at 25 °C (Hay & Finke, 1988). Carbon-cobalt bond homolysis has been shown to be an intermediate step in the mechanisms of enzymes that catalyze unusual carbon skeleton rearrangements (Dolphin, 1982) and in the mechanism of the enzyme that catalyzes ribonucleotide reduction (Tamao & Blakley, 1973). The turnover numbers of these enzymes vary from 2 to 300 s$^{-1}$, meaning that the enzymes must accelerate this process by a factor of at least $10^{10}$ to $10^{12}$. The mechanism of this rate acceleration for the AdoCbl-dependent ribonucleoside triphosphate reductase (RTPR) from Lactobacillus leichmannii is addressed in this paper.

RTPR catalyzes the conversion of nucleoside triphosphates to deoxynucleoside triphosphates. In the course of nucleotide reduction, a pair of cysteines at the active site is oxidized to a disulfide, making re-reduction of the enzyme by an external reducing system necessary. During catalysis, the carbon-cobalt bond of AdoCbl is homolyzed to generate cob(II)alamin with an apparent first order rate constant for cob(II)alamin formation catalyzed by RTPR is >200 s$^{-1}$ when a reducing system consisting of E. coli thioredoxin, E. coli thioredoxin reductase, and NADPH is used (S. Licht and J. Stubbe, unpublished results). Thus, RTPR accelerates carbon-cobalt bond homolysis by a factor of ~$1x10^{12}$, which corresponds to a transition state stabilization of ~15 kcal/mol.

Many hypotheses have been advanced to explain the large rate enhancements of carbon-cobalt bond cleavage by enzymes (Banerjee, 1997, Halpern, 1985). One hypothesis, based on structural and mechanistic studies of model organocobalt compounds (Chemaly & Pratt, 1980, Geno & Halpern, 1987, Kräutler et al., 1994, Randaccio et al., 1981, Schraufer & Grate, 1979), holds that enzymes induce a conformational change in the relatively flexible corrin ring, causing it to flex upward, applying a steric strain to the axial adenosyl moiety of AdoCbl. Catalysis thus results from the decrease in the
carbon-cobalt bond dissociation energy of AdoCbl bound to the enzyme. The three-dimensional structure of cob(II)alamin supports the hypothesis that carbon-cobalt bond homolysis is associated with upward flexing of the corrin ring (Kratky et al., 1995). Another view holds that modulation of the carbon-cobalt bond dissociation energy by the trans axial ligand of cobalt (either the dimethylbenzimidazole portion of AdoCbl or, as shown for methionine synthase (Drennan et al., 1994) and methylmalonyl-CoA mutase (Mancia et al., 1996), a histidine residue of the enzyme) is the key to catalysis. Model studies have shown that a weaker donor ligand causes the carbon-cobalt bond to become longer and the bond dissociation energy to decrease (De Ridder et al., 1996, Marzilli et al., 1985, Ng & Rempel, 1982). The enzyme could thus favor carbon-cobalt bond homolysis by constraining the cobalt-nitrogen distance to be long or by protonating the imidazole to make it a weaker donating ligand. However, FT-Raman (Puckett et al., 1996) and resonance Raman (Dong et al., 1996) studies have shown that for alkylcobalamins, carbon-cobalt bond stretching frequencies do not depend on the trans axial ligand, suggesting that the role of the axial ligand is more complex than simply modulating the carbon-cobalt bond strength.

Transition state stabilization may provide an additional mechanism to accelerate carbon-cobalt bond homolysis. The fact that the activation energy for carbon-cobalt bond homolysis in solution (~35 kcal/mol) (Hay & Finke, 1988) is not much greater than the bond dissociation energy suggests, however, that selective stabilization of the transition state alone will not be able to provide the ~15 kcal/mol of stabilization required, because the transition state is only ~2 kcal/mol higher in energy than the product (Finke & Hay, 1984, Halpern et al., 1984).

Finally, in both the enzymatic and model systems, entropic effects must be considered as a source of catalytic power. In the enzymatic case, solvent release from the enzyme or an increase in degrees of freedom of the cofactor and/or the enzyme could accompany progression along the carbon-cobalt bond homolysis reaction coordinate.

To distinguish between these hypotheses, the relevant thermodynamic parameters can be derived from the temperature dependence of the microscopic rate constant for carbon-cobalt bond homolysis. The presteady state kinetic analysis of carbon-cobalt bond homolysis in the presence of substrate is very complex, and work on deriving microscopic rate constants
from the observed kinetics is still in progress. However, as described in the accompanying manuscript, RTPR catalyzes exchange of $^3$H from [5'-$^3$H]-AdoCbl in the absence of substrate, requiring only a deoxynucleotide allosteric effector. Cob(II)alamin is an intermediate in this reaction as well, and, as our previous studies have revealed (Booker et al., 1994, Licht et al., 1996), this reaction is an excellent model for the early stages of the nucleotide reduction process itself, since both reactions involve cofactor-mediated formation of cob(II)alamin, 5'-deoxyadenosine (5'-dA), and an enzyme-based thyl radical. An understanding of this simple model system has now allowed us to study the concentration and temperature dependence of the observed kinetics of RTPR-catalyzed carbon-cobalt bond homolysis, and derive microscopic rate constants and thermodynamic parameters associated with this process. These results suggest that catalysis is largely entropic in nature.
Materials and Methods

General Methods

Nucleotides, nucleosides, and NADPH were obtained from Sigma. RTPR (specific activity of 1.4 μmol ATP reduced/min/mg RTPR), thioredoxin (specific activity of 300-700 μmol DTNB reduced/min/mg) and thioredoxin reductase (specific activity of 3000-7000 μmol DTNB reduced/min/mg) were isolated as previously described (Booker & Stubbe, 1993, Lunn et al., 1984, Russel & Model, 1985). UV-visible spectroscopy was performed on a Cary 3 or Hewlett-Packard 8452A. Centricon-30 microconcentrators were obtained from Millipore. All operations involving AdoCbl were carried out under dim light or red light.

Stopped-flow studies were carried out using an APL DX.17MV stopped-flow spectrophotometer from Applied Photophysics (Leatherhead, U. K.). Anaerobic conditions were not employed, as rates and amounts of cob(II)alamin formation were observed to be unaffected by the presence of atmospheric oxygen at times <1 s after mixing.

Concentrations of RTPR were measured spectrophotometrically (ε_{280} = 101000 M^{-1} cm^{-1}) (Blakley, 1978). Formation of cob(II)alamin was monitored using the decrease in absorption at 524 nm. For the conversion of AdoCbl to cob(II)alamin, ∆ε = 4800 M^{-1} cm^{-1} (ε_{524} of AdoCbl = 3000 M^{-1} cm^{-1}, ε_{524} of cob(II)alamin = 3200 M^{-1} cm^{-1}) (Tamao & Blakley, 1973).

Linear and non-linear least squares fits were carried out using either the Applied Photophysics system software or Kaleidagraph.

Stopped-flow Studies on the Exchange Reaction: Dependence of Rates and Amounts of Cob(II)alamin Formation on Concentration of AdoCbl and on Temperature

RTPR (6–80 μM), 20 μM TR, 1 μM TRR, 2 mM NADPH, and 1 mM dGTP in 200 mM sodium dimethylglutarate, pH 7.3 were mixed with an equal volume of the same reaction buffer containing variable amounts of AdoCbl (26 μM–960 μM) and 1 mM dGTP. The formation of cob(II)alamin was measured by monitoring the change in A_{524}. For each set of concentrations, experiments were carried out at temperatures ranging from 25 °C to 39 °C. Traces were fit to a single or double exponential using the Applied
Photophysics software. Double exponential fits were required for \([\text{AdoCbl}] \geq 200 \, \mu\text{M}\) when the temperature was \(\geq 37 \, ^\circ\text{C}\).

The temperature was determined using the temperature detector in the APL DX.17MV; this typically deviated from the bath temperature by \(~0.5\) \(^\circ\text{C}\). When the temperature was changed, the system was allowed to equilibrate for at least 10 min before kinetic traces were recorded. The order of the temperature changes had no effect on the rate constants or amplitudes of the kinetic traces.

**Global Analysis of Kinetic Data**

Global analysis was carried out using the program Dynafit.220 (Kuzmic, 1996). The rapid equilibrium assumption for AdoCbl binding (see previous paper for justification) was accommodated by fixing the association rate constant at \(10^8 \, \text{M}^{-1} \, \text{s}^{-1}\) (assumed to be the diffusion-controlled limit) (Jencks, 1969) and allowing the dissociation rate constant to vary to obtain the equilibrium constant. To accommodate the rapid equilibrium assumption for thiol radical formation in the stepwise mechanism, the rate constant for thiol radical formation was set at \(1000 \, \text{s}^{-1}\) (\(~100\)-fold greater than the rate constants for carbon-cobalt bond cleavage and re-formation), and the rate constant for re-formation of thiol was allowed to vary to obtain the equilibrium constant.

Because the rate and, especially, the amount of cob(II)alamin formed depend on the concentration of active enzyme, a systematic error is introduced into the analysis if the concentration of active sites is erroneously assumed to be equal to the enzyme concentration. Earlier studies had suggested that only 75–90\% of the protein is active (Booker et al., 1994, Chen et al., 1974). In accordance with these studies, the best global fits required entering the concentration of enzyme as \(~80\%\) of the protein concentration. In addition, adjusting for uncertainties in offsets and titration errors is often required to obtain good fits for SF UV-vis data (Kuzmic, 1996). Offsets of absorbance traces (i.e., the absorbance at time zero, which is subject to uncertainty due to the dead time of the instrument) and enzyme concentrations were thus allowed to vary to within 10\% of their input values. Global fitting was carried out using either the mechanism in Scheme 4.1 (Results section) or a modified mechanism which includes an alternate binding mode for AdoCbl (Scheme 4.2, Results section).
Determination of Equilibrium Constant for Carbon-Cobalt Bond Homolysis/Thiyl Radical Formation

RTPR (1 mM), 60 μM TR, 1.5 μM TRR, 2 mM NADPH, and 1 mM EDTA in 200 mM sodium dimethylglutarate, pH 7.3 were mixed in a septum-sealed Eppendorf tube which was purged with argon (blown over the solution) for 20 min while stirring at 0 °C. This mixture was transferred via gas-tight syringe to a septum-sealed, argon-purged cuvette (1 cm pathlength). The cuvette was maintained at 37 °C in a jacketed cell, and its UV-vis spectrum was recorded. This background spectrum was essential because at the high protein and NADPH concentrations used, these components had significant absorption (~0.1 absorbance units total) above 400 nm, where the spectra of the cobalamins of interest exhibit characteristic features. Degassed AdoCbl was then added to a concentration of 70 μM (measured spectrophotometrically), and the UV-visible spectrum was recorded. Finally, dGTP was added to a final concentration of 1 mM, and the UV-vis spectrum was again recorded (the entire process took approximately 5 min). Prolonged incubation was avoided as irreversible formation of cob(II)alamin was observed (Tamao and Blakley, 1973).

Derivation of Activation Parameters from the Temperature Dependence of k_{obs} as a Function of Concentration

The observed rate constants for cob(II)alamin formation (obtained from exponential fits to the kinetic traces) were plotted as a function of temperature in the form of an Arrhenius plot (k_{obs} vs. 1/T). These plots were generated for every [AdoCbl] tested. The linear functions that gave the best fits to these plots were used to interpolate k_{obs} values for a variety of temperatures, and the k_{obs} so obtained were plotted as a function of concentration for each temperature. K_1, k_{+2}, k_{-2} (Scheme 4.1, Results section) were derived from these plots using Eq. 4.1 (Bernasconi, 1976),

\[
k_{\text{obs}} = k_{+2} \left( \frac{K_1 [\text{AdoCbl}]_0}{K_1 [\text{AdoCbl}]_0 + 1} \right) + k_{-2} \quad \text{Eq. 4.1}
\]

where k_{obs} is the observed rate constant obtained from exponential fitting of the SF UV-vis trace, and [AdoCbl]_0 is the starting concentration of AdoCbl
(under the experimental conditions used, with AdoCbl in 4–10-fold excess over RTPR, [RTPR] does not contribute significantly to the magnitude of $k_{\text{obs}}$). This procedure has the effect of averaging out random errors in the determination of $k_{\text{obs}}$ as a function of temperature, since the best fit value, rather than $k_{\text{obs}}$ as determined at each individual temperature, is used.

For the large observed rate constants measured above 34 °C, precise fits to the concentration dependence of the calculated $k_{\text{obs}}$ could be obtained (Fig. 4.5, Results section). Below 34 °C, the rate constants predicted by the temperature dependences are too low to obtain precise fits of the concentration dependence, given the scatter in the data. Individual activation parameters could therefore be derived for a relatively narrow temperature range (35–40 °C). The data used to derive these parameters, however, was taken over a broader temperature range (25–40 °C).

Once $K_1$, $k_{+2}$, and $k_{-2}$ were known individually as a function of temperature, individual activation parameters were derived using Eyring theory. Thus, temperature dependence of rates was analyzed using the Arrhenius equation,

$$k = A \cdot \exp\left(-\frac{E_a}{RT}\right)$$

where $A$ is the Arrhenius pre-exponential factor, $E_a$ is the activation energy, $R$ is the universal gas constant, and $T$ is temperature (in K). Enthalpies of activation ($\Delta H^\ddagger$) were assumed to be equal to activation energies ($E_a$), and entropies of activation ($\Delta S^\ddagger$) were calculated from the Arrhenius pre-exponential factor according to Eyring rate theory (Tinoco et al., 1985), with

$$\Delta S^\ddagger = R \left( \ln \frac{A_h}{\kappa T_e} \right)$$

where $\kappa$ is the transmission coefficient, $h$ is Planck's constant and $e$ is Euler's constant.

**Derivation of Thermodynamic Parameters from the Temperature Dependence of [Cob(II)alamin]$_\infty$/[AdoCbl]$_0$ as a Function of Concentration**

An analogous approach was used to derive thermodynamic parameters from the variation of steady state amounts of cob(II)alamin formed as a function of temperature and concentration. The fraction of cofactor in the form of cob(II)alamin in the steady state ([cob(II)alamin]$_\infty$/[AdoCbl]$_0$, called $\nu$, was plotted as a function of temperature in the form of a van't Hoff plot ($\ln \nu$...
vs. 1/T). The linear functions that give the best fits to these plots were used to obtain values for v at a variety of temperatures. These values for v were then plotted as a function of [AdoCbl] for each temperature. \( K_1 \) and \( K_2 \) were derived from these plots using Eq. 4.2, which is obtained by calculating the energies of each of the three states in Scheme 4.1 (Results section) using the relation \( \Delta G = -RT\ln K \) (with the free enzyme plus free AdoCbl as the reference state), then calculating the probability of an enzyme molecule being in the thiol radical/cob(II)alamin/5'-dA form using the Boltzmann distribution (Eisenberg & Crothers, 1979).

\[
v = \frac{K_2 K_1 [\text{AdoCbl}]_0}{1 + K_1 [\text{AdoCbl}]_0 + K_2 K_1 [\text{AdoCbl}]_0}
\]

Eq. 4.2

This mechanism does not take into account the possibility of alternate modes of binding for AdoCbl (Scheme 4.2, Results section), which would have a significant effect on the amount of cob(II)alamin formed. The values obtained from the analysis using Eq. 4.2 must thus be considered estimates of the equilibrium constants, with the possibility that these values include contributions from equilibrium constants associated with alternate mode(s) of binding.

As was the case for \( k_{obs} \), the most precise fits to the concentration dependence of the calculated \( v \) were obtained with data at temperatures above 34 °C. Thermodynamic parameters were thus also derived for a relatively narrow temperature range (35–40 °C), although the data used to derive these parameters was taken over a broader temperature range (25–40 °C). To obtain enthalpies (\( \Delta H \)) and entropies (\( \Delta S \)) of reaction, the temperature dependence of the equilibrium constants \( K_1 \) and \( K_2 \) was analyzed according to the van't Hoff equation:

\[
\ln(K) = \frac{-\Delta H}{RT} + \frac{\Delta S}{R}
\]

The enthalpy and entropy calculated from this equation must also be considered estimates, as enthalpy and entropy associated with an alternate
mode of binding are likely to contribute to the values obtained by this method.
Results

Effect of AdoCbl Concentration on Kinetics of Cob(II)alamin Formation

Studies in the accompanying manuscript are most consistent with concerted formation of cob(II)alamin, 5'-dA, and thiol radical from reaction of AdoCbl with C408 of RTPR. The mechanism used to analyze this data (Scheme 4.1) begins with rapid, reversible binding of AdoCbl to RTPR, indicated by $K_1$.

\[
\text{SH} \quad E + \text{AdoCbl} \xrightleftharpoons{K_1} \text{EAdoCbl} \xrightarrow{k_2} \text{E}5'-\text{dA}\cdot\text{Cob(II)alamin}
\]

Scheme 4.1

The rate constant $k_2$ corresponds to the AdoCbl-mediated formation of cob(II)alamin, 5'-dA, and thiol radical, and $k_2$ is the rate constant for the reverse reaction, regeneration of the C408 thiol and re-formation of the carbon-cobalt bond of AdoCbl. $K_2$, the equilibrium constant for the carbon-cobalt bond homolysis/thiol radical formation reaction, is the ratio $k_2/k_2$. In order to derive values for $k_2$ and $k_2$ so that the temperature dependence of these rate constants could be evaluated, the effect of [AdoCbl] on the kinetics of cob(II)alamin formation was determined experimentally. As in the accompanying manuscript, dGTP is the allosteric effector, substrate is not present (meaning that the exchange reaction, rather than turnover is being examined), and RTPR is maintained in the reduced form by the TR/TRR/NADPH reducing system.

Tamao and Blakley (Tamao & Blakley, 1973) had also examined the rate of cob(II)alamin formation after mixing RTPR with AdoCbl in the presence of an allosteric effector and a reducing system. In their stopped-flow UV-vis studies, they found that the observed rate constant for cob(II)alamin exhibited a small [AdoCbl] dependence, varying by no more than 20% over [AdoCbl] from 25 to 150 μM (with [RTPR] at ~10 μM). These studies suggested that the $K_d$ for AdoCbl (1/$K_1$ in Scheme 4.1) is high, and provided a framework for the initial design of our experiments.

When [AdoCbl] is large compared to [RTPR] (pseudo-first order conditions), the observed rate constant for cob(II)alamin formation is given by
Eq. 4.1 (see Materials and Methods). In an effort to define the microscopic rate constants and activation parameters for the reaction, we examined cob(II)alamin formation by SF UV-vis spectroscopy over a range of AdoCbl concentrations varying from 10 to 500 µM. In each experiment, AdoCbl was initially present in 4- to 10-fold excess over RTPR (3–40 µM). Under these conditions at 37 °C, $k_{\text{obs}}$ obtained by exponential fits to SF UV-vis traces ranged from 30 to 60 s$^{-1}$ (Fig. 4.1). Above 200 µM AdoCbl, cob(II)alamin formation was biphasic, with the second kinetic phase having a rate constant of ~5 s$^{-1}$ and an amplitude of 15–20% of the first phase. In these cases, the data in Fig. 4.1 show the rate constant of the faster, larger amplitude phase. As discussed later, the second phase may represent an alternate conformation for the RTPR-AdoCbl complex that does not accelerate carbon-cobalt bond homolysis as efficiently (Scheme 4.2). At each set of concentrations, similar measurements were made over a temperature range of 25 to 39 °C, as discussed subsequently.

Non-linear least squares fits of kobs to Eq. 4.1 give $k_2 = 55±7$ s$^{-1}$, $k_2 = 27±2$ s$^{-1}$, and $K_1 = 5100±1700$ M$^{-1}$ at 37 °C. The equilibrium constant for the second step, $K_2$, is calculated to be 2.0±0.3. The error in $K_1$ is large because it was not possible to determine rates with a saturating concentration of AdoCbl; above ~500 µM, the $A_{524}$ of AdoCbl is too high for accurate measurement in the available cells. With the experimental conditions used, binding of AdoCbl in an alternate mode associated with a small amount of cob(II)alamin formation (Scheme 4.2) will have a minimal effect on the rate constant for the faster phase of cob(II)alamin formation, as only a small fraction of AdoCbl will be bound in the alternate mode compared to the amount free in solution.

While the magnitude of $K_1$ is not precisely defined by the data, the data suggest that $K_d$ for AdoCbl is >50 µM and probably 200–300 µM. This is in accord with the high observed $K_m$ for AdoCbl (60±20 µM) (Booker, 1994) in the exchange reaction and our inability to measure AdoCbl binding using a

---

1 Because the $K_d$ for AdoCbl is high, this approximation ([AdoCbl] = [AdoCbl]$_0$) holds even at ratios of [AdoCbl]/[RTPR] less than 10, as long as [AdoCbl]<<$K_d$. Under these conditions, most of the AdoCbl remains unbound at equilibrium, so its concentration is approximately constant over the course of the reaction. Analysis of the amounts of cob(II)alamin observed using Eq. 2 (see Materials and Methods) indicated that under all the conditions used, bound forms of AdoCbl (the Michaelis complex and cob(II)alamin + 5'-dA) represented ≤15% of the total [AdoCbl], verifying the validity of approximating [AdoCbl] as [AdoCbl]$_0$. 

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Fig. 4.1. Effect of [AdoCbl] on observed rate constants for cob(II)alamin formation. Observed rate constants obtained from stopped-flow traces are plotted as a function of starting concentration of AdoCbl ([AdoCbl]₀). Data are fit to Eq. 4.1. The temperature was 37 °C for this set of data. [RTPR] was varied from 3 to 40 µM, and [AdoCbl] varied from 12 to 480 µM. Exponential fits of kinetic traces were used to obtain kₐₖₑₑ₅, as described in the text.
The graph shows the relationship between \( k_{\text{obs}} \) (s\(^{-1}\)) and \([\text{AdoCbl}]_0\) (µM). The data is fit to the equation:

\[
y = \frac{m1\cdot m2\cdot m0}{(1+m1\cdot m0)} + m3
\]

The table below provides the fitted values and errors:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>m1</td>
<td>5.1244e+03</td>
<td>1.7464e+03</td>
</tr>
<tr>
<td>m2</td>
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<td>6.4177e+00</td>
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<tr>
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<td>Chisq</td>
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</tr>
<tr>
<td>( R^2 )</td>
<td>971.03e-03</td>
<td>NA</td>
</tr>
</tbody>
</table>
Scheme 4.2

A variety of standard methods (Booker et al., 1994). It also suggests that although the reaction studied here almost certainly mimics early events in nucleotide reduction, it is unlikely to be physiologically important, as the physiological concentrations of AdoCbl are not sufficiently high to allow much binding in the absence of substrate (Davis et al., 1952, Kashket et al., 1962).

The equilibrium constants $K_1$ and $K_2$ can also be used to determine a $\Delta G$ of $\sim -5$ kcal/mol associated with AdoCbl binding, and a $\Delta G$ of $-0.4\pm0.1$ kcal/mol associated with carbon-cobalt bond cleavage/thiyl radical formation. The latter value is consistent with significant stabilization of the cob(II)alamin/thiyl radical species at the enzyme active site, as the net reaction (homolytic cleavage of the carbon-cobalt bond and an S-H bond, formation of a C-H bond) would be expected to be $\sim 20$ kcal/mol uphill in solution (see Discussion). In addition, the rate constants $k_{+2}$ and $k_2$ allow calculation of free energies of activation of $15.7\pm0.1$ kcal/mol ($\Delta G_{+2}^{\dagger}$) and $16.1\pm0.1$ kcal/mol ($\Delta G_{-2}^{\dagger}$), respectively. These values will provide a check on the free energies of activation derived from the temperature dependence of $k_{obs}$ discussed subsequently.
Effect of AdoCbl Concentration on Amounts of Cob(II)alamin Formation

The stopped-flow experiments described above also show how the amount of cob(II)alamin formed depends on the concentration of AdoCbl. The amplitude of the absorbance change (ΔA_{524}) in these experiments (derived from exponential fits to the SF UV-vis traces) gives the amount of cob(II)alamin formed at equilibrium. As described above, two exponentials were needed to fit the time course of the absorbance change at [AdoCbl] ≥ 200 μM. The faster kinetic phase (80-85% of the total absorbance change) was used for analysis of the amplitudes, as it was for the observed rate constants.

For the concerted mechanism in Scheme 4.1, v, defined as [E-S• · 5′-dA · cob(II)alamin]/[E]_{total} (i.e., the fraction of total enzyme in the thyl radical/5′-dA/cob(II)alamin form) is given by Eq. 4.2 (see Materials and Methods) when AdoCbl is in large excess over RTPR. The equilibrium constants derived from this method, however, are subject to an uncertainty stemming from the uncertainty in the number of active sites present in solution in each experiment. The concentration of active sites might be assumed to be equal to the concentration of enzyme, measured spectrophotometrically. However, single turnover experiments show that ~ 1.5 of an equivalent of deoxynucleotide product is formed when reduced RTPR is incubated with substrate, allosteric effector, and AdoCbl, in contrast to the 2 equivalents expected from the two pairs of active site cysteines that can provide reducing equivalents (Booker et al., 1994). It is not known, however, whether this observation is due to 1/4 of the enzyme being inactive or to the delivery of reducing equivalents to the substrate being less than 100% efficient. Equilibrium binding studies on RTPR isolated from *L. leichmanii* (Chen et al., 1974) yielded an estimate of 0.85±0.04 binding sites for dATP/enzyme (although the number of sites for other effectors was measured to be ≥1), similar studies on RTPR overexpressed in *E. coli* (Booker et al., 1994) gave an estimate of 0.9±0.3 binding sites for dGTP/enzyme, consistent with some fraction of the enzyme being inactive. The single-turnover and allosteric effector binding results do not necessarily imply that the number of AdoCbl binding sites that can perform carbon-cobalt bond homolysis is also less than the protein concentration. Nonetheless, the uncertainty in the number of active sites can make a significant difference in the equilibrium constants derived.
Assuming a value of 0.8 active site per protein, plotting \( v \) against [AdoCbl], and fitting to the above equation gives a \( K_1 \) of 21000±5000 M\(^{-1}\) for AdoCbl, and \( K_2 \) of 0.65±0.05 (Fig. 4.2) at 37 °C, corresponding to free energies of \(~ -6 \text{ kcal/mol} \) and \(~ 0.03 \text{ kcal/mol} \), respectively. The value for \( K_1 \) is significantly higher than that obtained from \( k_{obs} \), and \( K_2 \) is significantly lower. For example, the equilibrium constants derived from the kinetic analysis predict formation of 0.6 eq. of cob(II)alamin when [AdoCbl] is 500 µM, compared to the 0.4 eq. observed. This observation might be explained by the existence one or more alternate modes of binding for AdoCbl that either do not allow cob(II)alamin formation or favor binding of AdoCbl over carbon-cobalt bond homolysis.

These data can also be fit to a mechanism incorporating an alternate binding mode (Scheme 4.2), although they cannot uniquely define the three equilibrium constants. The equation for \( v \) appropriate for this mechanism (Eq. 3, Scheme 4.2) is analogous to Eq. 4.2,

\[
v = \frac{K_2K_1[\text{AdoCbl}]_0}{1 + (K_1 + K'_1)[\text{AdoCbl}]_0 + K_2K_1[\text{AdoCbl}]_0}
\]

Eq. 4.3

with \( K'_1 \) defined as the affinity constant for AdoCbl binding in the alternate mode and the other equilibrium constants defined as in Eq. 4.2. Fitting the data to this equation gives \( K_1 \sim 6000 \), \( K'_1 \sim 15000 \), and \( K_2 \sim 2 \), although these equilibrium constants are not a unique fit. These data are thus not inconsistent with the kinetic data, provided an alternate binding mode for AdoCbl is taken into account. Analysis of the steady-state amounts of cob(II)alamin using Eq. 4.1 gives values for \( K_1 \) and \( K_2 \) that are likely to contain contributions from \( K'_1 \) and \( K'_2 \) (\( k_{+2} / k_{-2} \)) (Scheme 4.2), so the values obtained should be viewed as qualitative estimates.

Global fitting of the stopped-flow kinetic traces for cob(II)alamin formation at various [AdoCbl], which allows a combined analysis of both \( k_{obs} \) and steady-state cob(II)alamin formation, is also consistent with the hypothesis of an alternate binding mode. When the SF UV-vis were fit using a simple two-step mechanism (Scheme 4.1), the best fit had \( K_1 = 3100\pm1800 \text{ M}^{-1} \), \( k_{+2} = 46\pm10 \), and \( k_{-2} = 28\pm3 \) (Fig. 4.3a), similar to the results obtained
Fig. 4.2. Effect of [AdoCbl] on amounts of cob(II)alamin formation. Fraction of total enzyme in thyl radical/5′-dA/cob(II)alamin form (v) is plotted as a function of starting concentration of AdoCbl ([AdoCbl]₀). Data are fit to Eq. 4.2.
\[ y = \frac{m_1 m_2 m_0}{1 + (m_1 m_0) + (m_2 \ldots)} \]

<table>
<thead>
<tr>
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<th>Value</th>
<th>Error</th>
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<tbody>
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</tr>
<tr>
<td>Chisq</td>
<td>2.7202e-03</td>
<td>NA</td>
</tr>
<tr>
<td>R²</td>
<td>972.79e-03</td>
<td>NA</td>
</tr>
</tbody>
</table>
using the $k_{obs}$ data alone, although the second, slower kinetic phase was not well fit. This suggests that the mode of binding that leads to fast cob(II)alamin formation is the dominant contributor to the observed stopped-flow traces. The SF UV-vis traces were also fit using a mechanism that included an alternate mode of binding for AdoCbl (Scheme 4.2). This model provided a good fit (Fig. 4.3b), with rate constants of $K_1 = 5000\pm2500$ M$^{-1}$, $K_1' = 7500\pm3000$ M$^{-1}$, $k_{+2} = 67\pm35$, $k_{-2} = 35\pm1$, $k_{+2'} = 0.6\pm0.2$, $k_{-2'} = 1.2\pm1$. The standard errors on most of the rate constants were large, since there was not enough data with a second kinetic phase to define its concentration dependence adequately. Nonetheless, this global fitting shows that the data is also consistent with the alternate mode of binding suggested by the analysis of the steady-state amounts of cob(II)alamin. All the data is thus consistent with $K_1$ being 4000-5000 M$^{-1}$ and $K_2$ being $\sim2$ (Schemes 4.1 and 4.2).

**Determination of Cob(II)alamin Bound to RTPR when [RTPR] >> [AdoCbl]**

If the initial binding equilibrium is saturated, the ratio of cob(II)alamin formed in the steady-state to RTPR present should give $K_2$ directly (Scheme 4.1), providing another check on this value. Although measuring cob(II)alamin spectrophotometrically was not possible for [AdoCbl] > 500 μM (the available cells had pathlength ≥ 0.2 cm), the availability of large amounts of RTPR allowed the binding equilibrium to be measured under conditions where [RTPR]$_0$ >> [AdoCbl]$_0$, [RTPR]$_0$ > $K_d$. Under these conditions, the ratio of cob(II)alamin to AdoCbl should be approximately equal to $K_2$. This experiment was carried by mixing (under anaerobic conditions) 1 mM RTPR with 70 μM AdoCbl in the presence of a reducing system, initiating carbon-cobalt bond cleavage by the addition of the allosteric effector dGTP. The SF UV-vis studies had indicated that the system would come to equilibrium in <1 s, so the change in the UV-visible spectrum was measured directly after mixing by hand.\(^2\) We measured a ratio of cob(II)alamin to AdoCbl of 0.9±0.1, in reasonable agreement with the values obtained from the [AdoCbl] dependence of the amount of cob(II)alamin formed as measured by SF UV-vis spectroscopy.

---

\(^2\) Slow (−3×10$^{-4}$ s$^{-1}$) irreversible formation of cob(II)alamin catalyzed by RTPR can be a major contributor to the uncertainty in experiments carried out by hand mixing. This irreversible reaction would artifactualy increase the apparent value of $K_2$. Therefore, data obtained immediately after addition of dGTP to start the reaction was used for analysis.
Fig. 4.3. The SF UV-vis traces (shown in terms of cob(II)alamin formation) from which $k_{\text{obs}}$ values were derived (Fig. 4.1) were globally fit using the program DynaFit.220 and the mechanisms in Scheme 4.1 or 2. For the experimental data used, the temperature was 37 °C, [RTPR] was varied from 3 to 40 μM, and [AdoCbl] varied from 12 to 480 μM. All rate/equilibrium constants were allowed to vary in the global fitting. Solid lines are experimental traces, dashed lines are fits. A. Global analysis using the mechanism in Scheme 4.1. B. Global analysis using the mechanism in Scheme 4.2.
One contributor to systematic error in this experiment is the possibility that the binding of AdoCbl to RTPR ($K_1$) is not saturated. In this case, the ratio of cob(II)alamin to AdoCbl underestimates $K_2$. For example, if $K_1$ is 5100 M$^{-1}$, as estimated from the [AdoCbl] dependence of $k_{obs}$ (Eq. 4.1), and the concentration of active sites is 0.8 mM rather than the 1 mM suggested by the A280 measurement, use of an equation equivalent to Eq. 4.2 (except that [RTPR]$_0$ replaces [AdoCbl]$_0$) shows that ~10% of the AdoCbl would not be bound to RTPR. Thus, $K_2$ would be 1.1±0.1, rather than 0.9±0.1. The lower $K_1$ is, the greater the influence of this uncertainty will be.

Failure to saturate the binding associated with $K_1$ cannot account for the discrepancy between the $K_2$ calculated from this experiment and the $K_2$ calculated from the [AdoCbl] dependence of $k_{obs}$ (2.0±0.3). As described above, the existence of an alternate bound state (Scheme 4.2) would lead to an underestimate of $K_2$ in this experiment, since some of the bound AdoCbl would not be in equilibrium with the thyl radical/cob(II)alamin/5'-dA form of the enzyme. The value calculated from this experiment should thus be considered a qualitative estimate of $K_2$.

Temperature Dependence of Kinetics of Cob(II)alamin Formation

For two coupled reactions such as the two steps in the exchange reaction (Scheme 4.1), both the slope and the intercept of an Arrhenius plot contain contributions from both the forward and reverse steps of each reaction. To sort out the contributions from each microscopic step, and thereby obtain activation parameters for each of these steps, the temperature dependence of $k_{obs}$ must be measured as a function of concentration. As discussed above (Materials and Methods), plots analogous to Fig. 4.1 have been generated for temperatures ranging from 25 to 40 °C, allowing determination of microscopic rate constants at each temperature. Eyring theory can then be applied to each microscopic rate constant individually in order to ascertain thermodynamic parameters. Representative temperature dependence data for $k_{obs}$ is shown in Fig. 4.4, plotted in the form of an Arrhenius plot. Fig. 4.4 shows that the data is well fit to a line. Although the slope and intercept of this plot reflect contributions from a number of thermodynamic quantities, the large slope of the plot indicates that the reaction is strongly temperature dependent, suggesting that at least one of the
Fig. 4.4. Temperature dependence of $k_{obs}$. $[\text{RTPR}]_0 = 10 \ \mu\text{M}, [\text{AdoCbl}]_0 = 50 \ \mu\text{M}$. Similar plots were obtained for all concentrations of RTPR and AdoCbl used. Although this plot is in the form of an Arrhenius plot, the slope and intercept represent contributions from thermodynamic and activation parameters of forward and reverse reactions of both steps in Scheme 4.1. A linear least squares fit was used to determine the slope and intercept of this plot, which were used to calculate the best fit $k_{obs}$ for any given temperature under these conditions. Analogous plots were generated for each set of conditions.
steps is highly endothermic. In addition, the large value for the intercept (from which the pre-exponential factor is derived) suggests that a large positive entropy is associated with one or more of the steps.

Each concentration of AdoCbl tested gave an Arrhenius plot analogous to Fig. 4.4, except with a different slope and intercept, reflecting the varying concentration- and temperature-dependent contributions to $k_{obs}$ from $K_1$, $k_{+2}$, and $k_{-2}$. Because the fits of the data to lines are good ($R^2 \sim 0.99$), these plots allow accurate prediction of $k_{obs}$ at any temperature within the range measured. Plotting the calculated $k_{obs}$'s as a function of [AdoCbl] (Fig. 4.5) allows calculation of the microscopic rate constants $K_1$, $k_{+2}$, and $k_{-2}$ at different temperatures.

Plotting the derived value of $k_{+2}$ as a function of temperature in an Arrhenius plot (Fig. 4.6) allows calculation of an activation enthalpy ($\Delta H_{+2}^\ddagger$) of $46\pm7$ kcal/mol and an activation entropy ($\Delta S_{+2}^\ddagger$) of $96\pm12$ cal/mol K for this step. The large value for $\Delta H_{+2}^\ddagger$ was unexpected, since model studies have often emphasized the role of the enzyme in reducing $\Delta H_{+2}^\ddagger$. This result suggests that the enzyme does not function to strain or weaken the carbon-cobalt bond in the ground state enzyme-AdoCbl complex. The large value for $\Delta S_{+2}^\ddagger$ suggests that an increase in entropy in the transition state provides much of the transition state stabilization in catalysis. The free energy of activation ($\Delta G_{+2}^\ddagger$) can be calculated from the relation $\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$ to be $16\pm3$ kcal/mol at $37 \, ^\circ C$, in good agreement with the value of $15.7\pm0.1$ kcal/mol derived from the microscopic rate constant using Eyring theory.

A similar analysis of the temperature dependence of $k_{-2}$ (Fig. 4.7) allows calculation of $\Delta H_{-2}^\ddagger$ of $26\pm3$ kcal/mol and $\Delta S_{-2}^\ddagger$ of $25\pm12$ cal/mol K. The calculated $\Delta G_{-2}^\ddagger$ is $18\pm5$ kcal/mol, in good agreement with the value of $16.1\pm0.1$ kcal/mol obtained directly from the microscopic rate constant.

Calculation of the activation parameters for $k_{+2}$ and $k_{-2}$ allows determination of $\Delta H$ and $\Delta S$ for step 2: they are, respectively, $\Delta H_{+2}^\ddagger - \Delta H_{-2}^\ddagger$ and $\Delta S_{+2}^\ddagger - \Delta S_{-2}^\ddagger$. Thus, $\Delta H$ is calculated to be $20\pm8$ kcal/mol, and $\Delta S$ is calculated to be $70\pm17$ cal/mol K. The value of $20\pm8$ kcal/mol is approximately equal to the net enthalpy change expected from the bonds broken and formed during this reaction (carbon-cobalt bond broken, $\sim -30$ kcal/mol, S-H bond broken $\sim +90$ kcal/mol, C-H bond formed, $\sim -100$ kcal/mol) (Benson, 1978, Griller & Martinho Simoes, 1990, McMillen &
Fig. 4.5. [AdoCbl] dependence of $k_{\text{obs}}$ as a function of temperature. Using the slopes and intercepts from Fig. 4 and the analogous plots obtained at other concentrations, the expected $k_{\text{obs}}$ was determined as a function of concentration at a variety of temperatures. Fits to Eq. 4.1 provided $K_1$, $k_{+2}$, and $k_{-2}$ at each temperature.
Fig. 4.6. Arrhenius plot for $k_{+2}$. The fits shown in Fig. 4.5 provided values of $k_{+2}$ at a variety of temperatures. A linear least-squares fit is shown, with the slope of the line equal to the activation energy ($E_a$) and the intercept equal to the Arrhenius pre-exponential factor ($A$).
Fig. 4.7. Arrhenius plot for $k_2$. The fits shown in Fig. 4.5 provided values of $k_2$ at a variety of temperatures. A linear least-squares fit is shown, with the slope of the line equal to the activation energy ($E_a$) and the intercept equal to the Arrhenius pre-exponential factor ($A$).
\[ y = 42.178 \times 10^0 + -12.098 \times 10^3 x \quad R = 999.97 \times 10^{-3} \]
Golden, 1982). This is consistent with the idea that the enzyme does not act to exert strain on the carbon-cobalt bond. The large entropy of reaction indicates that an increase of degrees of freedom in the solvent, the protein, and/or the cofactor accompanies carbon-cobalt bond homolysis.

In principle, the enthalpy and entropy of AdoCbl binding could be determined from the temperature dependence of $K_1$. However, the error in $K_1$ is too large (up to 150%) to obtain reliable values. $K_1$ is observed to decrease with temperature, consistent with binding being exothermic (data not shown).

**Temperature Dependence of Amounts of Cob(II)alamin Formation**

The amplitude data from stopped-flow experiments provide an independent check on the values of $\Delta H$ and $\Delta S$ for step 2. The discrepancy between the equilibrium constants calculated from the amplitude data and the equilibrium constants calculated from the rate data suggests, as described above, that there is an alternate bound state for AdoCbl. While the data was analyzed by a method analogous to that used for the rate data, using Eq. 4.1 to calculate $K_1$ and $K_2$ over a range of temperatures, this method will yield thermodynamic parameters that include contributions from both modes of binding. These thermodynamic parameters are thus only an estimate of the true values for $\Delta H$ and $\Delta S$ for step 2.

Experimentally, the amplitude of the absorbance change was measured over a range of temperatures at each concentration of RTPR and AdoCbl used, giving the fraction of the enzyme (v) that is in the cob(II)alamin/thiyl radical/5'-dA form. The change in v as a function of temperature was plotted in the form of a van't Hoff plot (representative data in Fig. 4.8). For each concentration of RTPR and AdoCbl employed experimentally, the equations from linear least squares fits to the data were used to calculate values for v at several temperatures for each [AdoCbl] examined. These values were plotted to give the [AdoCbl] dependence of v at a variety of temperatures (Fig. 4.9). The equilibrium constants $K_1$ and $K_2$ were then calculated as a function of temperature. As was the case for the data derived from $k_{obs}$, the errors in $K_1$ are too large to obtain a reliable estimate of thermodynamic parameters associated with binding. However, a van't Hoff plot of $K_2$ as a function of temperature (Fig. 4.10) allows determination of $\Delta H_2=12 \pm 5$ kcal/mol, in agreement with the values predicted from analysis of $k_{obs}$ to within about one
Fig. 4.8. Temperature dependence of v (the fraction of total enzyme in thyl radical/5'-dA/cob(II)alamin form). [RTPR]o = 10 µM, [AdoCbl]o = 50 µM. Similar plots were obtained for all concentrations of RTPR and AdoCbl used. Although this plot is in the form of an van't Hoff plot, the slope and intercept represent contributions from thermodynamic parameters of both the steps. The slope and intercept obtained from a linear least-squares fit were used to calculate best-fit values for v at any arbitrary temperature for this set of conditions. Analogous plots were generated for each set of conditions.
\[ y = 21.684e+00 + -7.1479e+03x \quad R = 999.87e-03 \]
Fig. 4.9. [AdoCbl] dependence of v (the fraction of total enzyme in thyl radical/5'-dA/cob(II)alamin form) as a function of temperature. Using the slopes and intercepts from Fig. 4.8 and the analogous plots obtained at other concentrations of AdoCbl, the expected v was determined as a function of concentration at a variety of temperatures. The data are fit to Eq. 4.2.
Fig. 4.10. van't Hoff plot for $K_2$. The fits shown in Fig. 4.9 provided values of $K_2$ at a variety of temperatures. A linear least-squares fit is shown, with the slope equal to $\Delta H_2/R$, and the intercept equal to $\Delta S_2/R$. 
\(-\frac{\ln(K_2)}{1/T (1/K \times 10^3)}\)

---
y = 19.418e+00 + -6.1355e+03x R= 996.06e-03
standard deviation. $\Delta S_2$ is calculated to be $40\pm15$ cal/mol K, in agreement with the value obtained from the analysis of $k_{obs}$ to within about one standard deviation. The fact that these values are lower than those deriving from the analysis of $k_{obs}$ is consistent with negative enthalpy and negative entropy changes associated with the alternate mode of binding ($K_1$ in Scheme 4.2) contributing to the calculated values for $\Delta H_2$ and $\Delta S_2$. Further studies on binding of AdoCbl to the enzyme will be needed to resolve the question of an alternate binding mode and its thermodynamics. Nonetheless, both analysis of $k_{obs}$ and analysis of $v$ are consistent with $\Delta G_2$ being dominated by the entropic term $T\Delta S_2$.

The rate and equilibrium constants and thermodynamic parameters derived from analyses of rate constants and amplitudes of absorbance changes are summarized in Tables 4.1 and 4.2. As discussed subsequently, these thermodynamic parameters provide new insight into the mechanism by which RTPR catalyzes homolysis of the carbon-cobalt bond of AdoCbl and formation of a thyl radical at C408.
Table 4.1. Summary of rate and equilibrium constants measured at 37 °C.

<table>
<thead>
<tr>
<th></th>
<th>A.</th>
<th>B.</th>
<th>C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_1$ (M$^{-1}$)</td>
<td>5100±1700</td>
<td>21000±5000</td>
<td>-</td>
</tr>
<tr>
<td>$k_{+2}$ (s$^{-1}$)</td>
<td>55±7 s$^{-1}$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$k_{-2}$ (s$^{-1}$)</td>
<td>27±2 s$^{-1}$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$K_2$</td>
<td>2.0±0.3</td>
<td>0.65±0.05</td>
<td>0.9±0.1</td>
</tr>
</tbody>
</table>

A. Measured by [AdoCbl] dependence of kobs (Fig. 4.1). B. Measured by [AdoCbl] dependence of v (Fig. 4.2), calculated using Eq. 4.1. C. Measured by amount of cob(II)alamin formed when [RTPR]>>[AdoCbl].

Table 4.2. Thermodynamic parameters.

<table>
<thead>
<tr>
<th></th>
<th>A.</th>
<th>B.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ$G_1$ (kcal/mol)</td>
<td>~-5</td>
<td>~-6</td>
</tr>
<tr>
<td>Δ$G_{+2}$ (kcal/mol)</td>
<td>15.7±0.1</td>
<td>-</td>
</tr>
<tr>
<td>Δ$G_{-2}$ (kcal/mol)</td>
<td>16.1±0.1</td>
<td>-</td>
</tr>
<tr>
<td>Δ$G_2$ (kcal/mol)</td>
<td>-0.5±0.1</td>
<td>~-0.03</td>
</tr>
<tr>
<td>Δ$H_{+2}$ (kcal/mol)</td>
<td>46±7</td>
<td>-</td>
</tr>
<tr>
<td>Δ$H_{-2}$ (kcal/mol)</td>
<td>26±3</td>
<td>-</td>
</tr>
<tr>
<td>Δ$H_2$ (kcal/mol)</td>
<td>20±8</td>
<td>12±5</td>
</tr>
<tr>
<td>Δ$S_{+2}$ (cal/ mol K)</td>
<td>96±12</td>
<td>-</td>
</tr>
<tr>
<td>Δ$S_{-2}$ (cal/ mol K)</td>
<td>25±12</td>
<td>-</td>
</tr>
<tr>
<td>Δ$S_2$ (cal/ mol K)</td>
<td>70±17</td>
<td>40±15</td>
</tr>
</tbody>
</table>

A. Measured by [AdoCbl] dependence of kobs (Fig. 4.1). B. Measured by [AdoCbl] dependence of v (Fig. 4.2).
Discussion

RTPR catalyzes carbon-cobalt bond homolysis with a rate acceleration of ~10\textsuperscript{11}-fold over the uncatalyzed reaction. The high carbon-cobalt bond dissociation energy of AdoCbl poses a fundamental obstacle for the large rate acceleration accomplished by RTPR. Since the enthalpy (ΔH) for carbon-cobalt bond homolysis is ~30 kcal/mol (Finke & Hay, 1984, Halpern et al., 1984, Hay & Finke, 1988), and TΔS is no more than ~4 kcal/mol at 37 °C (Hay & Finke, 1988), ΔG for this reaction is ~25 kcal/mol. Stabilization of the transition state alone cannot decrease the activation free energy (ΔG\textsuperscript{‡}) to less than ~25 kcal/mol (Scheme 4.3a). However, the RTPR-catalyzed rate of carbon-cobalt bond homolysis corresponds to an activation free energy of ~15 kcal/mol (Scheme 4.3b). The enzyme must therefore destabilize the reactant state (ESH·AdoCbl) and/or stabilize the product state (ES·cob(II)alamin·5´-dA) such that ΔG\textsubscript{2} (the free energy change for step 2 in Scheme 4.1) is ≤15 kcal mol (Scheme 4.3b, showing a ΔG\textsubscript{2} ~ 0, as indicated by the experimental results). Only then can ΔG\textsuperscript{‡}\textsubscript{2} be ≤15 kcal/mol. An enzyme-mediated decrease in the homolytic bond dissociation energy could decrease ΔG\textsubscript{2} by making the reaction more enthalpically favorable (decreasing ΔH\textsubscript{2}). An increase in the entropy of reaction (ΔS\textsubscript{2}), through an increase in degrees of freedom of cofactor, protein and/or solvent, could also contribute to a decrease in ΔG\textsubscript{2}.

The observation that the equilibrium constant for homolysis of the carbon-cobalt bond and thiyl radical formation is on the order of one (2.0±0.3) demonstrates that the thermodynamics of carbon-cobalt bond homolysis are in fact highly perturbed at the enzyme active site. The observed perturbation puts the AdoCbl-bound state at roughly the same free energy as the cob(II)alamin/5´-dA-bound thiyl radical state (ΔG\textsubscript{2} ~ 0). This observation is in accord with theoretical expectations for the relative free energies of reactive intermediates in enzyme mechanims. For a concerted reaction, catalysis is predicted to be most efficient for reactions in which the intermediates are at equal energies, whether the reaction coordinate is analyzed using a Brønsted formalism (Albery & Knowles, 1976) or a Marcus formalism (Gerlt & Gassman, 1993).

To address the question of how RTPR solves the problem of efficient catalysis of a reaction that is highly endergonic in solution, the thermodynamics of carbon-cobalt bond homolysis on the enzyme were
Scheme 4.3
investigated. In the absence of substrate, the kinetics are simple enough to
determine microscopic rate constants for a concerted mechanism (Scheme
4.1). Measuring the rate and amount of cob(II)alamin formation as a function
of temperature addresses two related questions. First, it provides information
about how the highly perturbed equilibrium constant between the AdoCbl-
bound state and cob(II)alamin/5'-dA-bound thiyl radical state is achieved by
allowing calculation of ΔH and ΔS. Second, it allows calculation of activation
parameters (ΔH‡ and ΔS‡), thus defining the factors likely to be important in
transition state stabilization. On the basis of model studies showing how the
carbon-cobalt bond might be weakened, it has been hypothesized that AdoCbl
dependent enzymes catalyze carbon-cobalt bond homolysis by lowering the
bond dissociation energy of AdoCbl bound to the enzyme (Banerjee, 1997,
Halpern, 1985). However, in the case of RTPR, entropy appears to be the
dominant factor in establishing the thermodynamics and kinetics of carbon-
cobalt bond homolysis.

Entropy Drives Carbon-Cobalt Bond Cleavage/Thiyl Radical Formation

Part of the large perturbation in the thermodynamics of carbon-cobalt
bond can be explained by the coupling of carbon-cobalt bond homolysis to
thiyl radical formation. Formation of a thiyl radical with concomitant
formation of a C-H bond at the 5' position of the cofactor will clearly be
enthalpically favorable. According to the mechanism in Scheme 4.1, ΔH₂ is
the sum of four enthalpy changes: the bond dissociation enthalpy of the
carbon-cobalt bond on the enzyme, the bond dissociation enthalpy of the S-H
bond of the catalytically required cysteine residue (C408), the enthalpy of
formation of the C-H bond in 5'-dA, and any enthalpy changes associated
with any conformational changes of the enzyme. The measured enthalpy
change is ~12-20 kcal/mol. Using 30-35 kcal/mol (Hay & Finke, 1988) as the
bond dissociation enthalpy of the carbon-cobalt bond of AdoCbl, 88 kcal/mol
(the value for methanethiol) (Griller & Martinho Simoes, 1990, McMillen &
Golden, 1982) as the bond dissociation enthalpy of the RS-H bond, and 101
kcal/mol as the bond dissociation enthalpy of the C-H bond (Benson, 1978),
the enthalpy for the analogous non-enzymatic set of reactions can be
estimated to be 15-20 kcal/mol. This is approximately the same as the
measured value in the enzymatic reaction, suggesting that binding of AdoCbl
to RTPR does not result in a significant alteration of the carbon-cobalt bond.
dissociation energy. This is in contrast to the hypothesis, based on extensive structural and mechanistic studies of model cobalamins and cobaloximes, that AdoCbl-dependent enzymes would perturb the thermodynamics of carbon-cobalt bond homolysis by weakening the carbon-cobalt bond through steric strain (Chemaly & Pratt, 1980, Geno & Halpern, 1987, Kräutler et al., 1994, Randaccio et al., 1981, Schrauwer & Grate, 1979) and/or electronic effects (De Ridder et al., 1996, Marzilli et al., 1985, Ng & Rempel, 1982).

Recent work on the AdoCbl-dependent isomerases has also led to the hypothesis that these enzymes do not weaken the carbon-cobalt bond to effect catalysis. Chen and Marsh, studying glutamate mutase, found that the $K_d$ for binding of AdoCbl was similar to $K_i$ for cob(II)alamin in the presence of 5'-dA, suggesting that the post-homolysis intermediates are not bound to the enzyme much more tightly than AdoCbl (Chen & Marsh, 1997). Banerjee, Chance and co-workers performed EXAFS studies on AdoCbl bound to methylmalonyl-CoA mutase, showing no difference in carbon-cobalt bond length between free AdoCbl and AdoCbl bound to the enzyme (Scheuring et al., 1997). These workers hypothesize that substrate binding induces differences in binding of AdoCbl or carbon-cobalt bond length on the enzyme (Chen & Marsh, 1997, Scheuring et al., 1997). However, to date, there is no direct experimental evidence for weakening of the carbon-cobalt bond on binding of AdoCbl to any of the isomerases. The appropriate experiments are difficult to carry out, however, as the AdoCbl-bound state that is the immediate precursor to carbon-cobalt bond homolysis may be present only transiently. Our conclusions with RTPR may or may not be generalizable to other enzymes that use AdoCbl. There are clearly significant mechanistic differences between RTPR and the isomerases, as enzymes such as methylmalonyl-CoA mutase (Meier et al., 1996) and glutamate mutase (Marsh, 1995) do not appear to employ protein radicals in their catalytic mechanisms.

If $\Delta H$ is not perturbed greatly from what would be expected based on reactions in solution, the observed equalization of energies of the reactant and product states must result from entropic effects. In fact, the entropy change associated with the enzymatic carbon-cobalt bond cleavage/thiyl radical formation does differ greatly from the entropy change measured in solution for carbon-cobalt bond homolysis. Whereas values up to 14 cal mol$^{-1}$ K$^{-1}$ have been reported for the non-enzymatic reaction (Hay & Finke,
1988), the value measured for the enzymatic reaction is 40–70 cal mol\(^{-1}\) K\(^{-1}\). Although entropy associated with the conformational mobility of the propionamide sidechains of the corrin macrocycle (Brown et al., 1995, Brown et al., 1996) may contribute to this large positive \(\Delta S\), positive entropy changes of this magnitude in biological systems have usually been associated with changes in solvation, as described below.

Macromolecular binding processes that involve large desolvations can have high entropies of reaction, providing a potential model for the large entropy change observed with RTPR. The polymerization of tobacco mosaic virus protein (TVMP) has been shown to be an entropy-driven process. The \(\Delta S\) for addition of trimers of TVMP to an existing TMVP polymer is \(\sim 100\) cal mol\(^{-1}\) K\(^{-1}\), compensating for an unfavorable \(\Delta H\) of 25–30 kcal/mol (Banerjee & Lauffer, 1966, Lauffer, 1975). The large \(\Delta S\) is most likely due to release of about 100 molecules of water from the protein on polymerization (Lauffer, 1975). While polymerization of TMVP is an example of how large desolvations can lead to large \(\Delta S\), association of the 52 kD TMVP trimer with a growing polymer is likely to involve larger changes in solvation than could be achieved by RTPR.

A second example of an entropy-driven macromolecular binding process is the interaction between the \(trp\) RNA-binding attenuation protein of \(B.\ subtilis\) and \(trp\) leader RNA (Baumann et al., 1996). This interaction has an unfavorable \(\Delta H\) of \(\sim 15\) kcal/mol and a large positive \(\Delta S\) of \(\sim 100\) cal mol\(^{-1}\) K\(^{-1}\). In this case as well, the entropy change was interpreted as a release of bound water on binding.

By analogy to these results, the large positive \(\Delta S\) of carbon-cobalt bond homolysis/thiyl radical formation step might be associated with a change in enzyme conformation that results in desolvation of a hydrophobic region. One possibility is that the AdoCbl binding site of RTPR is desolvated during carbon-cobalt bond homolysis. As shown in the previous manuscript, binding and dissociation of AdoCbl are rapid, suggesting that the AdoCbl-binding site is easily accessible to the solvent. It seems unlikely, however, that the cofactor binding site would be solvent-accessible after carbon-cobalt bond homolysis, as oxidation of cob(II)alamin would cause destruction of the cofactor. It is therefore plausible that the cofactor binding site could be desolvated concomitant with carbon-cobalt bond cleavage. The recent three-dimensional structure of methylmalonyl-CoA mutase shows that the
substrate-binding domain of the $\alpha$ chain of the enzyme covers the corrin ring when the partial substrate desulpho-CoA is bound, conditions which are likely to mimic the closed state of the enzyme in which the cofactor is homolyzed (Mancia et al., 1996). Using a similar model for RTPR, it is possible that a conformational change in the protein could switch the enzyme from a state in which the cofactor binding site is open to solvent to a state in which the cofactor binding site is desolvated and covered by another domain of the protein.

Allosteric transitions in hemoglobin provide an alternative model for an entropy-driven process. By measuring the differences in dimer-dimer dissociation constants for ligated and unligated hemoglobin subunits, Ackers and co-workers have determined enthalpies and entropies for the conformational transitions associated with cooperative ligand binding (Huang & Ackers, 1995, Mills & Ackers, 1979). For both cyanomethemoglobin and oxyhemoglobin, the $\Delta H$ for the cooperative transition between the unliganded and fully liganded states has been shown to be $\sim 30$ kcal/mol, and the $\Delta S$ has been shown to be $\sim 80-90$ cal/mol K (Huang & Ackers, 1995). This allosteric transition is thus entropy driven. However, paradoxically, while cooperative binding of oxygen to hemoglobin is associated with a positive entropy, it is also associated with an increase in the number of water molecules bound to the protein. Parsegian and co-workers found that the oxygen affinity of hemoglobin decreased with increasing osmotic pressure, and calculated that $\sim 60$ additional water molecules bind to hemoglobin in the course of the transition between unliganded and fully liganded states. This is consistent with structural studies indicating a decrease of $\sim 700$ Å in solvent accessible surface area on the transition between deoxygenated and oxygenated hemoglobin (Lesk et al., 1985). The physical basis for the large positive change in entropy in this case thus remains a mystery. Conformational entropy and intramolecular vibrations of the protein may be involved (Sturtevant, 1977). Positive entropy changes (20-30 cal/mol K) have also been measured for allosteric transitions in carbamoyl phosphate synthetase, with an increase in the conformational entropy posited as the basis for the entropy change (Braxton et al., 1996).

The physical basis of $\Delta S_2$ in the case of RTPR could be further investigated by examining the osmotic pressure dependence of cob(II)alamin formation. If desolvation drives carbon-cobalt bond homolysis, increasing
the osmotic pressure should favor cob(II)alamin formation. While a meaningful interpretation of \( \Delta S_2 \) must await further experiments, its magnitude shows that the enzyme-mediated perturbation of the thermodynamics of carbon-cobalt bond homolysis required for catalysis to occur is primarily entropic.

\[ \Delta S^\ddagger \text{ Dominates Transition State Stabilization for Carbon-Cobalt Bond Cleavage/Thiyl Radical Formation} \]

While the role of the enzyme in stabilizing intermediate species is important for catalysis, rate acceleration is most directly dependent on stabilization of the transition state. The activation parameters calculated from the [AdoCbl]- and temperature-dependence of the observed rate constant for cob(II)alamin formation provide insight into what factors contribute to transition state stabilization in this reaction.

The activation enthalpy \( (\Delta H_2^\ddagger) \) for step 2 (Scheme 4.1), with a measured value of 46±7 kcal/mol, is greater than the bond dissociation enthalpy of the carbon-cobalt bond (30–35 kcal/mol) (Finke & Hay, 1984, Halpern et al., 1984, Hay & Finke, 1988). This result suggests that RTPR does not serve to weaken this bond in the AdoCbl-bound state. In addition, this result shows that an increase in enthalpically favorable enzyme-AdoCbl interactions (e.g., increased hydrogen bonding) on progressing to the transition state cannot account for the observed rate enhancement. Two factors may account for the observation of an activation enthalpy greater than the bond dissociation enthalpy for AdoCbl. First, the activation enthalpy of thiyl radical formation must be taken into account. Second, the enthalpy of "cage escape", the diffusion of radicals out of the solvent cage, may have an analog in enzymatic systems, which would contribute to the observed \( \Delta H_2^\ddagger \) (Garr & Finke, 1993).

As described above, the formation of a thiyl radical, which involves homolytic cleavage of an S-H bond and formation of a C-H bond, is exothermic. However, this does not necessarily imply that the activation barrier for this process is negligible. In solution, the activation energy (assumed to be roughly equivalent to \( \Delta H^\ddagger \)) of formation of thiyl radicals from carbon-centered radicals is \( \sim 4 \) kcal/mol (Zavitsas & Chagiliougolou, 1995). The activation barrier in the enzymatic reaction is likely to be significant as well, since kinetic isotope effects are observed with [5'-2H2]-AdoCbl and D2O.
(Chapter 3). Since carbon-cobalt bond formation and thyl radical formation occur in a concerted fashion (previous paper in this issue), the observed activation barrier will include both the activation barrier for carbon-cobalt bond cleavage and the activation barrier for thyl radical formation.

Cage effects represent another potential contributor to the observed $\Delta H_2\dagger$. In model systems, cage effects have also been shown to influence the measurement of both activation enthalpies and entropies (Koenig et al., 1988). Koenig and Finke have argued that when the efficiency of cage recombination is high, the observed activation enthalpy for a metal-ligand bond homolysis is approximately equal to the sum of the activation enthalpies for bond homolysis and for the escape of the free radicals from the solvent cage (Koenig, T.W. et al, 1988). Cage escape makes a positive contribution to activation enthalpy because it involves repulsive interactions between the escaping radical and the solvent surrounding it (Franck & Rabinowitch, 1934, Koenig et al., 1988), and because the escaping radical can disrupt favorable solvent-solvent solvent interactions such as hydrogen bonds and van der Waals interactions. The observation that the measured activation enthalpy for carbon-cobalt bond homolysis in ethylene glycol is ~4.5 kcal/mol higher than the values measured in water is consistent with a higher activation enthalpy of cage escape in ethylene glycol, the more viscous solvent (Hay & Finke, 1988).

Koenig et al. argue that the activation enthalpy for viscous flow can be used as an approximation for the activation enthalpy for cage escape in carbon-cobalt bond homolysis (Koenig et al., 1988). This is reasonable, as the viscosity of a solvent can be viewed as a measure of the resistance of solvent molecules to motion (Tinoco et al., 1985). The activation enthalpy for viscous flow of ethylene glycol is 4.4 kcal/mol over the temperature range studied in the non-enzymatic reaction (Raznjevic, 1976). This could account for the difference in the measured activation enthalpies for carbon-cobalt bond homolysis, since the measured value contains contributions from both the activation enthalpy of carbon-cobalt bond homolysis and the activation enthalpy of cage escape (Hay & Finke, 1988). It is difficult to estimate what the magnitude of the activation enthalpy for cage escape on the protein might be in the absence of detailed information about protein dynamics. However, an effect of comparable size to the putative cage effect observed for homolyis of
AdoCbl in ethylene glycol could make a significant contribution to the RTPR-mediated process.

Of course, the radical intermediates in the enzymatic reaction are not solvated by the bulk solvent, so it is fair to ask whether cage effects can be relevant in enzymatic systems. If the protein environment is viewed as, in effect, solvating the cofactor, conformation reorganization required for separation of the 5'-deoxyadenosyl moiety and the corrin ring could be viewed as analogous to the solvent reorganization of cage escape. Finke and co-workers have hypothesized that the AdoCbl binding sites of enzymes may serve as extremely efficient radical cages (which has the beneficial effect of safeguarding reactive intermediates), but also be able to adopt a conformation in which cage escape is highly efficient, allowing abstraction of hydrogen from the substrate or a protein residue (Garr & Finke, 1993). The activation energy of the conformational change required for efficient cage escape would thus contribute to the observed $\Delta H_2^\ddagger$. The activation enthalpy for cage escape in RTPR could be significant, as favorable interactions such as hydrogen bonds or salt bridges might be broken or weakened.

While the lifetimes of caged radical pairs are too short for these species to be detected directly by conventional SF UV-vis spectroscopy, magnetic field effect studies (Harkins & Grissom, 1994) have provided strong evidence that for ethanolamine ammonia lyase, the enzyme initially produces a caged radical pair, with partitioning between recombination of the radicals and cage escape. Magnetic field effect studies might help determine whether cage effects are significant in the RTPR system as well.

The difference between the $\Delta H_2^\ddagger$ measured in this study (46±7 kcal mol) and the activation enthalpy of homolytic carbon-cobalt bond cleavage in solution (30–35 kcal/mol) can thus be explained by taking into account the contribution from the activation enthalpies of S-H bond homolysis/C-H bond formation (~4 kcal/mol) and a potential contribution from conformational reorganization of the enzyme to allow separation of a radical pair species (~4 kcal/mol or greater). A decrease in $\Delta H_2^\ddagger$ thus cannot explain the rate acceleration of the enzymatic process. In fact, enthalpically unfavorable enzyme reorganization is likely to be required in order to help the reaction to progress.

Experimentally, $\Delta S_2^\ddagger$ appears to be the key to the observed rate acceleration. A very large $\Delta S_2^\ddagger$, ~95 cal/mol K, is measured, contributing
about -30 kcal/mol to $\Delta G_2^\ddagger$ at 37 °C. A variety of mechanistic studies on model and enzymatic systems suggest possible sources of this large activation entropy.

Many of the same factors that might influence the entropy change of the overall reaction might also influence the activation entropy. As discussed by Koenig, et al., cage effects in metal-ligand bond homolysis have, to a first approximation, the same effects on observed $\Delta S^\ddagger$ as they do on observed $\Delta H^\ddagger$ (Koenig et al., 1988). In the few model systems for which activation entropies of cage escape have measured, they are typically on the order of 5–10 cal/mol K (Herkes et al., 1969, Koenig et al., 1988). While these effects can be significant, they are probably not the major determinant of activation entropy in the case of RTPR.

The entropy associated with the conformational mobility of the propionamide side chains may also contribute to $\Delta S^\ddagger$. Studies from Brown's laboratory indicate that the $\Delta S^\ddagger$ measured for homolysis of neopentylcobalamin bound to haptocorrin is 4.3 cal mol$^{-1}$ K$^{-1}$ greater than that of free neopentylcobalamin, thus contributing to a more favorable free energy of activation (Brown et al., 1996). If this effect is similar in RTPR, it cannot by itself account for a major percentage of the observed $\Delta S^\ddagger$.

Halpern has shown a direct correlation between increasing $\Delta S^\ddagger$ and increasing $\Delta H^\ddagger$ for carbon-cobalt bond homolysis in a large group of organocobalt compounds. He ascribes this trend to increased endothermicity leading to transition states that increasingly resemble the products, non-planar cobalt (II) complexes that are expected to be less rigid than the starting complexes (Halpern, 1988). However, the recent crystal structure of cob(II)alamin suggests the carbon-cobalt bond homolysis in AdoCbl does not lead to a relaxation of octahedral geometry (Kratky et al., 1995). In addition, even for the most endothermic reaction he examined ($\Delta H^\ddagger = 35$ kcal/mol), $\Delta S^\ddagger$ is only 27 cal mol$^{-1}$ K$^{-1}$. Again, this effect may contribute to the activation entropy, but cannot by itself explain the magnitude of $\Delta S_2^\ddagger$.

Thus, as for $\Delta S_2$, the largest contributors to $\Delta S_2^\ddagger$ are likely to be a release of bound water from hydrophobic regions or an increase in conformational entropy. If the RTPR-AdoCbl complex leaves hydrophobic regions of the enzyme and/or AdoCbl partially solvated, a conformational change might allow contacts between these regions, leading to their desolvation. While the magnitude of the entropy change is large, the
amount of hydrophobic surface that would have to be desolvated is likely to represent a reasonably small fraction of the total surface area. A free energy change of 25-50 cal is associated with desolvation of 1 Å² of hydrophobic surface of a protein (Chothia & Janin, 1975, Williams, 1991). A transition state stabilization of 30 kcal/mol (to compensate for a ΔH‡ of ~45 kcal/mol) would thus require desolvation of ~1000 Å² of hydrophobic surface. A conformational change causing a change in solvent accessible area of this magnitude would not be unreasonable for an 82 kD protein such as RTPR. Hexokinase (MW = 100 kD) undergoes a conformational change on substrate binding that results in the release of ~65 water molecules (Rand et al., 1993), which, if each water molecule is assumed to take up 9–10 Å of surface (Colombo et al., 1992), would correspond to desolvation of ~600 Å² of protein surface.

Through the measurement of catalytic activities as a function of osmotic pressure, binding and release of bound water have been associated with catalysis in a number of enzymes, including cytochrome P450 camphor (Di Primo et al., 1992), cytochrome c oxidase (Kornblatt & Hui Bon Hoa, 1990) and the restriction endonucleases EcoRI, EcoRV, BamH1, and Pvu II (Robinson & Sligar, 1995, Robinson & Sligar, 1996), although the relationship between the release of solvent and entropy changes have not been characterized. For the reaction of methanol dehydrogenase with cytochrome c₅₃, SF UV-vis studies carried out at a range of hydrostatic pressures have allowed measurement both of ΔS‡ and ΔV‡, the volume change in the transition state (Heiber-Langer et al., 1992). At 4 °C, catalysis is entropically driven, with a ΔS‡ of ~30 cal/mol K. The measured value for ΔV‡ is also positive, at ~120 ml/mol. This positive ΔV‡ is consistent either with a conformational change or with release of bound water (Kornblatt & Hui Bon Hoa, 1990).

Another example of a large activation entropy in a biological reaction comes from the association of the ShB peptide, a synthetic peptide of the amino-terminal inactivation domain of the Shaker potassium channel, with the parent channel. This reaction exhibits a ΔS‡ of 60 cal mol⁻¹ K⁻¹ (Murrell-Lagnado & Aldrich, 1993a). Hydrophobic interactions between the peptide and the channel have been shown to be important for peptide binding (Murrell-Lagnado & Aldrich, 1993b), suggesting that desolvation of hydrophobic surfaces in the transition state of binding might contribute to the

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large entropy of activation. Interestingly, the ShB peptide is made up of only 20 amino acid residues, indicating that a large area of hydrophobic contact is not required for a large ΔS‡ in this case. Whether desolvation of hydrophobic regions of RTPR is a viable explanation for the observed activation parameters awaits determination of a three-dimensional structures of RTPR in apo- and cob(II)alamin/5′-dA-complexed forms.

In conclusion, steady-state and pre-steady state analysis of carbon-cobalt bond homolysis/thiyl radical formation catalyzed by RTPR in the presence of dGTP has allowed determination of rate constants using a minimal mechanism for the exchange reaction. Analysis of the temperature dependence of the rates and amounts of cob(II)alamin has allowed determination of the thermodynamic parameters for carbon-cobalt bond homolysis/thiyl radical formation. This analysis shows that entropic effects dominate catalysis. Differential solvation of the ground state and the transition state is suggested as a possible source of the observed entropic effects.
References


Kratky, K., Färber, G., Gruber, K., Wilson, K., Dauter, Z., Nolting, H.-F.,


Chapter 5:

Evidence that Carbon-Cobalt Bond Re-Formation Follows Each Turnover
Introduction

While RFQ EPR experiments have demonstrated that the thiol radical is a kinetically competent intermediate in catalysis (Chapter 2), many details of its role in the catalytic mechanism remain unknown. The experiments described in this chapter were designed to address some basic mechanistic questions concerning nucleotide reduction. The mechanism in Scheme 5.1 served as the basic framework for interpretation of experimental results. The mechanism in Scheme 5.1 posits re-formation of the carbon-cobalt bond after each turnover. This prediction may be counterintuitive, since carbon-cobalt bond re-formation after turnover is not required for a catalytic cycle, and might seem to detract from catalytic efficiency. However, experiments described in this chapter strongly support the hypothesis that carbon-cobalt bond follows each turnover. These experiments also allow conclusions to be drawn about the thiol radical's commitment to catalysis and about the nature of the rate-limiting step in the first turnover.

Scheme 5.1


**Ethanolamine Ammonia Lyase and Diol Dehydrase: AdoCbl-Dependent Enzymes with Mechanistic Similarities to RTPR**

The issues of rate-limiting step, chain length, and commitment to catalysis that will be addressed in this chapter for RTPR have been addressed, to varying extents, in published work on ethanolamine ammonia lyase and diol dehydratase. These AdoCbl-dependent enzymes catalyze reactions that are formally similar to ribonucleotide reduction (Scheme 5.2) (Abeles & Dolphin, 1976, Stubbe, 1990). For ethanolamine ammonia lyase and diol dehydratase, the rearrangements can be thought of as intramolecular redox reactions. Two carbon centers start at the alcohol oxidation state in the substrate, while in the product, one is oxidized to the aldehyde oxidation state, and the other is reduced to the methylene oxidation state. For RTPR, a similar phenomenon occurs initially, with the 3′ carbon center is transiently oxidized to the ketone oxidation state, and the 2′ carbon center reduced to the methylene oxidation state. However, active site cysteines act to reduce the 3′ keto intermediate (Mao et al, 1992, Booker et al, 1994), making the net redox chemistry intermolecular. In many cases, mechanistic similarities between the enzymes mirror the formal similarities in reactions catalyzed.

For all three enzymes, cob(II)alamin is observed as an intermediate, but in varying steady-state amounts. In addition, kinetic isotope effects implicate a hydrogen abstraction step for all three mechanisms, although the magnitudes of these effects, and whether they are observed on V/K or V_max, vary from enzyme to enzyme. Paramagnetic intermediates are also observed in all three enzymes, although the EPR lineshape varies among the enzymes and, in some cases, from substrate to substrate. Relevant experimental results are summarized in Tables 5.1 and 5.2.

**Binding of Substrates to RTPR**

Scheme 5.1 begins with binding of AdoCbl to the enzyme, which also binds substrate and allosteric effector. Little is known about the order of binding of substrates to RTPR. It has been observed that allosteric effector can bind to RTPR in the absence of AdoCbl (Chen et al, 1974, Booker et al, 1994). The allosteric effector dGTP, for example, binds with a K_d of ~10 μM at 22 °C and ~4 μM at 4 °C (Chen et al, 1974, Booker et al, 1994). The observation of relatively high affinity binding of the allosteric effector to RTPR in the absence of AdoCbl is consistent with, but does not prove,
Ethanolamine Ammonia Lyase

Diol dehydrase

Ribonucleotide Reductase

Scheme 5.2
Table 5.1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$D_V^{max}$ (deuterated substrate)</th>
<th>$D_V/K$ or $T_V/K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAL, ethanolamine$^a$</td>
<td>140</td>
<td>~10</td>
<td>~6 (T), overall</td>
</tr>
<tr>
<td>EAL, (S)-2-aminopropanol$^a$</td>
<td>~2</td>
<td>~6$^d$</td>
<td>~5 (D), overall</td>
</tr>
<tr>
<td>Diol dehydrase, propanediol$^b$</td>
<td>340</td>
<td>~10</td>
<td>20 (T), substrate -&gt; AdoCbl</td>
</tr>
<tr>
<td>RTPR, ATP$^c$</td>
<td>~2</td>
<td>-</td>
<td>~2 (T)</td>
</tr>
</tbody>
</table>

a. (Babior, 1982) and references therein
b. (Toraya & Fukui, 1982)
c. (Ashley et al, 1986)
d. (Diziol et al, 1980)
Table 5.2

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>cob(II)alamin, eq. (steady state, by UV-vis)</th>
<th>unpaired spin, eq.*</th>
<th>EPR spectrum*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAL, ethanolamine(^a)</td>
<td>0.6</td>
<td>0.05 (0.01 org.)</td>
<td>singlet, g = 2.0</td>
</tr>
<tr>
<td>EAL, (S)-2-amino-propanol(^a)</td>
<td>~1</td>
<td>1.3</td>
<td>doublet, g = 2.04, 1.96</td>
</tr>
<tr>
<td>Diol dehydrase, propanediol(^b)</td>
<td>~0.5</td>
<td>~1.1 (0.6–0.75 org.)</td>
<td>doublet, g = 2.04, 1.96</td>
</tr>
<tr>
<td>Diol dehydrase, chloracetaldehyde(^b)</td>
<td>~0.9</td>
<td>2</td>
<td>singlet, g = 2.0</td>
</tr>
<tr>
<td>RTPR, ATP(^c)</td>
<td>~0.5</td>
<td>~1 (calc. w/S = 1/2)</td>
<td>broad singlet, g = 2.12</td>
</tr>
<tr>
<td>RTPR, ATP w/monothiol reductants(^d)</td>
<td>-</td>
<td>0.02 (org.)</td>
<td>doublet, g = 2.03, 1.97</td>
</tr>
</tbody>
</table>

EAL = ethanolamine ammonia lyase

* With EAL and diol dehydratase, the EPR spectra also had a feature at g ~ 2.2, identified as arising from cob(II)alamin. The spin concentrations include both organic radical and cob(II)alamin. The contribution of the organic radical is shown where it has been specified.

a. (Babior, 1982) and references therein
b. (Toraya and Fukui, 1982) and references therein
c. (Orme-Johnson et al, 1974) and this work
d. (Hamilton et al, 1972)
as evaluated by V/K, the partitioning should minimize carbon-cobalt bond re-formation and maximize initiation of turnover.
binding of effector before AdoCbl in the catalytic mechanism. The large estimated $K_d$ (~200 μM) for AdoCbl in the exchange reaction (Chapter 4) suggests that substrate is required for high affinity binding of AdoCbl, and is consistent with the mechanism in Scheme 5.1. However, none of these experiments directly address the order of binding in the catalytic mechanism. Preliminary results discussed in this chapter suggest that ATP binding is slow compared to carbon-cobalt bond homolysis, which would be consistent with the order of binding shown in Scheme 5.1. Defining the order of binding and the order of product release still remains to be accomplished.

**Commitment to Catalysis of the Thyl Radical and Rate of a Single Turnover**

After AdoCbl binding, carbon-cobalt bond homolysis takes place, followed by initiation of nucleotide reduction by the thiyl radical. The mechanism in Scheme 5.1 illustrates that the thiyl radical can partition between initiating turnover ($k_{+3}$) and abstracting hydrogen from $5\'-dA$ to reform the carbon-cobalt bond ($k_{-2}$). This partitioning can be a critical component of the catalytic efficiency of the enzyme. For maximum efficiency, as evaluated by $V/K$, the partitioning should minimize carbon-cobalt bond re-formation and maximize initiation of turnover.

In thinking about this partitioning, it will be helpful to compare RTPR, ethanolamine ammonia lyase, and diol dehydratase (Abeles and Dolphin, 1976, Stubbe, 1990). For ethanolamine ammonia lyase and diol dehydratase, the commitment to catalysis appears to be low for the putative $5\'-dA•$ intermediate (the functional equivalent in these enzymes of the thiyl radical). Ethanolamine ammonia lyase exhibits a $V/K$ deuterium isotope effect of ~10 with deuterated ethanolamine (Babior, 1969, Weisblat & Babior, 1971, Babior, 1982). The magnitude of this effect is consistent with a fully expressed primary isotope effect, and thus a low commitment to catalysis for $5\'-dA•$. Diol dehydrase exhibits tritium isotope effects (which measure $V/K$) of 20 on transfer of hydrogen from substrate to cofactor and 250 on transfer of hydrogen from cofactor to product (Frey et al, 1967, Essenberg et al, 1971). While the detailed interpretation of these isotope effects, particularly the extremely large isotope effect of 250, is still a subject of debate (Cleland, 1982b), they indicate a large selection effect against tritiated substrate, and thus suggest a low commitment to catalysis.
The RTPR thiyl radical, in contrast, appears to exhibit a high commitment to catalysis. Tritium V/K isotope effects with [3'-3H]-NTPs range from 1.6 to 2.1 (Stubbe et al, 1981, Ashley et al., 1986). These effects are too large for a secondary isotope effect, but small enough that they are likely to represent an incompletely expressed primary isotope effect. These experiments were thus interpreted as evidence for 3' carbon-hydrogen bond cleavage occurring via a high commitment intermediate (Ashley et al., 1986). To date, these experiments have provided the best evidence that RTPR catalysis includes abstraction of the 3' hydrogen of substrate. Experiments described in this chapter on the presteady state kinetics of turnover and tritium washout from [5'-3H]-AdoCbl provide evidence for the hypothesis that commitment to catalysis is high for the intermediate that abstracts the 3' hydrogen (called X• in the earlier work, now formulated as a thiyl radical).

Abstraction of the 3' hydrogen is not the rate-limiting step in catalysis by RTPR. Studies on the ribonucleotide reductase from E. coli, which exhibits similar V/K isotope effects (Stubbe et al, 1983), indicate no \( V_{\text{max}} \) isotope effect using [3'-2H]-NDPs as substrate, indicating that 3' hydrogen abstraction is not rate-limiting in catalysis (Ator, 1984). Given the mechanistic similarities between these enzymes, the same is likely to be true for RTPR. This would be in contrast to results for ethanolamine ammonia lyase and diol dehydratase, where hydrogen atom transfer is proposed to be rate-limiting. In the case of ethanolamine ammonia lyase, transfer of hydrogen from 5'-dA to a putative product radical is proposed to be the rate-limiting step (Babior, 1982). With ethanolamine as substrate, the deuterium kinetic isotope effect on transfer of hydrogen from cofactor to product is 7.3, while the effect on hydrogen transfer from substrate to cofactor is <4.7 (Babior, 1969, Weisblat and Babior, 1971). Assuming similar transition state structures for the two hydrogen atom transfers, this result argues for the hydrogen atom transfer from cofactor to product being rate-limiting. Likewise, a deuterium \( V_{\text{max}} \) isotope effect of 10–12 is observed with diol dehydratase (Zagalak et al, 1966, Moore et al, 1979), indicating that hydrogen transfer, either from substrate to cofactor (Essenberg et al, 1971) or from cofactor to product (Moore et al., 1979) is rate-limiting.

This chapter describes rapid acid quench experiments that were carried out to determine whether nucleotide reduction is rate-limiting in catalysis of the first turnover by RTPR. These experiments show that neither hydrogen...
atom abstraction nor any other step in nucleotide reduction is rate-limiting in catalysis. This observation may help explain why the turnover number for RTPR (2 s\(^{-1}\)) is much lower than those observed for ethanolamine ammonia lyase (140 s\(^{-1}\) for aminoethanol) (Babior, 1982) or diol dehydratase (340 s\(^{-1}\) for with propanediol as substrate) (Toraya and Fukui, 1982). The basis for the difference in rate-limiting steps between RTPR and the isomerases may lie in the net redox character of the reaction catalyzed by ribonucleotide reductase. One possibility consistent with the kinetic data is that re-reduction of RTPR after turnover is rate-limiting.

Attempts to Trap a Substrate-Based Radical

The identity of the rate-limiting step has implications for the prospects of trapping substrate-based radical intermediates. In cases where hydrogen abstraction by a product radical is rate-limiting, large amounts of substrate-based radical can accumulate. To date, no substrate-based radicals have been observed during catalysis by RTPR, in which hydrogen atom abstraction is not rate-limiting. Substrate-based radicals have, however, been observed during catalysis by ethanolamine ammonia lyase and diol dehydratase, in which hydrogen abstraction steps are rate-limiting (Essenberg et al., 1971, Moore et al., 1979, Babior, 1982). The observation with RTPR of a non-kinetically competent radical under turnover conditions with poor reductants (or small amounts of good reductants) suggests a general strategy for trapping substrate-based radicals in this system. Perturbations of conditions that hinder reduction of a substrate-based radical by C119 and C419, but do not hinder 3'-hydrogen abstraction, should increase the concentration of substrate-based radical that accumulates in the presteady state. Three tactics have so far been attempted to carry out this strategy: site-directed mutagenesis, use of the substrate analog ara-ATP, and use of a large excess of pre-reduced RTPR over TR.

RFQ EPR experiments in which ethanolamine ammonia lyase and AdoCbl were mixed with the slow substrate aminopropanol demonstrated the production of a substrate-based radical species with a rate constant approximately twice that of substrate turnover (Babior et al., 1974, Graves et al., 1980). This radical was initially assigned as the 1-hydroxy-2-aminoethyl radical based on the effects of isotopic labeling (Babior et al., 1974). Later, the same radical was examined by ESEEM and re-formulated as an intermediate
radical in which the amino group had already been lost (Tan et al, 1986).\textsuperscript{1} EPR studies on reaction mixtures containing ethanolamine, in contrast, show only 0.01 equivalent of organic radical species per active site in the steady state (Babior et al, 1972). These experiments suggest that with aminopropanol, re-abstraction of hydrogen by a product-based radical is slow enough for this intermediate to accumulate. The results with ethanolamine are consistent with rapid substrate turnover precluding observation of a substrate- or product-based paramagnetic intermediate. However, the low spin quantitation compared to the cob(II)alamin formation measured in SF UV-vis experiments (~0.6 eq.) (Hollaway et al, 1978) suggests that RFQ EPR techniques might be required to trap paramagnetic intermediates, as is the case with RTPR (Orme-Johnson et al, 1974).

Paramagnetic reaction intermediates in catalysis by diol dehydratase have also been trapped (Finlay et al, 1973, Valinsky et al, 1974a, Valinsky et al, 1974b). A doublet signal with g ~ 2.0, corresponding to 0.6–0.75 molecules of paramagnetic species per molecule of holoenzyme, was trapped during steady state turnover of propanediol (Finlay et al, 1973, Toraya et al, 1979), and shown by RFQ EPR experiments to be formed with a rate constant >200 s\(^{-1}\) (compared to 340 s\(^{-1}\) for turnover) (Valinsky et al, 1974b). A singlet signal with g ~ 2.0, corresponding to 2 molecules of paramagnetic species per molecule of holoenzyme, was identified on incubation of diol dehydratase with AdoCbl and the mechanism-based inhibitor chloroacetaldehyde (Valinsky et al, 1974a). The kinetics of formation of this signal were not measured in detail, although it formed in <30 s. Incubation of diol dehydratase with AdoCbl and chloroacetaldehyde also led to the formation of ~0.9 eq. of cob(II)alamin with a rate constant of >0.25 s\(^{-1}\) (Finlay et al, 1972). The use of [U-\textsuperscript{13}C]-chloroacetaldehyde gives rise to broadened hyperfine features in this signal, demonstrating that unpaired spin density is present on the carbon skeleton of this substrate analog (Valinsky et al, 1974a). These studies provide evidence for a substrate-based radical as a kinetically competent intermediate in catalysis by diol dehydratase.

Since hydrogen atom abstraction in the case of RTPR is not rate-limiting, a substrate-based radical intermediate may react to give product as fast as it is formed. Thus, it is perhaps not surprising that kinetically

\textsuperscript{1} Other, more recent EPR experiments have cast doubt on the hypothesis that this radical has lost the amino group (G. Reed, personal communication).
competent substrate-based radicals have not yet been observed in this system. However, a novel, albeit non-kinetically competent, paramagnetic species has been observed on incubation of in the presence of RTPR with AdoCbl, nucleotide substrate, deoxynucleotide allosteric effector, and low amounts of reductant (or monothiols, which are inefficient reductants) (Hamilton et al, 1972). These conditions give rise to an EPR spectrum known in the literature as the "doublet signal" (Hamilton et al, 1972, Pilbrow, 1982). The spectrum consists of a broad absorbance at centered at $g = 2.3$ and a partially resolved doublet with a hyperfine splitting of 110 G centered at $g = 2$ (Hamilton et al, 1972). It is similar in lineshape to the doublet signals observed with ethanolamine ammonia lyase (Babior et al, 1974) and diol dehydratase (Finlay et al, 1973, Toraya et al, 1979).

The species responsible for the doublet signal observed with RTPR is not likely to be a catalytic intermediate. The spin concentration of the doublet species was low, corresponding to ~2% of the enzyme concentration. More importantly, the slow rate of formation of the doublet species ($t_{1/2} = 5-10$ min) compared to turnover ($k_{cat} \sim 2$ s$^{-1}$) indicates that the doublet species is not a kinetically competent intermediate in catalysis.

These observations are consistent with the hypothesis that the doublet signal results from an alternate reaction pathway branching from an intermediate in catalysis. Use of reducing systems that are unable to reduce the enzyme at the maximal rate could lead to accumulation of oxidized enzyme forms that would be expected to be unable to catalyze loss of 2' hydroxyl as water (since the thiol protons of the bottom face cysteines would be missing) and/or reduction of substrate-based radical intermediates. The observation of an unidentified product that accumulates in a time-dependent fashion on incubation of oxidized RTPR with substrate provides further evidence that oxidized RTPR can catalyze 3'-hydrogen abstraction (albeit slowly) (Booker et al, 1994). This "mystery peak" may derive from a substrate-based radical that undergoes further reaction to form the observed product (Booker et al, 1994).

One area of continuing experimental interest is determining the nature of the doublet species, and whether it is in fact a substrate-derived radical. It was reported that perdeuterated GTP gave rise to a doublet signal identical to that observed with unlabeled GTP, although spectra were not shown (Hamilton et al, 1972). However, this observation does not rule out
the doublet species being an off-pathway species with spin density at the 3' position. In this case, hyperfine contributions from deuterium atoms \( \beta \) to the unpaired electron might not be observed, especially if interactions with cob(II)alamin broaden the spectrum of the organic radical. If this species is an off-pathway reaction product, studies of its structure could prove mechanistically informative. The EPR spectrum of doublet species generated using [U-\( ^{13} \)C]-ATP was examined to address this question.

While the doublet signal is not kinetically competent, its observation suggests that interfering with RTPR's ability to deliver reducing equivalents might be a productive strategy for trapping substrate-based radicals. Using site-directed mutagenesis to deprive the enzyme of its ability to deliver reducing equivalents to the substrate is one way to prevent the complete nucleotide reduction process from taking place. To this end, RFQ EPR studies were carried out using the C419S mutant RTPR. This mutation abolishes the ability of the enzyme to deliver reducing equivalents to the substrate (Booker et al., 1994).

Another method of trapping a substrate-based radical would be to use a substrate analog in which abstraction of the 3' hydrogen atom was possible, but one or more subsequent steps were slow. Since ara-ATP is epimeric to the natural substrate at the 2' position, it is possible that RTPR will be able to catalyze the abstraction of its 3' hydrogen but unable to catalyze elimination of its 2' hydroxyl group. The abstraction of the 3' hydrogen of substrate may be driven both by deprotonation of the 3' hydroxyl and by coupling of hydrogen atom abstraction to loss of the 2' hydroxyl group (Chapter I).

Depending on the relative importance of these two factors, hydrogen atom abstraction might still be possible with ara-ATP. If it is, it is unlikely that loss of the 2' hydroxyl will be possible, since protonation of the 2' hydroxyl by a residue or residues at the \( \alpha \) face of the substrate (e.g., C119 and C419) is most likely to be required for facile loss of this group (Stubbe, 1990, Booker et al., 1994). This would be in contrast to results with 2'-halonucleotides, where the halo group leaves from either the ribo or the ara positions, presumably because it does not require protonation to become a good leaving group (Harris et al., 1987). Preliminary SF UV-vis studies were carried out to determine whether the substrate analog ara-ATP was likely to be processed to a substrate-based radical on the millisecond timescale, with the aim of
determining whether RFQ EPR studies with ara-ATP would allow trapping of a 3’ radical intermediate.

The third tactic, incubation of pre-reduced RTPR with a greatly sub-stoichiometric (<0.01 eq.) amount of TR, provides conditions similar to those which have previously been shown to result in formation of the doublet species. Oxidized enzyme may be able to generate substrate-based radicals that were not detected in the earlier studies (Hamilton et al., 1972) due to the method of quenching. SF UV-vis and RFQ EPR studies were therefore carried out to ascertain what, if any, substrate-based radicals were trappable.

Re-formation of the Carbon-Cobalt Bond After Turnover

The final mechanistic question addressed in this chapter is the fate of the thiyl radical intermediate after turnover. Halpern has emphasized the importance of the problem of the chain length of AdoCbl-dependent processes in the context of enzymatic rate acceleration of carbon-cobalt bond homolysis (Halpern, 1985). If a single carbon-cobalt bond homolysis is sufficient to initiate multiple cycles of catalysis, the rate of carbon-cobalt bond homolysis required to maintain a given steady state turnover rate will be less than if the carbon-cobalt bond must be re-formed after every turnover. In cases where carbon-cobalt bond homolysis is slow compared to turnover, increasing the chain length of the reaction would significantly increase the steady state rate of turnover. For the limiting case of an infinite chain length, the slow carbon-cobalt bond homolysis step will be bypassed in the steady state. However, an alternate possibility, which might have the advantage of minimizing the opportunities for enzymatic self-inactivation, is that the carbon-cobalt bond is re-formed after every turnover. As shown in Scheme 5.1, this is the mechanism favored for RTPR. Rapid acid quench studies on the kinetics of tritium washout from [5’-3H]-AdoCbl during turnover, described in this chapter, provide support for this hypothesis.

Re-formation of the carbon-cobalt bond after every turnover might help protect AdoCbl-dependent enzymes from oxidative inactivation. Rétey has suggested that enzymes such as RTPR and the AdoCbl-dependent isomerases, which employ highly reactive intermediates, catalyze reactions specifically by making non-productive reactions unfavorable, rather than through large transition state stabilizations, since the intermediates are
already reactive enough for the physiological reaction to proceed at a high rate (Rétéy, 1990). Oxidation of radical intermediates by dissolved oxygen is one of the major non-productive reaction pathways likely to pose a problem. The consequences of adventitious oxidation of the thiyl radical would be severe, as this would inactivate the enzyme as well as irreversibly trap the coenzyme in the cob(II)alamin/5'-dA form, which is inactive as a cofactor. Oxidation of cob(II)alamin to aquocobalamin would irreversibly inactivate the cofactor. Binding of substrate to the active site is likely to require exposure of the active site to bulk solvent. If 5'-dA• (or, for RTPR, the thiyl radical) and cob(II)alamin are still at the enzyme active site when it is exposed to bulk solvent, there is a risk of inactivation. However, if these radicals recombine to re-form AdoCbl, that risk would be avoided. The issue of the chain length for AdoCbl-dependent reactions is thus an important one from the point of view of control of reactivity.

A number of experiments which are relevant to the issue of the chain length of turnover have been performed on the AdoCbl-dependent isomerases, but thus far no definitive answer has been forthcoming. The issue of protection of reactive intermediates is likely to be an important one for ethanolamine ammonia lyase, since this enzyme exhibits both a high turnover number (140 s⁻¹ for aminoethanol) (Babior, 1982) and a high steady state concentration of cob(II)alamin. The enzyme must thus be able to maintain reactive intermediates in high concentration on average, while also allowing, at some point in the catalytic cycle, free access of substrate to the active site.

SF UV-vis studies have shown that when ethanolamine ammonia lyase (in slight excess over AdoCbl) and AdoCbl are mixed with the slow substrate aminopropanol (with aminopropanol and AdoCbl concentrations both much greater than the respective Kₘₛ), >90% of the coenzyme is converted into cob(II)alamin within the mixing time of the instrument used (3 ms). This essentially stoichiometric level of cob(II)alamin is maintained throughout the steady state phase of the reaction (Holloway et al., 1978). With aminoethanol (kᵣ = 140 s⁻¹, compared to 2 s⁻¹ for aminopropanol), ~60% of the coenzyme is converted to cob(II)alamin within the mixing time. This concentration of cob(II)alamin represents the steady state level, which is maintained until substrate has been exhausted (t₁/₂ = 200 s), when oxidation to aquocobalamin is observed.
Carbon-cobalt bond re-formation after every turnover would account for cob(II)alamin building up to a greater extent when aminopropanol is the substrate than when aminoethanol is the substrate. When aminopropanol as substrate, the effective rate constant of carbon-cobalt bond re-formation would be \( \sim 2 \text{ s}^{-1} \) if carbon-cobalt bond re-formation followed every turnover. This rate constant is much less than the \( k_{\text{obs}} \) of carbon-cobalt bond homolysis (>300 s\(^{-1}\)). In contrast, with aminoethanol as substrate, the net rate of carbon-cobalt bond re-formation (140 s\(^{-1}\)) would be closer to the net rate of carbon-cobalt bond homolysis.

Diol dehydrase also accumulates high steady state concentrations of radical intermediates while maintaining a high turnover number (340 s\(^{-1}\) for with propanediol as substrate) (Toraya and Fukui, 1982). Under turnover conditions, with enzyme in excess over AdoCbl and 1,2-propanediol as the substrate, the UV-visible spectrum of the steady state reaction mixture shows that \( \sim 45\% \) of the coenzyme is in the form of cob(II)alamin (Toraya et al, 1979). This result is also consistent with carbon-cobalt bond re-formation occurring after each turnover. It is known that radical intermediates are formed with a rate constant of \( >200 \text{ s}^{-1} \) (Valinsky et al, 1974b), compared with 340 s\(^{-1}\) for turnover. If the rate constant for carbon-cobalt bond homolysis is comparable to that of turnover, and carbon-cobalt bond re-formation follows each turnover, the net rate for cob(II)alamin formation will be approximately the same as the net rate for re-formation of the carbon-cobalt bond, and approximately 50% of bound cofactor will be in the form of cob(II)alamin in the steady state.

While these experiments are consistent with re-formation of the carbon-cobalt bond after every turnover in ethanolamine ammonia lyase and diol dehydratase, they do not prove that it occurs. Cleland has made an argument against re-formation of the carbon-cobalt bond occurring after every turnover in the mechanisms of ethanolamine ammonia lyase and diol dehydratase (Cleland, 1982a). He suggests that the extremely large tritium isotope effects on transfer of hydrogen from AdoCbl to product observed with ethanolamine ammonia lyase (\( k_{H}/k_{T} = 160 \) for tritiated ethanolamine) (Weisblat and Babior, 1971) and with diol dehydratase (\( k_{H}/k_{T} = 125 \) for tritiated propanediol) (Frey et al, 1967, Essenberge et al, 1971) could be explained by the formation of a protein radical that initiates catalysis over multiple turnovers before re-abstracting a hydrogen atom from 5'-dA,
allowing re-formation of the carbon-cobalt bond. In this case, transfer of hydrogen from AdoCbl to product would not occur on every turnover. Cleland notes that if the putative protein radical in ethanolamine ammonia lyase abstracts a hydrogen from 5'-dA once every 9 turnovers, the true tritium isotope effect would be ~18, a more reasonable magnitude for a primary tritium isotope effect. While this hypothesis can account for the data, it is unappealing on general principles, since it requires the existence of two radical species, one cofactor-derived and one protein-derived, doing the same reaction at comparable rates in the same enzyme mechanism.

In support of this hypothesis, however, Babior and co-workers discovered that when ethanolamine ammonia lyase is incubated with [5'-3H]-AdoCbl in the presence of ethanolamine as substrate, ~10% of the tritium label becomes exchangeable with solvent within 60 s (O'Brien et al., 1985). This observation is consistent with a mechanism for solvent exchange analogous to that proposed for RTPR (Chapter 2, Scheme 2.1). Abstraction of hydrogen from an exchangeable protein residue by 5'-dA• would form an X• that can serve to initiate multiple turnovers. Re-abstraction of hydrogen from 5'-dA by this X• would allow exchange of tritium with solvent. The observation that ~10% of the tritium in AdoCbl exchanges with solvent is qualitatively consistent with 5'-dA• initiating only one turnover before re-formation of the carbon-cobalt bond, but the putative X• initiating ~10 turnovers before abstracting a hydrogen from 5'-dA. This would mean that ~90% of the tritium flux would be through the product and ~10% through the exchangeable residue.

Given that in the steady state (with aminoethanol as product), ~40% of the bound cofactor is in the form of AdoCbl, the hypothesis that a protein radical initiates multiple turnovers in ethanolamine ammonia lyase predicts that on average, in the steady state, almost half of the enzyme active sites are quiescent, while the rest are initiating multiple rounds of catalysis without re-formation of the carbon-cobalt bond. This suggests the steady state k_{cat} actually underestimates the steady state rate of an active ethanolamine ammonia lyase molecule, because this k_{cat} represents the average over quiescent and activated enzyme molecules. In other words, the protein radical hypothesis suggests that only about half of the enzyme molecules are turning over substrate at any given time, but that they are turning it over twice as fast as predicted from the steady state k_{cat}. If this hypothesis is
correct, in a single turnover experiment with holoenzyme in large excess over substrate, the rate constant for formation of product will be larger than the steady state $k_{\text{cat}}$, even though product formation is the rate limiting step in the reaction. An experiment of this type has not yet been carried out with ethanolamine ammonia lyase or diol dehydratase.

Pending new information on the presteady state kinetics of substrate turnover and/or direct evidence for protein radicals, the issue of whether carbon-cobalt bond re-formation follows substrate turnover is unresolved for the AdoCbl-dependent isomerases. A number of aspects of the kinetics of the AdoCbl-dependent isomerase systems have made it difficult to determine whether carbon-cobalt bond re-formation follows each turnover. Because re-formation of the carbon-cobalt bond after turnover does not appear as a kinetic phase distinct from carbon-cobalt bond re-formation before turnover, it is difficult to distinguish between the two in SF-UV vis experiments. In addition, because carbon-cobalt bond homolysis and hydrogen atom abstraction have not been shown to be concerted in any of the AdoCbl-dependent isomerases (although carbon-cobalt bond homolysis and turnover have been shown to be coupled) (Padmin Kumar et al, 1997), it is difficult to link the SF UV-vis experiments with tritium exchange experiments to determine whether carbon-cobalt bond homolysis follows each turnover.

RTPR provides a more favorable system for addressing this question. For RTPR, two distinct kinetic phases are observed under turnover conditions, one corresponding to a net formation of cob(II)alamin, and the other corresponding to a net re-formation of the carbon-cobalt bond. Also, as described in Chapter III, carbon-cobalt bond homolysis and hydrogen abstraction from C408 are concerted for the exchange reaction of RTPR. Assuming that this also holds true for the turnover reaction (as is suggested by cofactor and solvent isotope effects described in this chapter), tritium washout in RTPR is a direct measure of carbon-cobalt bond re-formation. The links that can be drawn between different presteady state kinetic experiments allow the question of the chain length to be directly addressed.

The biphasic nature of cob(II)alamin formation was discovered in early SF UV-vis experiments on RTPR. Tamao and Blakley found that when RTPR was mixed with AdoCbl in the presence of nucleotide substrates and deoxynucleotide effectors, the kinetics of cob(II)alamin were biphasic (Tamao
& Blakley, 1973). The fast absorbance change at 525 nm (~40 s⁻¹) corresponding to net carbon-cobalt bond homolysis was followed by a slower phase (~10 s⁻¹) corresponding to net re-formation of the carbon-cobalt bond. Similar results with the recombinant enzyme are described in this chapter. These observations are consistent with net carbon-cobalt bond homolysis occurring to form the thiyl radical, followed by turnover and net re-formation of the carbon-cobalt bond.

A second observation of Tamao and Blakley suggests even more strongly that the thiyl radical/cob(II)alamin intermediate is a transient one that disappears after turnover. RTPR, AdoCbl, and deoxynucleotide effector were pre-incubated to form a steady-state level of thiyl radical/cob(II)alamin intermediate, then mixed with nucleotide substrate (Tamao and Blakley, 1973). This caused the cob(II)alamin concentration to decline to a new steady state level. Thus, it appears that the addition of substrate leads to the consumption of the thiyl radical/cob(II)alamin intermediate, as would be expected if turnover led to the re-formation of the carbon-cobalt bond.

In the absence of direct measurement of the single turnover rate, however, these results cannot directly link carbon-cobalt bond re-formation with turnover. To answer the question of whether carbon-cobalt bond homolysis occurs after each turnover catalyzed by RTPR, SF UV-vis measurements of carbon-cobalt bond homolysis were combined with rapid acid quench measurements of substrate turnover and washout of tritium from [5'⁻³H]-AdoCbl. Determination of the kinetics of a single turnover allows determination of whether the rate constant for turnover is larger than k_obs for the net re-formation of the carbon-cobalt bond as measured by SF UV-vis experiments. Tritium washout from [5'⁻³H]-AdoCbl provides an independent measure of re-formation of the carbon-cobalt bond, as washout cannot occur unless without abstraction of hydrogen from 5'-dA by the thiyl radical, which in turn leads to carbon-cobalt bond re-formation. Thus, comparison of the rate of tritium washout to the turnover rate is an independent test of whether carbon-cobalt bond re-formation follows every turnover.

Dissociation of AdoCbl from RTPR During Turnover

Related to the question of whether the carbon-cobalt bond is re-formed after each turnover is the question of whether AdoCbl dissociates from the
enzyme after each turnover. If AdoCbl is released after every turnover, the carbon-cobalt bond must be re-formed after every turnover for the reaction to be catalytic in AdoCbl. It is important to note, however, that a failure of AdoCbl to dissociate from the enzyme after each turnover does not imply that carbon-cobalt bond re-formation does not occur.

Release of AdoCbl from RTPR in the course of the catalytic cycle may also be important physiologically in *L. leichmannii*. *L. leichmannii* takes vitamin B12 up avidly at saturating AdoCbl (10 ng/ml of medium), incorporating ~10,000 molecules of cobalamin per cell (Kashket et al, 1962). *L. leichmannii* can also grow under conditions of sub-saturating AdoCbl, however, and under these conditions much less AdoCbl is taken up into cells, with only ~10 molecules of cobalamin present per cell in cells grown on medium containing 0.1 ng/ml vitamin B12 (Davis et al, 1952). RTPR is made in large quantities by *L. leichmannii*, and concentrations of RTPR in *L. leichmannii* can be estimated to be greater than the concentrations of AdoCbl under certain growth conditions. Goulian and Beck found that growing *L. leichmannii* in medium without vitamin B12 or without deoxyadenosine de-repressed RTPR expression, and that when withdrawal of deoxyadenosine was used to effect de-repression, ~20 mg of purified RTPR could be obtained from 130 g of cells (Goulian & Beck, 1966). Estimating the density of cells as 1 g/ml, and approximating the volume of an *L. leichmannii* cell as that of a cylinder 5 microns in length and 1 micron in diameter, based on the average size of bacilli (Atlas, 1986), the number of molecules of RTPR per cell can be approximated as ~10,000.

These observations suggest that under conditions of limiting AdoCbl, an *L. leichmannii* cell produces RTPR in molar excess over the AdoCbl it contains. If AdoCbl is tightly bound to RTPR throughout the catalytic cycle, this response would seem paradoxical, since in that case, increasing RTPR would not increase the amount of functional holoenzyme in the cell. However, AdoCbl may be able to dissociate from RTPR after nucleotide reduction but before the putatively rate-limiting re-reduction of the enzyme. If so, increasing the concentration of RTPR when AdoCbl was scarce could actually lead to an increase in the rate of nucleotide reduction. The presence of an excess of RTPR would allow a single molecule of AdoCbl to initiate catalysis by multiple molecules of RTPR, "hopping" from one RTPR molecule to another after each nucleotide reduction.
This behavior would be in marked contrast to the AdoCbl-dependent isomerases, which do not release AdoCbl in the course of turnover. Most of these enzymes bind AdoCbl tightly, and they can often be purified as the holoenzyme. Diol dehydrase, for example, binds AdoCbl so tightly that it can only be purified as the apoenzyme in the absence of potassium ions, which are required for cofactor binding (Toraya and Fukui, 1982). In the case of ethanolamine ammonia lyase and diol dehydratase, kinetic studies provide direct evidence that AdoCbl does not dissociate in the course of turnover. Weisblat and Babior measured the amount of tritium transfer from [5'-3H]-AdoCbl to product (with ethanolamine as substrate) when [5'-3H]-AdoCbl was in large excess over ethanolamine ammonia lyase. Finding that only a small fraction of the total radioactivity present in AdoCbl was transferred to product, they concluded that the AdoCbl bound to the enzyme is not in free exchange with AdoCbl in solution (Weisblat and Babior, 1971). For diol dehydratase, Abeles and co-workers performed a similar experiment, measuring the amount of tritium transfer from [5'-3H]-AdoCbl to product (with propanediol as substrate). They demonstrated that at sufficiently high concentration of [5'-3H]-AdoCbl, the amount of tritium transferred to product is no longer proportional to the concentration of [5'-3H]-AdoCbl (Essenberg et al., 1971). This experiment indicates that exchange of AdoCbl bound to diol dehydratase with free AdoCbl is slow compared to turnover (Essenberg et al., 1971, Weisblat and Babior, 1971). If exchange of bound AdoCbl with free AdoCbl were facile, the amount of tritium transferred to product would be proportional to the amount of [5'-3H]-AdoCbl present at all concentrations of [5'-3H]-AdoCbl.

Unlike the AdoCbl-dependent isomerases, RTPR does not bind AdoCbl tightly. It is isolated as the apoenzyme (Goulian and Beck, 1966, Booker & Stubbe, 1993) and SF UV-vis studies suggest that the $K_d$ for AdoCbl is $\sim 250$ $\mu$M in the absence of substrate (Chapter 4). However, binding of AdoCbl to the enzyme is rapid in the absence of substrate (Chapter 3). These observations are consistent with rapid dissociation of AdoCbl from the enzyme. They do not, however, furnish evidence that dissociation from the enzyme is rapid during the catalytic cycle.

One indication that dissociation from the enzyme is reasonably fast is the observation that RTPR can catalyze exchange of all the tritium (with a rate constant of $\sim 0.2$ s$^{-1}$) from [5'-3H]-AdoCbl, even when [5'-3H]-AdoCbl is in
large excess over RTPR. This is the opposite of what is observed with ethanolamine ammonia lyase and diol dehydratase. However, it is not possible to derive a rate constant for AdoCbl dissociation from this data, leaving open the question of whether this dissociation occurs every turnover.

To determine whether AdoCbl dissociates from RTPR during the catalytic cycle, the rate of turnover was measured when [RTPR] $>>[\text{AdoCbl}]>K_m$. Under these conditions, if AdoCbl remains bound to the enzyme throughout the catalytic cycle, the rate of turnover will be equal to the product of $k_{cat}$ and [AdoCbl], since the amount of holoenzyme present will be limited by [AdoCbl]. The observation that the rate of turnover exceeds the product of $k_{cat}$ and [AdoCbl] is consistent with AdoCbl dissociating from the enzyme after each turnover. A steady state kinetic analysis indicates that AdoCbl must dissociate from the enzyme after at least 85% of turnovers on average. This result confirms that the carbon-cobalt bond is re-formed after each turnover, and suggests that *L. leichmannii* may use the ability of AdoCbl to dissociate from the enzyme during the catalytic cycle to maximize the rate of nucleotide reduction under conditions of limiting AdoCbl.
Materials and Methods

Materials and General Methods

RTPR was purified as described in Chapter 2. [5'2H2]-AdoCbl was prepared as described in Chapter II. [U-13C]-ATP was a generous gift from Robert Batey and Prof. Jamie Williamson (MIT). Sep-Paks were from Millipore.

A Cary 3 spectrophotometer was used for kinetic studies. An HP 8452A diode array spectrophotometer was used to measure concentrations of RTPR, AdoCbl, and nucleotides spectrophotometrically. Deoxygenation of solutions for SF UV-vis and RFQ EPR experiments was carried out as described in Chapter 2. Fits of kinetic traces to single or double exponentials were carried out using either the Applied Photophysics system software or Kaleidagraph (Abelbeck Software). Other non-linear least squares fitting was carried out using Kaleidagraph.

SF UV-vis Studies on the Turnover Reaction: Formation of Cob(II)alamin

Deoxygenation of reaction mixtures and preparation of the stopped flow sample handling unit were carried out as described in Chapter II. Pre-reduced RTPR (120 μM), 2 mM dGTP, 2 mM ATP, 20 μM TR, 1 μM TRR, and 2 mM NADPH in 200 mM sodium dimethylglutarate, pH 7.3 were placed in one syringe and mixed with an equal volume of the same reaction buffer containing 100 μM AdoCbl, 2 mM dGTP, and 2 mM ATP. The formation of cob(II)alamin was measured by monitoring change in A525 at 37 °C. Changes in absorbance at wavelengths from 440–540 nm were recorded to assemble a point-by-point spectrum using the Applied Photophysics software. The biphasic kinetics observed were not well-fit by double exponentials. Therefore, the two phases (a rapid decrease and a slow increase in A525) were each fit separately to single exponentials.

SF UV-vis studies using [5'2H2]-AdoCbl and D2O: Determination of Kinetic and Equilibrium Isotope Effects on Cob(II)alamin Formation under Turnover Conditions

To prepare 0.5 M dimethylgluqutarate buffer (pD 7.3), the acid was dissolved in D2O and titrated to pH 6.9 (glass electrode) using 5 N NaOD. For studies in D2O, non-protein components of the reaction mixture were
lyophilized and re-dissolved in D₂O. RTPR (20-30 mg, ~200 μl) was exchanged into D₂O by dilution into 2 mL of deuterated 5 mM sodium dimethylglutarate (pD 7.3), and concentrated using a Centricon 30 apparatus. TR and TRR were exchanged the same way (~50 μl of protein solution in ~2 mL of deuterated buffer), except a Centricon 3 device was used. The total amount of H₂O introduced by this exchange procedure was calculated to be <5%. Reaction mixtures were deoxygenated by purging with D₂O-saturated argon.

RTPR (130 μM), 20 μM TR, 1 μM TRR, 2 mM NADPH, 1 mM dGTP, and 1 mM ATP in 200 mM sodium dimethylglutarate, pH 7.3 (or pD 7.3) were placed in one syringe and mixed with an equal volume from a second syringe of the same reaction buffer containing 100 μM AdoCbl (or [5'-²H₂]-AdoCbl), 1 mM dGTP, and 1 mM ATP. The formation of cob(II)alamin was measured by monitoring change in A₅₂₅ at 37 °C. The two phases (a rapid decrease and a slow increase in A₅₂₅) were each fit separately to single exponentials.

**SF-UV vis studies on the Turnover Reaction:** [AdoCbl]-Dependence of Cob(II)alamin Formation by RTPR in the Presence of CTP and dATP

RTPR (20-60 μM), 1 μM TR, 20 μM TRR, 2 mM NADPH, 1 mM CTP and 1 mM dATP in 200 mM sodium dimethylglutarate, pH 7.3 were placed in one syringe and mixed with an equal volume from a second syringe of the same reaction buffer containing 100-1000 μM AdoCbl, 1 mM ATP, and 1 mM dGTP. The formation of cob(II)alamin was measured by monitoring change of A₅₂₅ and A₄₇₅ at 37 °C. The kinetic traces were fit to two exponentials.

**SF UV-vis Studies on the Turnover Reaction:** Formation of Cob(II)alamin by Pre-Oxidized RTPR

Pre-reduced RTPR (0.1 μmol, pre-reduced as described in Chapter II) (Booker et al., 1994) was added to a reaction mixture which contained the following in a volume of 500 μl: 50 mM sodium dimethylglutarate, pH 7.3; 1 M sodium acetate; 10 mM ATP; 40 μM AdoCbl. The reaction mixture was incubated at 37 °C for 20 min, and the oxidized RTPR was separated from the small molecules by Sephadex G-50 chromatography (0.75x7 cm column, equilibrated in 5 mM sodium dimethylglutarate buffer, pH 7.3). The enzyme
was separated from the small molecules and concentrated using a Centricon-30 device.

Deoxygenation of reaction mixtures and preparation of the stopped flow sample handling unit were carried out as described in Chapter II. Pre-oxidized RTPR (70 μM), 3 mM dGTP, and 10 mM ATP in 200 mM sodium dimethylglutarate, pH 7.3 were mixed in the stopped-flow apparatus with an equal volume of the same reaction buffer containing 100 μM AdoCbl, 3 mM dGTP, 10 mM ATP, 150 μM TR, 2 μM TRR, 3 mM NADPH, and the formation of cob(II)alamin was measured by monitoring change in A_{525} at 37 °C.

A similar experiment was carried out in the absence of substrate. Pre-oxidized RTPR (50 μM), 100 μM AdoCbl, and 3 mM dGTP in 200 mM sodium dimethylglutarate, pH 7.3 were placed in one syringe and mixed with an equal volume from a second syringe of the same reaction buffer containing 3 mM dGTP, 150 μM TR, 2 μM TRR, and 3 mM NADPH. The formation of cob(II)alamin was measured by monitoring change in A_{525} at 37 °C. In the presence of dGTP alone, pre-oxidized RTPR was found to degrade AdoCbl slowly enough to allow these reagents to be incubated in the same syringe.

The experiment was also carried out using 30 mM DTT in place of the TR/TRR/NADPH reducing system. Pre-oxidized RTPR (45 μM), 70 μM AdoCbl, and 4.5 mM dGTP in 200 mM sodium dimethylglutarate, pH 7.3 were placed in one syringe and mixed with an equal volume from a second syringe of the same reaction buffer containing 4.5 mM dGTP, 150 μM TR, 2 μM TRR, and 3 mM NADPH. The formation of cob(II)alamin was measured by monitoring change in A_{525} at 37 °C.

Rapid Acid Quench Experiments Under Single Turnover Conditions: Fate of the 5'-Hydrogens and Rate of Single Turnover

A Kin-Tek model RQF-3 rapid quench apparatus was used. To allow reproducible loading in dim light, the sample loops were loaded using Luer-tip gas-tight syringes that had been calibrated so that the displacement required to fill each sample loop was marked on the barrel of the syringe.

Pre-reduced RTPR (120 μM), 20 μM TR, 1 μM TRR, 2 mM NADPH, 2 mM dGTP, 2 mM [U-14C]-ATP (5.2x10^6 cpm/μmol), and 100 mM sodium dimethylglutarate, pH 7.3 were placed in one syringe and rapidly mixed at 37 °C with an equal volume from a second syringe of [5'-3H]-AdoCbl (100 μM,
1.2x10^6 cpm/μmol), 2 mM dGTP, and 2 mM [U-14C]-ATP in the reaction buffer. After the specified time, the reaction was quenched with 2 percent perchloric acid (60 to 220 μl), collected in tubes containing 5'-dA (55 nmol) and 5', 8-cycloadenosine (15 nmol). Immediately after quenching, samples were neutralized with equal volumes of 0.4 M KOH and 0.5 M sodium dimethylglutarate, pH 7.3 (50 to 200 μl each). Neutralized samples were immediately quick-frozen in liquid nitrogen and stored on dry ice. The neutralized reaction mixtures were stable at -20 °C for up to a week. A zero time point was generated by omitting [5'-3H]-AdoCbl from the second syringe and putting it instead in the sample collection tube, so that the protein would be acid-precipitated before encountering it.

Samples were centrifuged for 45 s in an Eppendorf microcentrifuge. The supernatant was removed from the protein pellet, neutralized with equal volumes of 0.4 M KOH and 0.5 M sodium dimethylglutarate, and rapidly frozen.

Tritiated water was removed from the quenched samples by bulb-to-bulb distillation. All operations were carried out in a fume hood. Samples (~1 ml) were transferred to silanized 50 ml pear-shaped flasks and shell-frozen in dry ice/acetone. The bulb-to-bulb distillation apparatus (two pear-shaped flasks connected by a Y-shaped adapter equipped with a stopcock) was evacuated and kept under static vacuum. The flask containing the sample was removed from the dry ice/acetone bath, and the empty flask was placed in the dry ice/acetone bath. Lyophilization of the sample was usually complete in 45 minutes or less. In cases when lyophilization did not proceed to completion (due to an inadequate seal or shell-freezing in too thick a layer), the volume in each flask was measured in order to normalize the measured radioactivity.

The distillate was transferred to a scintillation vial, which was immediately capped. The flask containing the distillate was washed with 500 μl of water, which was also transferred to the scintillation vial. Scintillation fluid (Scint-A, 8.5 ml) was added to the vial, and the distillate was counted.

The lyophilized material was redissolved in 1 ml of water, frozen on dry ice, and stored at -20 °C. It was then thawed and loaded onto reverse phase C18 Sep-Paks. Nucleotides were eluted with 10 ml of water. Nucleosides and AdoCbl were eluted with 50% CH3OH/50% H2O (10 ml) and the solvent removed by lyophilization. The nucleoside-containing
residue was dissolved in 450 μl of H2O and analyzed by reversed phase-C18 HPLC (Econosil C18 10U, 4.6x250 mm), with a linear gradient. Solvent A was H2O, and solvent B was MeOH: 0-5 minutes, 0-20% B; 5-22 minutes, isocratic elution with 20% B; 22-32 minutes, 20-100% B; 32-40 minutes, 100% B. The flow rate was 1 ml/min, the elution profile was monitored by A260, and 1 ml fractions were collected. Adenine, 5', 8-cycloadenosine, and 5'-dA eluted at 12, 19 and 21 minutes, respectively. UV-vis spectroscopy (ε260 = 13400 M⁻¹ cm⁻¹, ε264 = 15100 M⁻¹ cm⁻¹, and ε260 = 15200 M⁻¹ cm⁻¹, respectively) was used to measure the recovery, and radioactivity was analyzed by scintillation counting (8.5 ml Scint-A per ml of eluate).

The nucleotide-containing water elution samples were lyophilized, then re-dissolved in 870 μl of water and combined with 100 μl of 10X alkaline phosphatase buffer (0.5 M Tris, 1 mM EDTA) and 30 units of calf intestinal alkaline phosphatase (30 μl). The reaction was incubated at 37 °C for 3 hours. The reaction mixture was analyzed by reversed phase-C18 HPLC, with a linear gradient. Solvent A was H2O and solvent B was MeOH: 0-2 minutes, isocratic elution with A; 2-7 minutes, 0-20% B; 7-30 minutes, 20% B. The flow rate was 1 ml/min, and the elution profile was monitored by A260. Adenine, adenosine and 2'-dA eluted at 12, 16.5 and 18 min, respectively. Fractions containing adenosine and 2'-dA were pooled and lyophilized. Because this HPLC method only effected partial separation of adenosine and 2'-dA, the 2'-dA-containing fractions were chromatographed by the method of Cory, et al. (Cory et al, 1973) to separate 2'-dA from adenosine more efficiently. Borate columns (Dowex AG 1X2, exchanged into the borate form using sodium tetraborate) were poured into glass wool-stoppered 5.75 inch Pasteur pipets, washed with 20 ml of water, then equilibrated by washing with 1 mM sodium tetraborate (2 ml). The lyophilized samples were re-dissolved in 1 ml of water and loaded onto the borate columns. The columns were washed with 2 ml of 1mM sodium tetraborate, and 2'-dA was eluted with 12 ml of 1mM sodium tetraborate. UV-vis spectroscopy (ε260 = 15200 M⁻¹ cm⁻¹ for 2'-dA) was used to measure the recovery. The sample was lyophilized and re-dissolved in 1 ml water, and radioactivity was analyzed by scintillation counting (8.5 ml Scint-A per ml of eluate).

The protein pellets were washed with 2 portions (100 μl each) of water and re-pelleted in an Eppendorf microcentrifuge. The pellets were suspended in 100 μl of water, and an alkaline solubilizing agent (Soluene-
350, 1 ml) was added to each tube. The tubes were sealed and incubated at 50°C for 3 hours. Radioactivity was analyzed by scintillation counting (Hionic Fluor, 10 ml/sample).

Similar studies were carried out to measure the presteady state rate of reduction of CTP. RTPR (65 μM), 30 μM TR, 1.5 μM TRR, 2 mM NADPH, 2 mM dATP, 2 mM [2-14C]-CTP (7.4x10^6 cpm/μmol), 2 mM MgCl2 and 100 mM sodium dimethylglutarate, pH 7.3 were placed in one syringe and rapidly mixed at 37°C with an equal volume from a second syringe of AdoCbl (300 μM, 1.2x10^6 cpm/μmol), 2 mM dATP, and 2 mM [2-14C]-CTP in the reaction buffer. Quenching, collection of protein pellets, neutralization, and storage of samples were carried out as described above. Nucleotides were dephosphorylated with calf intestinal alkaline phosphatase as described above. Nucleosides were applied to borate columns (Dowex AG 1X2, exchanged into the borate form using sodium tetraborate), which had been poured into glass wool-stoppered 5.75 inch Pasteur pipets and washed with 20 ml of water. 2'-dA was eluted with 10 ml of water. A 1 ml aliquot was analyzed for radioactivity by scintillation counting (8.5 ml Scint-A per ml of eluate).

**Rate of Turnover in D2O and with [5'-2H2]AdoCbl: Determination of a V/K Isotope Effect**

An assay mixture containing 1 mM dGTP, 1 mM ATP, and 0.2 mM NADPH in 200 mM sodium dimethylglutarate (pH 7.3) was prepared, lyophilized, and re-dissolved in D2O or H2O. RTPR (3 μM), 40 μM TR, and 0.4 μM TRR were combined with the reconstituted assay mixture in a total volume of 496 μl and incubated in a quartz cuvette for 1-2 min at 37 °C. The background rate of NADPH consumption was measured using the ΔA_{340} (Δε_{340} = 6220). The reaction was initiated by the addition of AdoCbl (0.07-10 μM) or [5'-2H2]AdoCbl (0.06-10 μM), and the ΔA_{340} recorded.

**SF UV-vis Studies on the Turnover Reaction: Order of Addition**

Deoxygenation of reaction mixtures and preparation of the stopped flow sample handling unit were carried out as described in Chapter 2. Pre-reduced RTPR (100 μM), 1 mM dGTP, 20 μM TR, 1 μM TRR, 2 mM NADPH in 200 mM sodium dimethylglutarate, pH 7.3 were placed in one syringe and mixed with an equal volume from a second syringe of the same reaction
buffer containing 100 µM AdoCbl, 1 mM dGTP, and 1 mM ATP, and the formation of cob(II)alamin was measured by monitoring change in $A_{525}$ at 37 °C. The two kinetic phases (a rapid decrease and a slow increase in $A_{525}$) were each fit separately to single exponentials.

**SF UV-vis studies using C419S and C119S: Formation of Cob(II)alamin under Turnover Conditions**

C419S or C119S RTPR (40–90 µM), 20 µM TR, 1 µM TRR, 2 mM NADPH, 1 mM dGTP, and 1 mM ATP in 200 mM sodium dimethylglutarate, pH 7.3 were placed in one syringe and mixed with an equal volume from a second syringe of the same reaction buffer containing 100 µM AdoCbl (or [5′-2H$_2$]-AdoCbl), 1 mM dGTP, and 1 mM ATP, and the formation of cob(II)alamin was measured by monitoring change in $A_{525}$ at 37 °C.

**SF UV-vis studies using C419S and [5′-2H$_2$]-AdoCbl: Kinetic Isotope Effects on Cob(II)alamin Formation under Turnover Conditions**

Pre-reduced C419S RTPR (400 µM), 20 µM TR, 1 µM TRR, 2 mM NADPH, 1 mM ATP and 1 mM dGTP in 200 mM sodium dimethylglutarate, pH 7.3 were placed in one syringe and mixed with an equal volume from a second syringe of the same reaction buffer containing 400 µM AdoCbl (or [5′-2H$_2$]-AdoCbl), 1 mM ATP, and 1 mM dGTP. The formation of cob(II)alamin was measured by monitoring change of $A_{525}$ at 37 °C.

**RFQ EPR: Paramagnetic Intermediate Generated by C419S RTPR Under Turnover Conditions**

Pre-reduced C419S RTPR (500 µM), 20 µM TR, 1 µM TRR, 2 mM NADPH, 1 mM ATP, 1 mM dGTP in 200 mM sodium dimethylglutarate, pH 7.3 were mixed with an equal volume of the same reaction buffer containing 500 µM AdoCbl, 1 mM ATP, and 1 mM dGTP. Drive syringes and loops were maintained at 37 °C. The ram velocity was 1 cm/s. The length of the push was 0.3–0.55 cm. Reaction mixtures were quenched at times ranging from 20 ms to 3 s.

EPR spectra were recorded at 100 K with spectrometer frequency 9.44 GHz; microwave power, 0.01-10 mW; modulation frequency, 100 kHz; modulation amplitude, 4 G; time constant, 1.3 s; and scan time, 671 s. Spectra
were also recorded at 20 K with microwave power of 0.1-1 mW. Spin quantitation was carried out by double integration and comparison with a CuSO_4 standard (Malmström et al, 1970), as described in Chapter 2.

**SF UV-vis studies using Pre-Reduced RTPR in Large Excess over TR: Effect on the Kinetics of Cob(II)alamin Formation**

Pre-reduced RTPR (510 μM), 1.5 μM TR, 1 μM TRR, 2 mM NADPH, 1 mM ATP and 1 mM dGTP in 200 mM sodium dimethylglutarate, pH 7.3 were mixed with an equal volume of the same reaction buffer containing 400 μM AdoCbl (or [5'-2H_2]-AdoCbl), 1 mM ATP, and 1 mM dGTP. The formation of cob(II)alamin was measured by monitoring change of A_525 at 37 °C. The experiment was also carried out using 100 μM RTPR and 100 μM AdoCbl.

**RFQ EPR: Paramagnetic Intermediate Generated by Pre-Reduced RTPR in Large Excess over TR**

Pre-reduced RTPR (450 μM), 1.5 μM TR, 1.3 μM TRR, 2 mM NADPH, 1 mM EDTA, 1 mM ATP and 1 mM dGTP in 200 mM sodium dimethylglutarate, pH 7.3 were mixed with an equal volume of the same reaction buffer containing 400 μM AdoCbl, 1 mM EDTA 1 mM ATP, and 1 mM dGTP. Drive syringes and loops were maintained at 37 °C. The ram velocity was 1 cm/s. The length of the push was 0.3-0.55 cm. Reaction mixtures were quenched at times ranging from 20 ms to 500 ms. The experiment was also carried out with enzyme that had been chromatographed on a G-50 column, but not pre-reduced.

EPR spectra were recorded at 100 K with spectrometer frequency 9.44 GHz; microwave power, 0.01-10 mW; modulation frequency, 100 kHz; modulation amplitude, 4 G; time constant, 1.3 s; and scan time, 671 s.

**SF UV-vis Studies of the Kinetics of Cob(II)alamin Formation by wt RTPR in the Presence of ara-ATP**

RTPR (100 μM), 20 μM TR, 1.5 μM TRR, 2 mM NADPH, 0.5 mM ara-ATP and 1 mM dGTP in 200 mM sodium dimethylglutarate, pH 7.3 were mixed with an equal volume of the same reaction buffer containing 200 μM AdoCbl, 0.5 mM ara-ATP, and 1 mM dGTP. The formation of cob(II)alamin was measured by monitoring change of A_525 and A_475 at 37 °C.
Effect of $[^{13}\text{C}]-\text{ATP}$ on the EPR-Active "Doublet Species"

Sodium dimethylglutarate (0.3 M, pH 7.3), dGTP (1.5 mM), AdoCbl (0.3 mM), and NADPH (3.75 mM) were combined in a total volume of 600 µl, degassed, and transferred to an septum sealed, argon-purged cuvette via gas-tight syringe. RTPR (1.3 mM), unlabeled ATP or $[^{13}\text{C}]-\text{ATP}$ (3.7 mM), TR (11 µM) and TRR (2.12 µM) were combined in a total volume of 150 µl, degassed, and combined with the solution in the cuvette via gas-tight syringe. After cob(II)alamin formation was complete as monitored by UV-vis spectroscopy (~10 min), the solution was transferred into a septum-sealed, argon-purged EPR tube via gas-tight syringe and frozen in liquid nitrogen. EPR spectra were recorded at 100 K with spectrometer frequency 9.44 GHz; microwave power, 0.01-10 mW; modulation frequency, 100 kHz; modulation amplitude, 4 G; time constant, 1.3 s; and scan time, 671 s.

[AdoCbl] Dependence of the Rate of Turnover when [RTPR] $>>$ [AdoCbl]: Measurement of the Rate of AdoCbl Dissociation from RTPR

Assays contained in a volume of 500 µl: 200 mM sodium dimethylglutarate (pH 7.3), 1 mM dGTP, 1 mM ATP, 0.2 mM NADPH, AdoCbl (0.1-1.25 µM), 20 µM TR, 0.45 µM TRR, and RTPR (1-10 µM). RTPR was present in 10-fold excess over AdoCbl. All components except AdoCbl were mixed in a 500 µl quartz cuvette and incubated at 37 °C for 1-2 min. A background rate of NADPH consumption was measured by monitoring $A_{340}$. AdoCbl was added to the cuvette, and the rate of NADPH consumption was measured.

Under conditions where [RTPR] $>>$ [AdoCbl] $>>$ $K_m$, SF UV-vis methods were required to measure the rate of NADPH consumption. RTPR (40 µM), 1 mM dGTP, 1 mM ATP, 50 µM TR, and 0.2-0.3 mM NADPH in 200 mM sodium dimethylglutarate, pH 7.3 were placed in one syringe and mixed with an equal volume from a second syringe of the same reaction buffer containing 4 µM AdoCbl, 1 mM dGTP, 1 mM ATP, and 1.5 µM TRR, and the consumption of NADPH was measured by monitoring change in $A_{340}$ at 37 °C ($\Delta\varepsilon_{340} = 6220$).

Similar experiments were also carried out with [RTPR] $<$ [AdoCbl]. Assays contained in a volume of 500 µl: 200 mM sodium dimethylglutarate (pH 7.3), 1 mM dGTP, 1 mM ATP, 0.2 mM NADPH, AdoCbl (0.6–4 µM), and RTPR (0.1–0.75 µM). AdoCbl was present in 5-fold excess over RTPR.
The rate of NADPH consumption when \([\text{RTPR}]<[\text{AdoCbl}]>\) was also measured in the SF UV-vis apparatus. RTPR (4 or 40 \(\mu\)M), 1 mM dGTP, 1 mM ATP, 50 \(\mu\)M TR, and 0.15–0.5 mM NADPH in 200 mM sodium dimethylglutarate, pH 7.3 were placed in one syringe and mixed with an equal volume from a second syringe of the same reaction buffer containing 50 \(\mu\)M AdoCbl, 1 mM dGTP, 1 mM ATP, and 1.5 \(\mu\)M TRR, and the formation of cob(II)alamin was measured by monitoring change in \(A_{340}\) at 37 °C.
Results

SF UV-vis Studies on the Turnover Reaction

The kinetics of cob(II)alamin formation during turnover were first investigated by Tamao and Blakley (Tamao and Blakley, 1973), who found that when RTPR was mixed with AdoCbl in the presence of nucleotide substrate, deoxynucleotide allosteric effector, and the dithiol reductant dihydrolipoate, cob(II)alamin was formed with a rate constant of \( \sim 40 \text{ s}^{-1} \), about the same as observed in the absence of substrate. However, in the presence of substrate, cob(II)alamin formation reached a maximum (0.3–0.5 eq.), then declined to a steady-state level (0.1–0.4 eq.) with a rate constant of 10–40 s\(^{-1}\), depending on the nucleotide present.

This experiment was repeated for the present study using recombinant RTPR and the TR/TRR/NADPH reducing system. When dGTP is the allosteric effector and ATP is the substrate, cob(II)alamin increases rapidly to a maximum of \( \sim 0.6 \) equivalent, then declines more slowly to a steady-state concentration of \( \sim 0.2 \) equivalent (Fig. 5.1). The kinetic trace was not well fit to two or even three exponentials. To get a qualitative idea of the rates, the rapid increase in cob(II)alamin (the first 15–20 ms after mixing) was fitted to a single exponential, as was the slow decrease in cob(II)alamin that followed (fits were from 50–200 ms after mixing to minimize the contribution of the first phase). This procedure gave rate constants of \( \sim 250 \text{ s}^{-1} \) for the increase of cob(II)alamin to the maximum and \( \sim 30 \text{ s}^{-1} \) for the decline to the steady-state concentration. While the kinetics are complex, it is likely that only two chromophoric species (AdoCbl and cob(II)alamin) are present. The point-by-point spectrum shows \( A_{524} \) decreasing with time and \( A_{477} \) increasing with time, with the isosbestic point at 490 nm expected for a two-state transition between AdoCbl and cob(II)alamin (Fig. 5.2).

This kinetic behavior is consistent with the mechanism in Scheme 5.1, with the first kinetic phase corresponding to initial carbon-cobalt bond homolysis, and the second kinetic phase corresponding to carbon-cobalt bond re-formation after turnover. The experiments described in this chapter were designed to test various aspects of this mechanism, with a particular focus on the rate-limiting step in catalysis and the question of whether carbon-cobalt bond re-formation follows each turnover.
Fig. 5.1. Formation of cob(II)alamin (measured by SF UV-vis spectroscopy) on mixing RTPR (120 μM), in one syringe with dGTP (2 mM), ATP (2 mM), and TR/TRR/NADPH, with AdoCbl (100 μM) in a second syringe, which also contained dGTP (2 mM) and ATP (2 mM). The trace represents the average of three trials. Both initial phase (5–20 ms) and the final phase (50–200 ms) were fit individually to single exponentials (fits are shown as dashed lines).
Fig. 5.2. Point-by-point spectrum of intermediates under turnover conditions. Experimental conditions were the same as described for Fig. 5.1. Kinetic traces were recorded at wavelengths ranging from 440–540 nm (in increments of 5 nm). A. Spectrum at 5 ms. B. Spectrum at 50 ms. C. Spectrum at 100 ms. Point-by-point spectra were assembled using the Applied Photophysics software. A cubic spline has been applied to the data to make peaks and troughs easier to see. The sloping baseline is likely to be due to the end absorbance of NADPH (present at 2 mM). Further studies will be required to determine the reproducibility and significance, if any, of the small feature at 505 nm.
SF UV-vis studies using [5'-2H₂]AdoCbl and D₂O: Determination of Kinetic and Equilibrium Isotope Effects on Cob(II)alamin Formation under Turnover Conditions

Qualitative predictions about kinetic and equilibrium effects of isotopic substitutions can be made using the model in Scheme 5.1. For the purposes of predicting isotope effects, the most important aspect of the model for turnover is that, just as in the model for the exchange reaction (Chapter 3), cobalt-carbon bond homolysis and thiyl radical formation occur in a concerted fashion. If this model pertains to turnover, it predicts that isotope effects qualitatively similar to those observed for the exchange reaction should be observed. When [5'-2H₂]-AdoCbl and/or D₂O are present in the reaction mixture, for example, primary isotope effects should alter the rates of all carbon-cobalt bond homolysis or re-formation reactions, because carbon-cobalt bond homolysis and re-formation will be coupled to transfer of deuterium between 5'-dA and the C408 thiol. In addition, as discussed in Chapter 3, equilibrium isotope effects should be observed due to the influence on thiyl radical formation of the low fractionation factor associated with thiol groups. However, if isotope-insensitive reactions such as turnover (k₄₃, Scheme 5.1) or some as-yet uncharacterized conformational change is slow compared to step 2 or step 4, large isotope effects would not be expected. Preliminary results on isotope effects in turnover observed in experiments analogous to those performed on the exchange reaction are consistent with cob(II)alamin formation and thiyl radical formation occurring in a concerted fashion, and with isotope-sensitive steps being rate-limiting in cob(II)alamin formation and AdoCbl re-formation.

Tamao and Blakley, using GTP, no allosteric effector, and dihydrolipoate as reductant, reported that the rate of the initial cob(II)alamin formation using [5'-2H₂]-AdoCbl was similar to that using unlabeled AdoCbl, but that the maximum amount of cob(II)alamin formed was greater, and the decline from this maximum was slower (Tamao and Blakley, 1973). By comparing the half-time for the decline from the maximum with [5'-2H₂]-AdoCbl to that with unlabeled AdoCbl, they computed a kinetic isotope effect of 2.2 for this process. In the present study, ATP was used with dGTP as an allosteric effector and the TR/TRR/NADPH reducing system. In contrast to the earlier experiments, an apparent kinetic isotope effect was observed on the initial rapid formation of cob(II)alamin as well as on its decline.

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The $k_{\text{obs}}$ for initial formation of cob(II)alamin with [5'-2H$_2$]-AdoCbl, calculated from a single exponential fit over the first 20 ms of the reaction can be estimated as $\sim$150 s$^{-1}$, compared to $\sim$250 s$^{-1}$ for unlabeled AdoCbl (Fig. 5.3). This corresponds to an isotope effect of $\sim$1.7, comparable to that observed in the exchange reaction (Chapter 3), although in this case, the speed of the reaction makes precise measurement of the rate constant difficult, as the dead time of the instrument is on the order of the calculated half-time of the reaction. This isotope effect would, as argued in Chapter 2, be consistent with carbon-cobalt bond cleavage occurring in a concerted fashion with thyl radical formation, and reversible transfer of deuterium between the cofactor and the C408 thiol during this step ($k_{+2}$, $k_{-2}$, Scheme 5.1).

The $k_{\text{obs}}$ for the slower re-formation of cob(II)alamin with [5'-2H$_2$]-AdoCbl, calculated from a single exponential fit of the data from 50 to 200 ms, can be estimated as $\sim$15 s$^{-1}$ (Fig. 5.3), compared to $\sim$35 s$^{-1}$ for unlabeled AdoCbl, corresponding to an isotope effect of $\sim$2.5. This isotope effect is comparable to the kinetic isotope effect of 2.2 reported by Tamao and Blakley for GTP as a substrate, and is consistent with the reversible transfer of deuterium from cofactor to the C408 thiol during re-formation of the carbon-cobalt bond ($k_{+4}$, $k_{-4}$, Scheme 5.1).

The observation of kinetic isotope effects $\sim$2 with [5'-2H$_2$]-AdoCbl for both kinetic phases of the reaction suggests that an isotope-insensitive step such as nucleotide reduction is not rate-limiting in the first turnover. According to the mechanism in Scheme 5.1, if nucleotide reduction were rate-limiting, the second kinetic phase (net carbon-cobalt bond re-formation) would be dominated by $k_{+3}$, which would not be expected to be sensitive to isotopic substitution in AdoCbl. This finding is consistent with rapid acid quench experiments described later in this chapter.

When [5'-2H$_2$]-AdoCbl is substituted for unlabeled AdoCbl, the cob(II)alamin formation in the steady-state observed with [5'-2H$_2$]-AdoCbl is expected to be identical to that observed with unlabeled AdoCbl. However, at 200 ms, these quantities are still significantly different (Fig. 5.3). This is consistent with the intrinsic rate of washout from the thiol group to bulk solvent being slow, as demonstrated for the exchange reaction (Chapter
Fig. 5.3. Kinetic isotope effect of [5'-2H2]-AdoCbl on formation of cob(II)alamin (measured by SF UV-vis spectroscopy). RTPR (130 μM), in one syringe with dGTP (1 mM), ATP (1 mM), and TR/TRR/NADPH, was mixed with AdoCbl or [5'-2H2]-AdoCbl (100 μM) in a second syringe, which also contained dGTP (1 mM) and ATP (1 mM). The traces represent averages of 3–4 trials. Both initial phase (5–20 ms) and the final phase (50–200 ms) were fit individually to single exponentials (fits are shown as dashed lines). A. Unlabeled AdoCbl. B. [5'-2H2]-AdoCbl. The inset shows the first 40 ms of the reaction, with the data plotted as the ratio of cob(II)alamin formed at time t to maximum cob(II)alamin formed, showing how the approach to the maximum is faster for unlabeled AdoCbl.
3). One might expect the rate of washout to be even slower under turnover conditions than in the exchange reaction, as a nucleotide in the active site could shield C408 from solvent. SF UV-vis experiments will have to be carried out on the time scale of seconds to determine how fast isotope exchange reaches a steady state.

The mechanism in Scheme 5.1 predicts a primary solvent kinetic isotope effect on both the initial cob(II)alamin formation (step 2) and on the re-formation of the carbon-cobalt bond (step 4), since these steps involve transfer of deuterium between the thyl radical and 5'-dA. Solvent kinetic isotope effects are in fact observed during turnover. The $k_{\text{obs}}$ for the initial increase of cob(II)alamin to the maximum is $\sim 120 \text{ s}^{-1}$ (Fig. 5.4, B), corresponding to a kinetic isotope effect of $\sim 2$. The $k_{\text{obs}}$ for the re-formation of the carbon-cobalt bond is $\sim 20 \text{ s}^{-1}$, also corresponding to an isotope effect of $\sim 2$. These effects are of a magnitude consistent with primary isotope effects.

For the exchange reaction, kinetic isotope effects in D$_2$O with [5'-2H$_2$]-AdoCbl were larger than those observed either in D$_2$O with unlabeled AdoCbl or in H$_2$O with [5'-2H$_2$]-AdoCbl The turnover reaction was also run in D$_2$O with [5'-2H$_2$]-AdoCbl (Fig. 5.4, C). In this experiment, the $k_{\text{obs}}$ for the initial rapid formation of cob(II)alamin is $\sim 90 \text{ s}^{-1}$, corresponding to a kinetic isotope effect of 2.7, which is larger than that observed with either D$_2$O or [5'-2H$_2$]-AdoCbl alone. Similarly, the half-time for the slow carbon-cobalt bond re-formation is $\sim 10 \text{ s}^{-1}$ (Fig. 5.4, C), corresponding to a kinetic isotope effect of 3.5, significantly larger than those observed with either D$_2$O or [5'-2H$_2$]-AdoCbl alone. Thus, as observed for the exchange reaction, the kinetic isotope effects are larger in D$_2$O with [5'-2H$_2$]-AdoCbl than observed with D$_2$O or [5'-2H$_2$]-AdoCbl alone. Due to the complexity of the turnover reaction, full interpretation of these kinetic isotope effects would require more mechanistic information than is currently available, in conjunction with global analysis (Chapter 3). Nonetheless, these results are consistent with carbon-cobalt bond homolysis and thyl radical occurring in a concerted fashion during turnover, as they do in the exchange reaction.

The exchange reaction exhibits an equilibrium isotope effect of $\sim 2$ in D$_2$O with [5'-2H$_2$]-AdoCbl (Chapter 3), consistent with the fractionation factor of 0.4–0.5 associated with thiols (Schowen & Schowen, 1982). For the turnover reaction, the mechanism in Scheme 5.1 predicts that there are
Fig. 5.4 Kinetic and equilibrium isotope effects in D$_2$O on formation of
co(II)alamin (measured by SF UV-vis spectroscopy). RTPR (130 µM), in
one syringe with dGTP (1 mM), ATP (1 mM), and TR/TRR/NADPH, was
mixed with AdoCbl (100 µM) in a second syringe, which also contained
dGTP (1 mM) and ATP (1 mM). The traces represent averages of 3–4 trials.
The traces represent averages of 3–4 trials. Both initial phase (5–20 ms) and
the final phase (50–200 ms) were fit individually to single exponentials (fits
are shown as dashed lines). A. Unlabeled AdoCbl, H$_2$O. B. Unlabeled
AdoCbl, D$_2$O. C. [5'-2H$_2$]-AdoCbl, D$_2$O.
two isotope sensitive equilibria, step 2 and step 4, leading to cob(II)alamin formation. The observed equilibrium isotope effect might thus be larger than that predicted for the single equilibrium involved in the exchange reaction. For the turnover reaction in D$_2$O with [5'-2H$_2$]-AdoCbl, ~0.29 equivalents of cob(II)alamin are present after 500 ms (Fig. 5.4, C), compared to ~0.08 equivalent observed in H$_2$O with unlabeled AdoCbl. This corresponds to an equilibrium isotope effect of ~3.6, larger than that observed in the exchange reaction (~2, Chapter 2). This equilibrium isotope effect is consistent with the low fractionation factor of the C408 thiol favoring the thiol radical form over the thiol in both step 2 and step 4, the isotope-sensitive carbon-cobalt bond homolysis equilibria in Scheme 5.1. However, these experiments must be repeated to determine the reproducibility of the amounts of cob(II)alamin formed and whether the difference in equilibrium isotope effect is significant.

**SF-UV vis studies on the Turnover Reaction: [AdoCbl]-Dependence of Cob(II)alamin Formation by wt RTPR**

For the mechanism in Scheme 5.1, the observed rate of cob(II)alamin formation will increase with the concentration of AdoCbl, until, at saturating concentrations of AdoCbl, the $k_{obs}$ will be a function only of unimolecular rate constants ($k_2$, $k_{-2}$, $k_{+3}$, $k_{+4}$, $k_{-4}$). Measurement of the observed rate constant of cob(II)alamin as a function of [AdoCbl]$_0$ (the initial concentration of AdoCbl) therefore provides information about the affinity of AdoCbl for RTPR in the presence of substrate and allosteric effector (i.e., K$_1$) and the rate constants for steps subsequent to AdoCbl binding ($k_2$, $k_{-2}$, $k_{+3}$, $k_{+4}$, $k_{-4}$). Investigations of the [AdoCbl]$_0$ dependence of the kinetics of cob(II)alamin formation were therefore undertaken, with the preliminary results allowing estimation of effective binding and rate constants for the initial formation of cob(II)alamin.

In these experiments, dATP was used as the allosteric effector and CTP was used as the substrate. The kinetics of cob(II)alamin formation were qualitatively similar to those observed with dGTP and ATP, with a fast initial increase in cob(II)alamin and a slower re-formation of the carbon-cobalt bond. In this case, however, the kinetics were well fit to a double exponential (Fig. 5.5), allowing more meaningful quantitative analysis of the
Fig. 5.5. Formation of cob(II)alamin (measured by SF UV-vis spectroscopy) during turnover of CTP. RTPR (30 μM), in one syringe with dATP (1 mM), CTP (1 mM), and TR/TRR/NADPH, was mixed with AdoCbl (130 μM) in a second syringe, which also contained dATP (1 mM) and CTP (1 mM). The data was fit to a double exponential (dashed line).
kinetics. As shown in Fig. 5.6, the apparent first order rate constants of cob(II)alamin formation (the first kinetic phase) vary from 112—258 s\(^{-1}\) (a factor of ~2.5) when [AdoCbl]\(_0\) varies over a range of 20–500 μM (a factor of 25). This suggests that in the case of the turnover reaction, the binding of AdoCbl to RTPR is becoming saturated (Johnson, 1992).

A full analytical expression for the predicted concentration dependence of the observed rate constant would be difficult to derive due to the complexity of the mechanism. However, the two-step binding and homolysis model described in Chapter 3 is likely to describe the kinetics of the first phase of this reaction to a first approximation (Scheme 5.1). The equation that describes the [AdoCbl] dependence of \(k_{\text{obs}}\) is

\[
k_{\text{obs}} = k_{+2} \left( \frac{K_1 [\text{AdoCbl}]}{K_1 [\text{AdoCbl}]_0 + 1} \right) + k_{-2}
\]

Eq. 5.1.

Use of this model depends on a number of assumptions. First, binding of dATP (1 mM) and CTP (1 mM) are assumed to be rapid, and these binding sites are assumed to be saturated. Second, the rate constant for turnover (\(k_{+3}\)) is assumed to be slow compared to \(k_{+2}\) and \(k_{-2}\). If this is true, \(k_{+3}\) will not make a significant contribution to \(k_{\text{obs}}\). As discussed later, this rate constant is at least 20 s\(^{-1}\), as judged by preliminary results on the single turnover rate constant for CTP reduction. If, however, nucleotide reduction includes early steps with rate constants much greater than 20 s\(^{-1}\), these steps will contribute significantly to \(k_{\text{obs}}\), invalidating this assumption. This analysis also assumes that the reaction is pseudo-first order in AdoCbl. However, at the lowest concentration of AdoCbl used (20 μM), AdoCbl is present in only two-fold excess over RTPR. Thus, this analysis provides only qualitative estimates for the rate constants.

To obtain estimates for the rate constants for the initial rapid carbon-cobalt bond homolysis, the data in Fig. 5.6 was fit to Eq. 1, giving a value of ~200 s\(^{-1}\) (the maximum \(k_{\text{obs}}\)) for the forward rate constant for carbon-cobalt bond homolysis (\(k_{+2}\)), and a value of ~75 s\(^{-1}\) (the intercept of the plot on the y-axis) for the reverse rate constant. This allows calculation of \(K_2\) (i.e., \(k_{+2}/k_{-2}\)), the equilibrium constant for the initial carbon-cobalt bond homolysis.
Fig. 5.6 Effect of [AdoCbl] on observed rate constants for the first kinetic phase of cob(II)alamin formation. RTPR (10–30 μM), in one syringe with dATP (1 mM), CTP (1 mM), and TR/TRR/NADPH, was mixed with AdoCbl (50–500 μM) in a second syringe, which also contained dATP (1 mM) and CTP (1 mM). Observed rate constants obtained from stopped-flow traces are plotted as a function of starting concentration of AdoCbl ([AdoCbl]₀). Double exponential fits of kinetic traces were used to obtain kₜₜ, as described in the text. Each kₜₜ is the average of 3–5 trials. Error bars represent 1 standard deviation. Data are fit to Eq. 5.1.
$k_{\text{obs}}$, 1st phase

$y = m_1 + \frac{(m_2 m_3 m_0)}{(m_2 m_0 + 1)}$

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</table>

[AdoCbl] (µM)
bond homolysis to be ~2, consistent with the observation of a maximum of 0.8 eq. of cob(II)alamin (Fig. 5.7). If an early step in nucleotide reduction (included in $k_{+3}$ in Scheme 5.1) is fast, the value of 200 s$^{-1}$ would represent contributions both from that step and from $k_{+2}$, rather than $k_{+2}$ alone. This alternative cannot be ruled out, and rapid acid quench experiments with ATP (described below) suggest that this is in fact the case. This analysis is nonetheless useful for approximating the net forward and reverse rate constants for the initial carbon-cobalt bond homolysis/thiol radical formation.

The amount of cob(II)alamin formed in the first phase can also provide information about the equilibrium constants $K_1$ and $K_2$. However, the amount of cob(II)alamin formed in the first phase (as measured by the amplitude of the exponential term corresponding to this phase) increases with increasing [AdoCbl]$_0$ at low [AdoCbl]$_0$ (<50 μM) and decreases with increasing [AdoCbl]$_0$ at higher [AdoCbl]$_0$ (Fig. 5.7). This behavior is not expected from the mechanism in Scheme 5.1. One possibility is that an alternate, non-productive, binding mode exists. Depending on what the order of binding actually is, another possibility is that the exchange reaction, in which no transient maximum in cob(II)alamin formation is observed, may begin to compete with turnover at high [AdoCbl], decreasing the size of the observed maximum.

As discussed later in this chapter, the second kinetic phase observed is likely to represent re-formation of the carbon-cobalt bond following nucleotide reduction (Scheme 5.1). Rate constants and amounts of AdoCbl re-formed during this phase were examined to get an idea of the magnitudes of $k_{+4}$ and $k_4$. As shown in Fig. 5.8, the rate constant of this phase is independent of [AdoCbl]$_0$ over the range tested (20–500 μM). A linear fit is plotted on these data to show that the rate constants are not correlated with [AdoCbl]$_0$ ($R^2 \sim 0.05$, compared to $>0.9$ for a significant correlation). As argued in the Discussion, this result is consistent with the rate of approach to equilibrium of this step being dominated by $k_4$.

The amount of AdoCbl re-formed in the second phase is expected to reflect the amount originally formed in the first phase. Thus, if competition from the exchange reaction decreases the amount of cob(II)alamin formed in the first phase, less AdoCbl can be re-formed in the second phase. As
Fig. 5.7. Effect of [AdoCbl] on amount of cob(II)alamin formed in the first kinetic phase. Experimental conditions were as described for Fig. 5.4. Fraction of total enzyme in thiol radical/5′-dA/cob(II)alamin form (calculated from the decrease in $A_{524}$ corresponding to the first amplitude in the double exponential fit) is plotted as a function of starting concentration of AdoCbl ([AdoCbl]₀). Each point is the average of 3–5 trials. Error bars represent 1 standard deviation. The curve shown is an interpolation to guide the eye.
Fig. 5.8. Effect of $[\text{AdoCbl}]$ on observed rate constants for the second kinetic phase (net re-formation of AdoCbl). Experimental conditions were as described for Fig. 5.4. Observed rate constants obtained from stopped-flow traces are plotted as a function of starting concentration of AdoCbl ($[\text{AdoCbl}]_0$). Double exponential fits of kinetic traces were used to obtain $k_{obs}$, as described in the text. Each point is the average of 3–5 trials. Error bars represent 1 standard deviation. The $k_{obs}$ are fit to a line, showing the lack of correlation.
expected, the amount of AdoCbl formed in the second phase (as measured by the amplitude of the exponential term corresponding to this phase) follows the same trend as observed in the first phase. It increases with $[\text{AdoCbl}]_0$ at low $[\text{AdoCbl}]_0$ (<50 μM) and decreases with $[\text{AdoCbl}]_0$ at higher $[\text{AdoCbl}]_0$ (Fig. 5.9).

**SF UV-vis Studies on the Turnover Reaction: Formation of Cob(II)alamin by Pre-Oxidized RTPR**

The mechanism in Scheme 5.1 predicts that the initial rapid formation of cob(II)alamin results from reaction of reduced RTPR with AdoCbl before the first turnover, while the slower net re-formation of AdoCbl is catalyzed after dNTP formation by oxidized RTPR ($E_{\text{ox}}$). This hypothesis implies that turnover is associated with a conformational change that interferes with the ability of RTPR to catalyze carbon-cobalt bond homolysis. In support of this hypothesis, Tamao and Blakley reported that when RTPR, dGTP, and AdoCbl are mixed with the dithiol dihydrolipoate, no cob(II)alamin formation is observed over 120 ms (Tamao and Blakley, 1973). Oxidation of C419 and C119 to a disulfide, which occurs concomitantly with substrate turnover (Booker et al., 1994), may prevent the enzyme from catalyzing carbon-cobalt bond cleavage efficiently.

To investigate this hypothesis further, SF UV-vis experiments were carried out in which pre-oxidized enzyme, in one syringe with ATP as substrate and dGTP as allosteric effector, was mixed with AdoCbl and TR/TRR/NADPH in a second syringe. The rate of cob(II)alamin formation was measured. Preliminary results showed that mixing of after a lag phase of ~25 ms, cob(II)alamin is formed with a rate constant of 20 s⁻¹ (Fig. 5.10). However, only ~0.04 equivalents of cob(II)alamin was present in the steady state, compared to ~0.2 eq. observed when RTPR and TR/TRR/NADPH were present in the same syringe. These experiments were performed with different concentrations of dGTP and ATP present (3 mM dGTP and 10 mM ATP were used in the former case, compared to 2 mM of each in the latter case), so the significance of the amount of cob(II)alamin remains to be determined. A further complication for this experiment was that AdoCbl was found not to be stable to incubation with pre-oxidized RTPR, dGTP and ATP (data not shown), suggesting that pre-oxidized RTPR can catalyze carbon-cobalt bond homolysis when both substrate and allosteric effector are
Fig. 5.9. Effect of [AdoCbl] on the magnitude of the decrease in cob(II)alamin (i.e., amount of AdoCbl re-formed) in the second kinetic phase. Experimental conditions were as described for Fig. 5.4. Decrease in fraction of total enzyme in thiy radical/5'-dA/cob(II)alamin form (calculated from the increase in A_{524} corresponding to the second amplitude in the double exponential fit) is plotted as a function of starting concentration of AdoCbl ([AdoCbl]_0). Each point is the average of 3–5 trials. Error bars represent 1 standard deviation. The curve shown is an interpolation to guide the eye.
Fig. 5.10. Formation of cob(II)alamin (measured by SF UV-vis spectroscopy) under turnover conditions with pre-oxidized RTPR. Pre-oxidized RTPR (70 μM), in one syringe with dGTP (3 mM) and ATP (10 mM), was mixed with with AdoCbl (100 μM) and TR/TRR/NADPH in a second syringe, which also contained dGTP (3 mM) and ATP (10 mM). The trace represents the average of three trials. Cob(II)alamin formation after the lag phase (~25 ms) is fit to a single exponential.
present. Since the observed cob(II)alamin formation could be associated with either the reduced enzyme or the oxidized enzyme, the results under turnover conditions are difficult to interpret.

As an alternative to the turnover conditions, the exchange reaction was once again used as a simplified model reaction. Degradation of AdoCbl on incubation with pre-oxidized RTPR in the presence of dGTP alone was found to be negligible on the time scale of minutes, indicating that reaction of oxidized enzyme with AdoCbl would not contribute significantly to cob(II)alamin formation on the millisecond timescale, and any cob(II)alamin formation could be ascribed to reduced enzyme. SF UV-vis experiments were carried out in which pre-oxidized RTPR, AdoCbl, and dGTP in one syringe were mixed with TR/TRR/NADPH or DTT in a second syringe. These experiments were similar to those carried out by Tamao and Blakley (Tamao and Blakley, 1973) using untreated RTPR (i.e., not pre-oxidized) and dihyroliolate as reductant. In our experiments, no cob(II)alamin formation is observed through 35 ms for either reductant (Figs. 5.11 and 5.12). However, in contrast to the observations of Tamao and Blakley, slow cob(II)alamin formation occurs after 35 ms. This cob(II)alamin formation is well fit by a single exponential, and the rate constant for cob(II)alamin formation was much slower than that observed with reduced RTPR (~40 s⁻¹, Chapter 3): 5±0.5 s⁻¹ when TR/TRR/NADPH is the reductant (Fig. 5.11), and 3±0.5 s⁻¹ when DTT is the reductant (Fig. 5.12). These rate constants are close to the turnover number of the enzyme (~2 s⁻¹). The steady-state concentrations observed are, as expected, consistent with those observed when enzyme and reductant are in the same syringe: ~0.2 equivalent when TR/TRR/NADPH is the reductant, and ~0.1 equivalent when DTT is the reductant. The slow formation of cob(II)alamin is consistent with a slow reduction of oxidized enzyme allowing rapid formation of cob(II)alamin. The lag phase might indicate that a change in the conformation of oxidized RTPR is required for reduction to take place. These experiments are consistent with disulfide re-reduction being at least partially rate-limiting in turnover, and being required for rapid carbon-cobalt bond homolysis.
Fig. 5.11. Formation of cob(II)alamin (measured by SF UV-vis spectroscopy) under exchange reaction conditions with pre-oxidized RTPR and TR/TRR/NADPH as reductant. Pre-oxidized RTPR (50 μM), in one syringe with dGTP (3 mM) with AdoCbl (100 μM) was mixed with TR/TRR/NADPH in a second syringe, which also contained dGTP (3 mM). The trace represents the average of three trials. Cob(II)alamin formation after the lag phase (~35 ms) is fit to a single exponential.
Fig. 5.12. Formation of cob(II)alamin (measured by SF UV-vis spectroscopy) under exchange reaction conditions with pre-oxidized RTPR and DTT as reductant. Pre-oxidized RTPR (45 μM), in one syringe with dGTP (4.5 mM) with AdoCbl (70 μM) was mixed with DTT (30 mM) in a second syringe, which also contained dGTP (3 mM). The trace represents the average of three trials. Cob(II)alamin formation after the lag phase (~35 ms) is fit to a single exponential.
Rapid Acid Quench Experiments Under Single Turnover Conditions: Fate of the 5'-Hydrogens and Rate of Single Turnover

The biphasic kinetics of cob(II)alamin formation studied in the SF UV-vis experiments described above are generally consistent with re-formation of the carbon-cobalt bond following the first turnover. This would imply that the first turnover was complete in less than 200 ms. However, the steady state rate constant for turnover is \( \sim 2 \text{ s}^{-1} \) (Vitols et al, 1967, Booker et al, 1994). If the first turnover is complete on the millisecond timescale, turnover must occur considerably faster than the steady state rate of dNTP production. This would be consistent with the SF UV-vis experiments on oxidized RTPR, which suggested that disulfide re-reduction and/or an associated conformational change is rate-limiting in steady state turnover. In order to test the hypothesis that dNTP formation itself is not rate-limiting in catalysis, the presteady state rate of turnover was measured using rapid acid quench techniques, monitoring formation of \([^{14}\text{C}]-\text{dNTP}\).

Rapid acid quench techniques also furnish a second way to investigate the question of carbon-cobalt bond re-formation during turnover. As discussed for deuterium exchange in Chapter 3, exchange of tritium from \([5^-^{3}\text{H}]-\text{AdoCbl}\) occurs as a consequence of carbon-cobalt bond re-formation (\(k_2\) and \(k_{+4}\), Scheme 5.1). Therefore, the extent of tritium washout to solvent from \([5^-^{3}\text{H}]-\text{AdoCbl}\) is a measure of re-formation of the carbon-cobalt bond either prior to or subsequent to turnover. Measuring the rate of tritium washout thus provides information about whether carbon-cobalt bond re-formation occurs after each turnover (through step 4), and whether a significant amount of carbon-cobalt bond re-formation occurs before turnover (through the reverse reaction of step 2).

Rapid acid quench experiments were carried out in which RTPR, in one syringe with \([^{14}\text{C}]-\text{ATP}, \text{dGTP}\) and TR/TRR/NADPH, was mixed with \([5^-^{3}\text{H}]-\text{AdoCbl}\) in a second syringe (which also contained \([^{14}\text{C}]-\text{ATP}\) and \(\text{dGTP}\)). The apparent first-order rate constant for the formation of \(\text{dATP}\) is \(55 \pm 10 \text{ s}^{-1}\) (Fig. 5.13). This is faster than the steady state turnover rate by an order of magnitude. A total of 0.6-0.7 eq. of product is formed during this fast reaction, suggesting that some fraction of the enzyme is not able to carry out turnover. As discussed in Chapter 4, this observation is consistent with the observation of only 1.5 eq. of product formed in single turnover experiments (Booker et al, 1994) and the observation of 0.85–0.9 \(\text{dGTP}\).
Fig. 5.13. Formation of $[^{14}\text{C}]-2'$-dATP. RTPR (120 $\mu$M), in one syringe with dGTP (2 mM), [U-$^{14}$C]-ATP (2 mM) and TR/TRR/NADPH, was mixed with [5'-$^{3}$H]-AdoCbl in a second syringe, which also contained dGTP (2 mM) and [U-$^{14}$C]-ATP (2 mM), followed by quenching with acid. The data from replicate experiments are shown. Each data point represents one trial. The data are fit to a single exponential (solid line).
\[ y = m1*(1 - \exp(-m2*m0)) \]

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binding sites per protein (Chen et al., 1974, Booker et al., 1994). These results indicate that the nucleotide reduction reaction itself is not rate limiting in catalysis.

Studies were also initiated to characterize the rate-limiting step in CTP reduction, since, as described above, the kinetics of cob(II)alamin formation are more manageable with this substrate. Preliminary results suggest that in the presteady state, \( \sim 0.7 \) eq. of dCTP is formed with a rate constant of 20\( \pm 3 \) s\(^{-1}\) (Fig. 5.14). However, it has not yet been demonstrated that the labeled product isolated is 100% dC, rather than cytidine. It should also be noted that the data exhibited low numbers of counts (200–500 cpm) and high backgrounds (200 cpm).

As alluded to above, tritiated water release has also been observed and quantified on the timescale of the first turnover (Fig. 5.15), under conditions identical to those used to quantify dATP production. The initial rate of this process is 0.6 eq. \([^3H]-H_2O/s\), comparable to the value observed in the presence of dGTP alone (0.3 eq./s, see Chapter 2). Unlike the kinetics observed in the absence of substrate, which were linear, the kinetics can in this case be fit to a single exponential, with a rate constant of 10\( \pm 3 \) s\(^{-1}\) (Fig. 5.15). This is consistent with only the reduced form of the enzyme being able to catalyze rapid carbon-cobalt bond homolysis and re-formation (Booker et al., 1994). As the enzyme is converted into the oxidized state in the course of the first turnover, the release of tritiated water should slow down, leading to the observed exponential kinetics.

According to Scheme 5.1, the tritiated water observed presumably derives from carbon-cobalt bond re-formation occurring before turnover \( (k_{-2}) \) and/or after turnover \( (k_{+4}) \). To analyze how much of the tritium release occurs after nucleotide reduction, the ratio of tritiated water release to \([2-^{14}C]\)-dATP was determined as a function of time. If the bulk of tritiated water were formed before turnover, this ratio would decrease with time, illustrating a burst of tritiated water release followed by slower dATP production. If turnover took place before the bulk of tritiated water release, however, this ratio would increase with time, illustrating a burst of product formation. As shown in Fig. 5.16, this ratio increases with time, suggesting that release of tritiated water following nucleotide reduction predominates. After 300 ms, 0.6 equivalent of \([2-^{14}C]\)-dATP is formed and 0.06 equivalent of trititated water is released, for a ratio of 1/10th of an
Fig. 5.14. Formation of $[^{14}\text{C}]-2'$-dCTP. RTPR (65 $\mu$M), in one syringe with dATP (2 mM), [2-$^{14}$C]-CTP (2 mM) and TR/TRR/NADPH, was mixed with AdoCbl in a second syringe, which also contained dATP (2 mM) and [2-$^{14}$C]-CTP (1 mM), followed by quenching with acid. Each data point represents one trial from a single experiment. The data are fit to a single exponential (solid line).
Fig. 5.15. Formation of $[^3\text{H}]-\text{H}_2\text{O}$ on mixing RTPR with $[5'[^3\text{H}]}$-AdoCbl in the presence of dGTP, $[^{14}\text{C}]-\text{ATP}$, and TR/TRR/NADPH, followed by quenching with acid. Experimental conditions were as described for Fig. 5.12. The data from replicate experiments are shown. Each data point represents one trial. The data are fit to a single exponential (solid line).
\[ y = m1 \times (1 - \exp(-m2 \times t)) \]

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Fig. 5.16. Ratio of $[^3\text{H}]$-H$_2$O formed to $[^{14}\text{C}]$-2′-dATP formed on mixing RTPR with $[5′-^3\text{H}]$-AdoCbl in the presence of dGTP, $[^{14}\text{C}]$-ATP, and TR/TRR/NADPH, followed by quenching with acid. The data are from Figs. 5.12 and 5.13. The data from replicate experiments are shown. Each data point represents one trial. The data are fit to a single exponential.
\[ y = m1 \cdot \left(1 - \exp(-m2 \cdot m0)\right) \]

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</table>

- \( y \) is the concentration of \( ^3H \)-H₂O/dATP.
- \( m0 \) is the initial concentration.
- \( m1 \) and \( m2 \) are the parameters of the function.
- \( R^2 \) is the coefficient of determination.

The graph shows the concentration of \( ^3H \)-H₂O/dATP over time (s).
equivalent of tritiated water released per equivalent of deoxynucleotide (Fig. 5.16). Taking into account a selection effect and a kinetic isotope effect acting against abstraction of tritium, this value is also consistent with re-formation of the carbon-cobalt bond occuring after each turnover, as described in the Discussion.

Rapid Acid Quench Experiments: Quenching Artifacts and Possible Trapping of a Substrate-Based Intermediate

The reactivity of intermediates is often sensitive to the methods used to quench reactions. One salient example from the literature on AdoCbl-dependent isomerases is the observation that the amount of 5'-dA released during catalysis by ethanolamine ammonia lyase is dependent on the denaturant used to stop the reaction. In the RTPR system, the thyl radical intermediate is observable when the reaction mixture is sprayed into liquid isopentane, but not when the reaction mixture is frozen in liquid nitrogen (Orme-Johnson et al, 1974). Two observations suggest that the thyl radical/cob(II)alamin intermediate in RTPR-catalyzed nucleotide reduction partitions between the catalytic modes of reactivity (carbon-cobalt bond reformation and 3' hydrogen abstraction) and alternate modes of reactivity that occur artifactually as a result of acid quenching.

First, 14C was observed in the precipitated protein pellets (Fig. 5.17). Like cob(II)alamin, 14C labeling increased rapidly to a maximum value, then decreased slowly to a steady-state value. The proportion of the total 14C observed at maximum formation of the unknown species varied widely from trial to trial, making accurate measurement of the rate of increase impossible. However, the decrease in [14C] label could be fit to a single exponential with a rate constant of 30±10 s⁻¹. Precise quantification of this species that labels the protein would require knowledge of the number of 14C-labeled carbons it contains. If the species labeling the protein contained all the carbons originally present in the substrate, it would represent (as shown in Fig. 5.17) ~0.6 equivalents of labeling species at the maximum and ~0.05 equivalents in the steady state. While variable amounts of adenine were detected (0.1-0.3 eq.), this did not occur in a reproducible fashion from experiment to experiment. The issue of how adenine release relates to labeling of the protein is thus still unresolved.
Fig. 5.17. Accumulation of $^{14}$C in the protein pellet formed on mixing RTPR with [5'-$^3$H]-AdoCbl in the presence of dGTP, $^{14}$C-ATP, and TR/TRR/NADPH, followed by quenching with acid. Experimental conditions were as described for Fig. 5.12. Equivalents of $^{14}$C in the pellets are calculated assuming that all the carbons originally present in the substrate label the protein. The data from replicate experiments are shown. Each data point (triangles) represents one trial. The data are fit to a single exponential. The data on 2'-dATP formation from Fig. 5.12 is shown (circles), with fit, for comparison.
These results are consistent either with covalent linkage to the protein of a true reactive intermediate occurring as an artifact of quenching or with generation a new reactive species, not an intermediate in nucleotide reduction, as an artifact of quenching, and covalent labeling of the protein by this new species. In either case, the observation that the rate constant for decrease in $[^{14}\text{C}]$-labeling of the protein pellet is close to the rate constant for nucleotide reduction suggests a link between these processes. The decrease in $[^{14}\text{C}]$-labeling could represent a decrease in the concentration of an intermediate in nucleotide reduction (e.g., the thiol radical or a substrate-based radical) as nucleotide reduction progresses.

The second result suggesting that the reactivity of the thiol radical intermediate can be affected by quenching artifacts is a discrepancy observed between the amount of 5'-$\text{dA}$ detected using rapid acid quench methods and the amount of cob(II)alamin detected using SF UV-vis methods. In the rapid acid quench experiments, [5'-$^{3}\text{H}$]-5'-$\text{dA}$ formation is observed with kinetics qualitatively similar to those of cob(II)alamin formation, a rapid increase to a maximum followed by a decline to a steady state value (Fig. 5.18). However, in contrast to the results in the exchange reaction (Chapter 2), the maximum amount of [5'-$^{3}\text{H}$]-5'-$\text{dA}$ is less than the amount of cob(II)alamin measured in SF UV-vis experiments. Although the scatter in the data is relatively large, the maximum [5'-$^{3}\text{H}$]-5'-$\text{dA}$ formed is $\sim0.3$ eq., compared to $\sim0.6$ eq. of cob(II)alamin. In contrast to the exchange reaction conditions, tritiated water washout under turnover conditions cannot explain this gap, since tritiated water release is only $\sim0.01$ eq. at the peak of cob(II)alamin formation (Fig. 5.12). Cyclization of 5'-$\text{dA}$ to form 5', 8'-cycloadenosine, which might also explain this discrepancy, does not occur. In fact, no tritiated products other than [5'-$^{3}\text{H}$]-5'-$\text{dA}$, tritiated water, and [5'-$^{3}\text{H}$]-AdoCbl are observed.

The source of this gap may be interconversion between 5'-$\text{dA}$ and AdoCbl. This could occur if the thiol radical intermediate can partition between being quenched by perchloric acid and abstracting a hydrogen from 5'-$\text{dA}$ to re-form the carbon-cobalt bond. Cob(II)alamin formation, as measured by SF UV-vis spectroscopy, is not subject to partitioning on quenching, while 5'-$\text{dA}$ formation, as measured by rapid acid quench experiments, is. Such a partitioning would be analogous to that partitioning that Babior, Abeles, and co-workers hypothesize to be responsible for the
Fig. 5.18. Formation of $[5'{}^{-3}{}^H]-5'$-dA on mixing RTPR with $[5'{}^{-3}{}^H]$-AdoCbl in the presence of dGTP, $[{}^{14}{}^C]$-ATP, and TR/TRR/NADPH, followed by quenching with acid. Experimental conditions were as described for Fig. 5.12. The data from replicate experiments are shown. Each data point represents one trial. The data on cob(II)alamin formation from Fig. 5.1 is shown, with fit, for comparison.

**Rate of Turnover in D<sub>2</sub>O and with [5'-2H<sub>2</sub>]AdoCbl: Investigation of a V/K Isotope Effect**

Experiments were performed to address the issue of V/K solvent/cofactor isotope effects. These isotope effect measure the commitment to catalysis for bound AdoCbl, or, in other words, the probability that carbon-cobalt bond homolysis (step 2, Scheme 5.1) will occur as opposed to dissociation of AdoCbl from the enzyme (the reverse reaction in step 1). They also measure the commitment to catalysis for the thyl radical formed in step 2, or the probability that turnover will occur as opposed to carbon-cobalt bond re-formation. Both D<sub>2</sub>O and [5'-2H<sub>2</sub>]-AdoCbl were used in these experiments, to avoid complications arising from washout of hydrogen from cofactor. The rate of turnover was measured in both H<sub>2</sub>O and D<sub>2</sub>O at two concentrations (Fig. 5.19): ~10 μM (>10X the K<sub>m</sub> in H<sub>2</sub>O) and ~0.1 μM (<0.5 the K<sub>m</sub> in H<sub>2</sub>O). While two concentrations are inadequate to obtain a reliable K<sub>m</sub>, especially considering the scatter in the data, the preliminary result is that there is no difference between V/K for in H<sub>2</sub>O and in D<sub>2</sub>O. This would be consistent with a high commitment to catalysis.

c3.SF UV-vis Studies on the Turnover Reaction: Order of Addition

The experiments described above address mechanistic questions primarily from a kinetic standpoint. Trapping and direct chemical characterization of substrate-based radical intermediates is also an area of great interest. Various perturbations of protein structure, substrate structure, and experimental conditions have been tested for their ability to produce a substrate-based radical intermediate trappable by RFQ techniques.

Even when the primary goal of RFQ studies is chemical characterization of an intermediate, however, a knowledge of the kinetics of the reaction is important for the design of experiments. The rate of binding of substrate to RTPR was investigated due to its potential impact on RFQ EPR studies. If a binding step or conformational change associated with it is slow compared to covalent reactions, order of addition can have a profound effect on the rate constants for formation of intermediates (Hollaway et al ,

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Fig. 5.19. Effect of D$_2$O/[5'-2H$_2$]-AdoCbl on V/K$_m$, AdoCbl for ATP turnover with dGTP as allosteric effector. Assay mixtures contained RTPR (3 μM), AdoCbl (0.07–10 μM) or [5'-2H$_2$]AdoCbl (0.06–10 μM), dGTP (1 mM), ATP (1 mM), and TR/TRR/NADPH. Circles: H$_2$O, unlabeled AdoCbl. Triangles: D$_2$O, [5'-2H$_2$]-AdoCbl. The data are fit to lines.
1978) and on the concentrations of intermediates formed in the presteady state. Unfortunately, since RTPR catalyzes the breakdown of AdoCbl to cob(II)alamin and 5'-dA, it is not possible to measure kinetics of cob(II)alamin formation when RTPR and AdoCbl are in the same syringe before mixing with substrate. However, the contribution of substrate binding to the rate of the reaction can be evaluated by comparing the kinetics of cob(II)alamin formation in two experiments: first, mixing of RTPR, dGTP, ATP, and TR/TRR/NADPH, in one syringe, with AdoCbl in a second syringe (which also contained ATP and dGTP); and second, mixing of RTPR, dGTP, and TR/TRR/NADPH, in one syringe, with ATP and AdoCbl in a second syringe (which also contained dGTP).

In the limit of fast binding of substrate to RTPR, the kinetics of cob(II)alamin formation should be independent of the order of addition. If binding of substrate is slow compared to subsequent steps, adding substrate after AdoCbl should decrease the observed rate of cob(II)alamin formation. The experimentally observed kinetics (Fig. 5.20), using ATP as substrate) are consistent with binding of AdoCbl being slow compared to subsequent steps. When RTPR is pre-incubated with ATP (Fig. 5.20 A), the \( k_{\text{obs}} \) for the initial cob(II)alamin formation (obtained from fitting the initial decline in A524 to a single exponential) is \( \sim 250 \, \text{s}^{-1} \), whereas when RTPR is mixed with AdoCbl and ATP simultaneously (Fig. 5.20 B), the \( k_{\text{obs}} \) for the initial rapid cob(II)alamin formation to a maximum is \( \sim 70 \, \text{s}^{-1} \). The \( k_{\text{obs}} \) for the re-formation of AdoCbl when RTPR is pre-incubated with ATP is \( \sim 20 \, \text{s}^{-1} \), and \( \sim 10 \, \text{s}^{-1} \) when RTPR is mixed with ATP and AdoCbl simultaneously.

The magnitude of the transient increase in cob(II)alamin also depends on the order of addition. When RTPR is pre-incubated with ATP (Fig. 5.20, A), a maximum of \( \sim 0.4 \, \text{eq. of cob(II)alamin is formed, whereas when RTPR} \) is mixed with ATP and AdoCbl simultaneously (Fig. 5.20, B), a maximum of \( \sim 0.2 \, \text{eq. of cob(II)alamin is formed. At long times (>300 ms), however, cob(II)alamin formation is independent of order of addition (~0.1 eq. cob(II)alamin).} \)

One very important caveat is that the final concentration of ATP was lower (0.5 mM) when RTPR was mixed with ATP than when RTPR and ATP were pre-incubated (1 mM), and the [ATP] dependence of the kinetics is as yet uncharacterized. The observed kinetic effects might come about in part because ATP binding is not saturated. Whatever the source of these results,
Fig. 5.20. Effect of order of addition of ATP on formation of cob(II)alamin.
(measured by SF UV-vis spectroscopy). Pre-incubation of RTPR and ATP;
i.e., RTPR (120 µM), in one syringe with dGTP (1 mM), ATP (1 mM), and
TR/TRR/NADPH, was mixed with AdoCbl (100 µM) in a second syringe,
which also contained dGTP (1 mM) and ATP (1 mM). B. RTPR and ATP
not pre-incubated; i.e., RTPR (100 µM), in one syringe with dGTP (1 mM)
and TR/TRR/NADPH, was mixed with AdoCbl (100 µM) and ATP (1 mM)
in a second syringe, which also contained dGTP (1 mM). For both A and B,
the trace represents the average of three trials. Both initial phase (5–20 ms)
and the final phase (50–200 ms) were fit individually to single exponentials
(fits are shown as dashed lines).
however, they have implications for planning order of addition and substrate concentration to maximize concentrations of paramagnetic intermediates in RFQ EPR experiments.

**SF UV-vis studies using C419S and C119S: Formation of Cob(II)alamin under Turnover Conditions**

In order to determine whether it was likely that a long-lived substrate-based radical could be observed during mechanism-based inhibition by C419S RTPR, the kinetics of cob(II)alamin formation by this mutant enzyme in the presence of substrate were measured when C419S RTPR, in one syringe with ATP, dGTP, and TR/TRR/NADPH, was mixed with AdoCbl in a second syringe (which also contained ATP and dGTP). As shown in Fig. 5.21a, cob(II)alamin formation at 37 °C reaches a maximum corresponding to ~0.1 eq. in ~500 ms, then declines to a minimum (0.03 eq.) over ~10 s. The kinetic trace can be fit by two exponentials with rate constants of 10 s⁻¹ (increase of cob(II)alamin) and 0.2 s⁻¹ (decline in cob(II)alamin). This data is consistent with a substrate-based radical being present in amounts large enough (a maximum of ~0.1 eq.) to be detected by RFQ EPR. In contrast, when the same experiment is carried out using C119S RTPR, cob(II)alamin increases to a maximum of only ~0.05 eq. over 200 ms, (data not shown), making it a less attractive candidate for RFQ EPR studies.

In an effort to determine whether the biphasic kinetics represent a slower version of events similar to those observed for the wild-type enzyme, the isotope sensitivity and temperature dependence of the kinetics were measured (with [AdoCbl] = 200 μM to minimize the contribution of bimolecular steps to the kinetics). Kinetic isotope effects with [5⁻²H₂]-AdoCbl were measured (Fig. 5.21b). In preliminary findings, a double exponential fit gave a rate constant of 6.7 s⁻¹ for the initial increase in cob(II)alamin formation, corresponding to an isotope effect of ~1.3. No isotope effect was observed on the rate of decline in cob(II)alamin. The maximum cob(II)alamin formed was 0.2 eq. (compared to 0.1 eq. with unlabeled AdoCbl), declining to 0.03 eq.) Preliminary results on the temperature dependence show that at 32 °C, the kinetic trace was fit by two exponentials with rate constants of 7 s⁻¹ and 0.2 s⁻¹, compared to 10 s⁻¹ and 0.2 s⁻¹ at 37 °C. The amounts of cob(II)alamin formed were the same as at 37 °C. These observations suggest that the faster phase, which is isotope sensitive.
Fig. 5.21a. Formation of cob(H2)alamin (measured by SF UV-vis spectroscopy) with C419S RTPR. C419S RTPR (400 μM), in one syringe with dGTP (1 mM), ATP (1 mM), and TK/TRR/NADPH, was mixed with AdoCbl (400 μM) in a second syringe, which also contained dGTP (1 mM) and ATP (1 mM). The trace represents the average of 3–4 trials. The fit to a double exponentials is shown (dashed lines).
Fig. 5.21b Isotope effects on formation of cob(II)alamin when [5'-2H2]-AdoCbl (400 μM) is substituted for AdoCbl. The traces represent the average of 3–4 trials. The fits to double exponentials are shown (dashed lines).
and highly temperature sensitive, corresponds to carbon-cobalt bond homolysis/thyl radical formation, which has also been shown to be isotope- and temperature-sensitive (Chapter 4). The slower phase, which is isotope insensitive and not highly temperature sensitive, may represent oxidation of cob(II)alamin after quenching of a radical intermediate, or reaction of cob(II)alamin with an active site thiol group (D. Silva, C. Lawrence, J. Stubbe, unpublished results). Spectral characterization of intermediates using diode array SF UV-vis spectroscopy could help to distinguish between these alternatives.

**RFQ EPR: Paramagnetic Intermediate Generated by C419S RTPR Under Turnover Conditions**

The SF UV-vis studies described above suggested that a substrate-based radical might be observable in the reaction of C419S RTPR with ATP. In RFQ EPR studies, C419S RTPR, in one syringe with ATP, dGTP, and TR/TRR/NADPH, was mixed with AdoCbl in a second syringe (which also contained ATP and dGTP). The EPR spectrum of a reaction mixture quenched at 250 ms is shown in Fig. 5.22. Similar spectra were observed for samples quenched at times ranging from 20 ms to 3 s (data not shown). The broad, low amplitude signal at \( g = 2.1-2.3 \) is difficult to interpret with confidence, as intensity of this magnitude could be an artifact of baseline subtraction. The feature appearing at \( g \sim 2.0 \) is likely to represent an organic radical, possibly a substrate-based radical. Features spaced \( \sim 30 \text{ G} \) apart are apparent on the high field side of the spectrum. Double integration of the entire signal gave a spin concentration of \( \sim 20 \text{ \mu M} \), assuming a packing factor of 0.7. This represents \( \sim 0.1 \text{ eq.} \) of radical, in rough agreement with what might be expected based on the results of SF UV-vis experiments. The \( g = 2.0 \) signal by itself, however, accounts for only \( \sim 3 \text{ \mu M} \), or \( \sim 0.02 \) equivalent. These preliminary studies must be followed up to characterize the lineshape and spin concentration with more confidence.

**SF UV-vis studies using Pre-Reduced RTPR in Large Excess over TR: Effect on the Kinetics of Cob(II)alamin Formation**

Another potential means of prolonging the lifetime of a substrate-based radical is to carry out substrate turnover with wt RTPR in large excess over TR. As long as the TR/TRR/NADPH reducing system is initially
Fig. 5.22. EPR spectrum observed on mixing C419S RTPR with AdoCbl in the presence of dGTP, ATP, and TR/TRR/NADPH, followed by freeze-quenching (250 ms). C419S RTPR (500 μM), in one syringe with dGTP (1 mM), ATP (1 mM), and TR/TRR/NADPH, was mixed with AdoCbl (500 μM) in a second syringe, which also contained dGTP (1 mM) and ATP (1 mM). The EPR spectrum was recorded at 100 K with spectrometer frequency 9.44 GHz; microwave power, 1 mW; modulation frequency, 100 kHz; modulation amplitude, 4 G; time constant, 1.3 s; and scan time, 671 s. The spectrum shown is the sum of 8 scans. A cubic function was used as the baseline and subtracted. The three arrows point to potential hyperfine features spaced ~30 G apart.
present with RTPR, the enzyme will start out in the reduced state. However, after mixing the enzyme (incubated with substrate, allosteric effector, and TR/TRR/NADPH) in one syringe with AdoCbl in a second syringe (which also contained substrate and allosteric effector) in the presence of substrate, the enzyme will be oxidized in the course of the first turnover. Delivery of external reducing equivalents to the active site will be slow, so the radical intermediates in the second turnover that are ordinarily reduced by the active site cysteines C419 and C119 may exhibit longer lifetimes. SF UV-vis experiments were carried out in order to determine whether the amount of cob(II)alamin-containing species (i.e., radical intermediates) formed under these conditions would be sufficient to observe by RFQ EPR (Fig. 5.23). Fitting the trace to two single exponentials gives a $k_{\text{obs}} \sim 160 \text{ s}^{-1}$ for the initial formation of cob(II)alamin and a $k_{\text{obs}} \sim 13 \text{ s}^{-1}$ for the net re-formation of AdoCbl. These rate constants are similar to those observed with larger TR concentrations. The decline in A524 corresponds to formation of $\sim 0.25 \text{ eq. of cob(II)alamin}$. Radical intermediates should thus be present in high enough concentrations to follow the reaction by RFQ EPR and determine whether substrate based radicals can be trapped following the first turnover, when RTPR cannot be efficiently reduced.

**RFQ EPR: Paramagnetic Intermediate Generated by wt RTPR with Pre-Reduced RTPR in Large Excess over TR:**

To determine whether substrate-based radicals could be trapped after the first turnover with RTPR in large excess over TR, RFQ EPR experiments were carried out. RTPR, in one syringe with ATP, dGTP and TR/TRR/NADPH (0.003 eq. per eq. of RTPR), was mixed with AdoCbl in a second syringe (which also contained ATP and dGTP). The EPR spectrum of a freeze-quenched reaction mixture (quenched at 250 ms) containing sublimiting TR (Fig. 5.24) is essentially identical in lineshape to spectra of samples containing sufficient TR to support turnover at the maximum rate (compare with Fig. 2.9, Chapter 2). Similar lineshapes are observed for samples quenched at times ranging from 20 ms to 45 s (data not shown). This observation indicates that these conditions do not support the formation of detectable amounts of long-lived substrate-based radicals.
Fig. 5.23. Formation of cob(II)alamin (measured by SF UV-vis spectroscopy) on mixing pre-reduced RTPR with AdoCbl in the presence of dGTP, ATP, and a sub-limiting amount of TR/TRR/NADPH reducing system (~0.003 eq. of TR/eq. of RTPR). Pre-reduced RTPR (500 μM), in one syringe with dGTP (1 mM), ATP (1 mM), and TR/TRR/NADPH, was mixed with AdoCbl (500 μM) in a second syringe, which also contained dGTP (1 mM) and ATP (1 mM). The trace is the average of three trials. Both initial phase (5–20 ms) and the final phase (50–200 ms) were fit individually to single exponentials (fits are shown as dashed lines).
Fig. 5.24. EPR spectrum observed on mixing pre-reduced RTPR with AdoCbl in the presence of dGTP, ATP, and a sub-limiting amount of TR/TRR/NADPH reducing system (~0.003 eq. of TR/eq. of RTPR), followed by freeze-quenching (250 ms). RTPR (510 μM), in one syringe with dGTP (1 mM), ATP (1 mM), and TR/TRR/NADPH, was mixed with AdoCbl (400 μM) in a second syringe, which also contained dGTP (1 mM) and ATP (1 mM). The EPR spectrum was recorded at 100 K with spectrometer frequency 9.43 GHz; microwave power, 0.1 mW; modulation frequency, 100 kHz; modulation amplitude, 5 G; time constant, 1.3 s; and scan time, 168 s. The spectrum shown is the sum of 33 scans.
SF UV-vis Studies of the Kinetics of Cob(II)alamin Formation by wt RTPR in the Presence of ara-ATP

Use of the substrate analog ara-ATP is another potential way to find a trappable nucleotide-based radical. In SF UV-vis experiments where RTPR, in one syringe with ara-ATP and dihydrolipoate, was mixed with AdoCbl in a second syringe (which also contained ara-ATP), Tamao and Blakley showed that ara-ATP can act analogously to allosteric effectors in stimulating rapid carbon-cobalt bond homolysis (Tamao and Blakley, 1973). This result can be interpreted in terms of the mechanism in Scheme 5.1. RTPR should be able to abstract the 3'-hydrogen from this molecule (step 2), but subsequent steps in catalysis should be retarded, since the 2'-hydroxyl will presumably not be in contact with the protein residues that participate in loss of the 2'-hydroxyl from ribonucleotide substrates. While the 3'-radical is not likely to be stable at the active site, it might react to form a more stable nucleotide-based radical. In order to design RFQ EPR experiments that might trap a nucleotide-based radical, SF UV-vis experiments similar to those of Tamao and Blakley were carried out with ara-ATP (Fig. 5.25). RTPR, in one syringe with ara-ATP, dGTP, and TR/TRR/NADPH, was mixed with AdoCbl in a second syringe (which also contained ara-ATP and dGTP). Preliminary results show that there is an initial fast phase of cob(II)alamin formation in which ~0.1 eq. of cob(II)alamin is formed at a rate constant of ~100 s⁻¹ (fitting to a single exponential). Unlike kinetics observed with the natural substrate, re-formation of the carbon-cobalt bond does not in this case manifest itself as a discrete kinetic phase. This is consistent with the idea that a relatively stable nucleotide-based radical is being formed in this reaction, and that this radical cannot re-abstract a hydrogen atom from C408 in order to initiate re-formation of the carbon-cobalt bond. Ara-ATP is thus a good candidate for RFQ EPR spectroscopy; however, the low amount of cob(II)alamin observed will require high concentrations of RTPR and AdoCbl for the radical to become detectable.

Effect of [¹³C]-ATP on the "Doublet Signal"

To investigate whether the doublet signal (Hamilton et al., 1972, Buettner & Coffman, 1977) might be a substrate-based radical, reaction mixtures containing either unlabeled ATP or [U-¹³C]-ATP were frozen by
Fig. 5.25. Formation of cob(II)alamin (measured by SF UV-vis spectroscopy) on mixing RTPR with AdoCbl in the presence of dGTP, ara-ATP, and TR/TRR/NADPH. RTPR (100 μM), in one syringe with dGTP (1 mM), ara-ATP (0.5 mM), and TR/TRR/NADPH, was mixed with AdoCbl (500 μM) in a second syringe, which also contained dGTP (1 mM) and ara-ATP (0.5 mM). The trace is the average of three trials. The fit to a single exponential is shown.
hand, and EPR spectra were recorded. The signal observed with unlabeled ATP (Fig. 5.26) was qualitatively similar but not identical to the signal reported by Hamilton et al. (Hamilton et al., 1972). Like the signal reported by Hamilton, et al., the observed signal had a feature at g = 2 and a broad absorbance at g = 2.3. However, while the previously reported g = 2 feature was a well-resolved doublet with a splitting of 100 G and an isotropic lineshape, the observed g = 2 feature was a broad (~300 G) singlet with an anisotropic lineshape. As the conditions used to generate the sample were the same as reported by Hamilton, et al., the reason for the differences in the spectra are not clear.

Substitution of [U-\textsuperscript{13}C]-ATP for unlabeled ATP produced no detectable change in the EPR spectrum (Fig. 5.26), indicating that the trapped species does not bear unpaired spin density on a carbon derived from the substrate. It is possible that the spectrum represents a thyl radical generated at the bottom face of the active site (i.e., on C419 or C119) by a substrate-derived radical.

\textbf{[AdoCbl] Dependence of the Rate of Turnover when [RTPR]>[AdoCbl]}

As described above, intermediate events in nucleotide reduction are of great interest, as they provide insight into how radical species perform their functions. However, events occurring after nucleotide reduction are of equal interest, as they relate to how radical species avoid adventitious reactions that could prevent them from carrying out their functions. One major unresolved question is whether AdoCbl is released from the enzyme after every turnover or whether it remains bound for multiple turnovers. This question relates to the issue of whether carbon-cobalt bond re-formation occurs after every turnover. Release of AdoCbl from RTPR after every turnover would imply that the carbon-cobalt bond is re-formed after every turnover, although it is important to note that no conclusion about carbon-cobalt bond re-formation can be drawn from an observation that AdoCbl does not dissociate. In that case, the cofactor might be bound either as AdoCbl or in the homolyzed state. To address these questions, the rate of turnover was measured both under the standard Michaelis-Menten conditions, where [AdoCbl]>[RTPR], and under conditions where [RTPR]>[AdoCbl].

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Fig. 5.26. Effect of $^{13}$C labeling of ATP on the EPR spectrum of the species observed on long (~10 min) incubation of RTPR with AdoCbl, dGTP, ATP, and low concentrations of TR/TRR/NADPH (~0.008 eq. TR/eq. RTPR). The final reaction mixture contained RTPR (1.3 mM), dGTP (1.5 mM), unlabeled ATP or $[^{13}$C]-ATP (3.7 mM), AdoCbl (0.3 mM), and TR/TRR/NADPH. These are the conditions reported to produce the doublet species (Hamilton et al., 1972). The EPR spectra was recorded at 20 K with spectrometer frequency 9.44 GHz; microwave power, 0.3 mW; modulation frequency, 100 kHz; modulation amplitude, 5 G; time constant, 164 ms; and scan time, 167 s.
When [RTPR] >> [AdoCbl], the rate of turnover (expressed as \( v / [\text{AdoCbl}] \)) will be independent of [AdoCbl] if AdoCbl does not dissociate from the enzyme after turnover. Because [RTPR] >> \( K_m \), AdoCbl, all the AdoCbl should be bound to the enzyme in some form in the steady state. Therefore, the concentration of enzyme that can turn over substrate (holoenzyme) will be equal to [AdoCbl], since AdoCbl is the limiting reagent. The rate of turnover will thus vary directly with [AdoCbl], so \( v / [\text{AdoCbl}] \) will be constant. In fact, however, \( v / [\text{AdoCbl}] \) varies in a hyperbolic fashion with [AdoCbl] (Fig. 5.27). By fitting this curve to the Michaelis-Menten equation, a \( K_m \) of 0.4±0.07 \( \mu \text{M} \) and a \( k_{\text{cat}} \) of 14±1 s\(^{-1}\) can be calculated. This is larger than the \( k_{\text{cat}} \) of 2 s\(^{-1}\) measured under conventional conditions ([AdoCbl] >> [RTPR]).

The optimal conditions for coupling RTPR to TR/TRR/NADPH when [RTPR] >> [AdoCbl] have yet to be determined. When [AdoCbl] < 1 \( \mu \text{M} \), the amounts of TR and TRR used are sufficient to give maximal rates. However, in preliminary results, when [AdoCbl] = 2 \( \mu \text{M} \), increasing [NADPH] from 100 \( \mu \text{M} \) to 150 \( \mu \text{M} \) increases the apparent turnover number (V/[AdoCbl]\(_0\)) from 12 to 20 s\(^{-1}\). The true turnover number under these conditions could thus be ≥20 s\(^{-1}\). It is clear, however, that this turnover number is greater than the turnover number (V/[E]\(_0\)) measured under conventional conditions ([AdoCbl] >> [RTPR]) (which, at [RTPR] = 2 \( \mu \text{M} \), did not increase when NADPH was increased from 65 to 250 \( \mu \text{M} \)).

It might seem counterintuitive that the \( k_{\text{cat}} \) measured when [RTPR] >> [AdoCbl] is larger than that measured under conventional conditions. This observation can be explained by the hypothesis that AdoCbl does dissociate from RTPR, with dissociation taking place after each nucleotide reduction event but before re-reduction of the enzyme by TR/TRR/NADPH. After dissociation, a molecule of AdoCbl can initiate another cycle of catalysis on an enzyme molecule that has already been reduced. This hypothesis posits that the rate-limiting step in substrate turnover when [RTPR] >> [AdoCbl] is dissociation of AdoCbl, and \( k_{\text{cat}} \) measures the dissociation of AdoCbl from RTPR, rather than subsequent steps.

The presteady state kinetics of NADPH consumption also support this mechanistic hypothesis. The model in Scheme 5.1 predicts that in the presteady state, there will be a lag in NADPH consumption, since before
Fig. 5.27. Dependence on [AdoCbl] of rate of turnover of ATP ([RTPR]:[AdoCbl] is ~10:1). Assay mixtures contained RTPR (0.1–0.75 μM), 1 mM dGTP, 1 mM ATP, TR/TRR/NADPH, and AdoCbl (0.6–4 μM). The data with [AdoCbl] = 2 μM was collected in the SF UV-vis spectrophotometer, mixing RTPR with AdoCbl in the presence of ATP, dGTP, and TR/TRR/NADPH. Other data was collected by hand-mixing. The data is fit to the Michaelis-Menten equation. Each data point represents a single trial.
nucleotide reduction, the enzyme is reduced and thus not a substrate for TR. The length of the lag phase should be comparable to the inverse of the rate constant for the slowest step preceding re-reduction of the enzyme (Laidler & Bunting, 1973). In this case, based on the experiments described above, the slowest step preceding re-reduction of the enzyme is expected to be dissociation of AdoCbl. The experimentally observed lag phase (Fig. 5.28) is ~125 ms when [AdoCbl]>>RTPR. The lag phase is consistent with dissociation of AdoCbl (~15-20 s⁻¹) being the slowest step before disulfide re-reduction. As expected, the transient phase of cob(II)alamin formation also occurs in the first ~100 ms (Fig. 5.29) when [AdoCbl]>>[RTPR], consistent with carbon-cobalt bond homolysis, a single nucleotide reduction, and a re-formation of the carbon-cobalt bond occurring before dissociation of AdoCbl. A longer lag phase of ~200 ms is observed when [RTPR]>>[AdoCbl] (Fig. 5.30). It is possible that a bimolecular step is partially rate-limiting in the pre-steady state under these conditions, since [AdoCbl] is only 2 μM and [RTPR] is 20 μM. Nonetheless, the lag phases are consistent with AdoCbl dissociation occurring at 5–10 s⁻¹.
Fig. 5.28. Consumption of NADPH (measured by SF UV-vis spectroscopy) on mixing RTPR (20 μM) with AdoCbl (25 μM) under $V_{\text{max}}$ conditions. RTPR (20 μM), in one syringe with dGTP (1 mM), ATP (1 mM), TR, and NADPH, was mixed with AdoCbl (25 μM) in a second syringe, which also contained dGTP (1 mM) and TRR. The trace is the average of three trials. The inset is the progress curve through 4 s. The main figure is an expansion of the boxed region in the inset. The dotted line shows the extrapolation of the linear steady state progress curve.
$y = -4.8321 + 38.237x \quad R^2 = 0.98671$
Fig. 5.29. Formation of cob(II)alamin (measured by SF UV-vis spectroscopy) on mixing RTPR (20 μM) with AdoCbl (25 μM) under $V_{\text{max}}$ conditions. Experimental conditions were the same as for Fig. 5.28. The trace is the average of four trials.
Fig. 5.30. Consumption of NADPH (measured by SF UV-vis spectroscopy) on mixing RTPR (20 μM) with AdoCbl (2 μM) under turnover conditions. RTPR (20 μM), in one syringe with dGTP (1 mM), ATP (1 mM), TR, and NADPH, was mixed with AdoCbl (4 μM) in a second syringe, which also contained dGTP (1 mM) and TRR. The trace is the average of four trials. The inset is the progress curve through 10 s. The main figure is an expansion of the boxed region in the inset. The dotted line shows the extrapolation of the steady state progress curve.
\[
\begin{align*}
y &= -3.9526 + 21.185x \\
R^2 &= 0.96499
\end{align*}
\]
Discussion

SF UV-vis Studies on the Turnover Reaction

SF UV-vis studies were carried out to determine the kinetics of cob(II)alamin formation during turnover. The ultimate goal of these studies is to interpret these kinetics in terms of individual steps in the mechanism. However, unlike the exchange reaction, in which the kinetics of cob(II)alamin formation are typically well described by a single exponential (Chapter 3), the kinetics of cob(II)alamin formation under turnover conditions are highly complex. When ATP is the substrate and dGTP is the allosteric effector, the time course up to 500 ms is not well fit by two exponentials. In all likelihood, there are multiple enzyme species binding AdoCbl or cob(II)alamin under these conditions. Scheme 5.1 shows two cob(II)alamin-bound species and two AdoCbl-bound species, and more could be included, depending on the order of binding of cofactor, effector, substrate, and product. Each interconversion of two of these species could in principle contribute another exponential term to the observed kinetics of cob(II)alamin (Johnson, 1992). Many different chemical steps are thus likely to contribute to the observed kinetics.

Qualitatively, however, the observed kinetics are consistent with the mechanism in Scheme 5.1. The rapid net formation of cob(II)alamin (Fig. 5.1), occurring with a $k_{\text{obs}} \sim 250 \text{ s}^{-1}$, can be interpreted as an initial carbon-cobalt bond homolysis before turnover, and the slower net carbon-cobalt bond re-formation, occurring with a $k_{\text{obs}} \sim 30 \text{ s}^{-1}$, can be interpreted as a re-formation of AdoCbl to remove radical intermediates when they are no longer in use. To test the mechanism in Scheme 5.1 further, a variety of SF UV-vis experiments were carried out. These included isotope effect studies using $[5^\prime-2^\text{H}_2]$-AdoCbl and $D_2O$, investigations of the [AdoCbl] dependence of the observed kinetics, and experiments on the effect of RTPR oxidation state on its ability to homolysize the carbon-cobalt bond.

SF UV-vis studies using $[5^\prime-2^\text{H}_2]$-AdoCbl and $D_2O$: Determination of Kinetic and Equilibrium Isotope Effects on Cob(II)alamin Formation under Turnover Conditions

Isotopic perturbations of the kinetics of cob(II)alamin formation under turnover conditions provide a useful means for testing the mechanism in
Scheme 5.1. The complexity of the kinetics observed during turnover presents difficulties for the interpretation of isotope effects. However, presteady state kinetic isotope effects on cob(II)alamin formation using [5'-2H2]-AdoCbl and D2O have been studied extensively in the absence of substrate (Chapter 3), conditions under which the kinetics are much simpler. In that case, isotope effects can be interpreted in terms of a mechanism in which carbon-cobalt bond homolysis and thiol radical are concerted, and both primary isotope effects on hydrogen abstraction and the low fractionation factor of the thiol group are taken into account. Using the experiments performed in the absence of substrate as a model, isotope effects observed under turnover conditions can be interpreted qualitatively to address the basic questions of whether a thiol radical is an intermediate in catalysis and whether it is formed in a stepwise or concerted fashion. These isotope effects also provide information about the identity of the rate-limiting step.

The equilibrium solvent isotope effects observed (Fig. 5.4) provide strong evidence that a thiol radical acts as an intermediate in catalysis. As previously discussed (Chapter 3), the observation that more cob(II)alamin is formed in the steady state in D2O than in H2O is consistent with the AdoCbl-bound thiol form of the enzyme being destabilized in D2O relative to the cob(II)alamin-bound thiol radical form, due to the low fractionation factor of the thiol group (Schowen and Schowen, 1982). However, the observed effect of 3.6-fold more cob(II)alamin in D2O with [5'-2H2]-AdoCbl than in the all-protonated case is larger than might be expected from the fractionation factor of a thiol, since a fractionation factor of ~0.5 would correspond to an equilibrium isotope effect of ~2.5. This observation is consistent with two isotope-sensitive equilibria affecting the amount of cob(II)alamin formed. One of these equilibria corresponds to the rapid net formation of cob(II)alamin (k+2/k-2 in Scheme 5.1), and the other corresponds to the slower net re-formation of AdoCbl (k+4/k-4 in Scheme 5.1). Both of these equilibria will be shifted toward formation of cob(II)alamin when the C408 thiol bears deuterium. The net effect of deuterated solvent on the amount of cob(II)alamin formed could thus be greater than the equilibrium isotope effect on either step individually. These data thus support the hypothesis that the two observed kinetic phases observed correspond to the net formation of an enzyme-based thiol radical coupled to carbon-cobalt bond
homolysis and the net regeneration of a thiol coupled to carbon-cobalt bond re-formation.

The observation of large kinetic isotope effects on both kinetic phases and of a large solvent isotope effect on the amount of cob(II)alamin is consistent with concerted carbon-cobalt bond cleavage and thyl radical formation. Similar isotope effects are observed in the absence of substrate, where quantitative analysis strongly supports a concerted mechanism. In this case, the quantitative analysis that might allow a concerted mechanism to be distinguished from a stepwise mechanism would require more information about mechanism and rate constants than is currently available. Qualitatively, however, the stepwise mechanism would be expected to give rise to smaller isotope effects, because the observed rate and equilibrium constants contain significant contributions from relatively isotope-insensitive steps. The sizable isotope effects observed thus provide a qualitative argument in favor of a concerted carbon-cobalt bond homolysis/thyl radical formation under turnover conditions.

The observation that the slow net re-formation of AdoCbl exhibits a kinetic isotope effect of ~2.5-fold with [5'-2H2]-AdoCbl suggests that carbon-cobalt bond re-formation is slower than the steps preceding it and puts a lower limit on the rate constant for the first turnover. As discussed in detail later in this chapter, carbon-cobalt bond re-formation is thought to occur after every turnover, as shown in Scheme 5.1. If the nucleotide reduction reaction itself were much slower than the net carbon-cobalt bond re-formation, no kinetic isotope effect would be observed, since nucleotide reduction would be rate-limiting in the overall process of carbon-cobalt bond re-formation. The observation of a kinetic isotope effect thus suggests that nucleotide reduction is either as fast as or faster than the second kinetic phase of the reaction (k_{+4}, k_{-4} in Scheme 5.1). If the first turnover precedes and is faster than carbon-cobalt bond re-formation, the rate constant for carbon-cobalt bond re-formation is a lower limit for the rate of the first turnover. Thus, for ATP and dGTP, the first turnover occurs with a rate constant of at least ~30 s⁻¹, and for CTP and dATP, the first turnover occurs with a rate constant of at least ~15 s⁻¹.
SF-UV vis studies on the Turnover Reaction: [AdoCbl]-Dependence of Cob(II)alamin Formation by wt RTPR

The relationship between the observed kinetics and individual steps in Scheme 5.1 was also investigated by studying how these kinetics of cob(II)alamin formation changed with varying [AdoCbl]. For these studies, CTP and dATP were used as substrate and allosteric effector, rather than ATP and dGTP. This substitution was made because with CTP and dATP present, the kinetics are simpler, being reasonably well fit to two exponentials (120–250 s\(^{-1}\) and \(\sim 15\) s\(^{-1}\), Figs. 5.6 and 5.8). It is possible that the relative rates of the different steps shown in Scheme 5.1 differ for different substrates and allosteric effectors. Rates of cob(II)alamin formation (Tamao and Blakley, 1973) and tritium washout from [5\(^{-3}\)H]-AdoCbl (Hogenkamp et al., 1968) have been shown to vary depending on the nucleotides used as allosteric effector and/or substrate. The complexity of the observed kinetics could be highly dependent on the relative rates of the steps in Scheme 5.1. If all the rate constants are of approximately equal magnitude, the observed kinetics will be very complex, as each step will contribute another exponential term to the kinetics (Johnson, 1992). If, however, one or two steps are much slower than the others, these steps will dominate the observed kinetics and make them relatively simple. Since the SF UV-vis kinetics are simpler than those observed with ATP and dGTP, and both initial formation of cob(II)alamin and the slower re-formation of AdoCbl are of sufficient magnitude to measure by absorbance changes, the conditions with CTP and dATP might be the best ones for future kinetic studies of the turnover reaction.

Even the reaction with CTP and dATP exhibits unexpected kinetic complications. The observation of two kinetic phases, corresponding to a rapid net carbon-cobalt bond homolysis and a slower net carbon-cobalt bond re-formation, is in qualitative agreement with the mechanism in Scheme 5.1. However, that mechanism predicts that both the rates and the amounts of cob(II)alamin formed will increasing with increasing [AdoCbl]. While the observed rate constant does increase with increasing [AdoCbl] (Fig. 5.6), the amount of cob(II)alamin formed first increases, then decreases with [AdoCbl] (Fig. 5.7). This is unexpected, since increasing [AdoCbl] would be expected to increase the fraction of enzyme in cob(II)alamin-bound states, pulling both
carbon-cobalt bond homolysis equilibria \((k_{+2}/k_{-2} \text{ and } k_{+4}/k_{-4})\) toward cob(II)alamin. One possible explanation for this is that AdoCbl can bind to the reduced form of the enzyme in a non-productive fashion, as described in Chapter 4 for the exchange reaction. In this case, increasing [AdoCbl] would saturate both the productive binding mode, leading to an increase in \(k_{\text{obs}}\) with [AdoCbl], and the non-productive binding mode, leading to a decrease in the amount of cob(II)alamin formed. The existence of an alternate, non-productive binding mode for AdoCbl would seem to be detrimental to physiological functioning of the enzyme. However, as shown in Fig. 5.7, the effects of this putative alternate binding mode are only significant for [AdoCbl]>30 \(\mu\)M, a concentration which is unlikely to be accessible in vivo (Davis et al., 1952, Kashket et al., 1962).

Another possibility is that the order of binding is not the one assumed in Scheme 5.1. Steady state kinetic experiments, the conventional means of determining the order of binding of substrates, have proven difficult to interpret, in part because the product is also an allosteric effector (Vitols et al., 1967). The order of binding is thus not yet known. If, for example, the order of binding is dATP, then AdoCbl, then CTP, RTPR bound to both dATP and AdoCbl could partition between binding CTP, which would lead to a transient maximum in cob(II)alamin formation, and homolytic cleavage of the carbon-cobalt bond in the absence of substrate (i.e., the exchange reaction), which would not lead to a transient maximum in cob(II)alamin formation. Thus, depending on the order of binding, the exchange reaction may begin to compete with turnover at high [AdoCbl], decreasing the size of the observed transient maximum in cob(II)alamin formation.

Qualitatively, the kinetics of the initial rapid carbon-cobalt bond homolysis are consistent with a fast but freely reversible initial carbon-cobalt bond homolysis. An approximate analysis (see Eq. 5.1 in the Results section) in which steps 1 and 2 (Scheme 5.1) are treated independently of subsequent steps suggests that the forward rate constant \((k_{+2})\) is \(~200 \text{ s}^{-1}\), and the reverse rate constant \((k_{-2})\) is \(~75 \text{ s}^{-1}\). These rate constants indicate that tritium washout associated with step 2 could in principle be significant, as carbon-cobalt bond re-formation might compete effectively with turnover. Taking the rate constant of net carbon-cobalt bond re-formation \((\sim15 \text{ s}^{-1})\) as a lower limit for the turnover rate constant, it appears that re-formation of the
carbon-cobalt bond, leading to tritium washout, might compete effectively with turnover.

While these results are consistent with the prediction of significant washout through step 2, they do not require it. An early step in nucleotide reduction, such as 3' hydrogen abstraction, may be fast, with a later step being rate limiting. In this case, tritium washout may not compete effectively with a fast step in nucleotide reduction, even though nucleotide reduction as a whole is slower. The presteady state tritium washout experiments that would allow investigation of these questions have not yet been carried out with CTP as substrate. However, rapid acid quench experiments with ATP as substrate (described below) suggest that an early step in catalysis is in fact fast, and that exchange through step 2 is in fact low.

The observation that $k_{obs}$ for the second phase of the reaction is independent of [AdoCbl]₀ (Fig. 5.8) is consistent with the cob(II)alamin/thiyl radical state being favored over the AdoCbl-bound state after turnover, so that carbon-cobalt bond re-formation is driven by release of product or cofactor. The argument for this depends on the principle that both the forward and reverse rate constants in an equilibrium contribute to the the rate of approach to equilibrium. In reactions requiring binding of a ligand, the contribution of the forward step to the overall rate of approach to equilibrium is in general dependent on the bimolecular step, and the contribution of the reverse rate constant is not. For example, Eq. 5.1 shows that for an equilibrium coupled to binding of a ligand, the contribution of the forward step (in this case, $k_{+2}$) to $k_{obs}$ is weighted by the fractional saturation of binding ($K_1[AdoCbl]_0/K_1[AdoCbl]_0+1$), while the contribution of the reverse step ($k_{-2}$ in Eq. 1) is independent of whether binding is saturated. Thus, the more the reverse step dominates the rate of approach to equilibrium, the less dependent $k_{obs}$ will be on the initial concentration of ligand.

The same principle applies to the approach to equilibrium of the second kinetic phase. In this case, while the contribution of $k_{+4}$ to $k_{obs}$ will be weighted by $k_{+2}$, $k_{-2}$, and $k_{+3}$ as well as by the fractional saturation of AdoCbl binding, the contribution of $k_{-4}$ will be independent of the fractional saturation of AdoCbl binding (assuming AdoCbl does not bind significantly to the oxidized form of the enzyme). Thus, if $k_{-4} >> k_{+4}$, $k_{obs}$ would be, as observed, insensitive to [AdoCbl]₀. However, if $k_{-4} >> k_{+4}$, the equilibrium in
step 4 lies toward cob(II)alamin. In this case, the net carbon-cobalt bond reformation would have to be driven by a step subsequent to step 4. Product release, which, under the experimental conditions, is approximately irreversible, might serve this purpose. This hypothesis implies that oxidation of RTPR impairs its ability to catalyze carbon-cobalt bond homolysis and re-formation, since \( k_{42} \) and \( k_{2} \) are estimated (from Eq. 5.1, Results) to be \( \sim 200 \text{ s}^{-1} \) and \( \sim 75 \text{ s}^{-1} \), and \( k_{44} \) and \( k_{4} \) are estimated to be \( << 15 \text{ s}^{-1} \) and \( 15 \text{ s}^{-1} \) (to account for a \( k_{\text{obs}} \) of \( \sim 15 \text{ s}^{-1} \) for the second kinetic phase).

So far, these kinetic experiments with CTP as substrate have raised more questions than they have answered. Nonetheless, the questions raised are important ones. The issues of commitment to catalysis for the thiyl radical and ability of the oxidized enzyme to catalyze carbon-cobalt bond cleavage will be addressed subsequently in this chapter in studies with ATP as substrate.

*SF UV-vis Studies on the Turnover Reaction: Formation of Cob(II)alamin by Pre-Oxidized RTPR*

The SF UV-vis kinetic experiments carried out on RTPR initially in the reduced state are consistent with the oxidation of the enzyme during the course of turnover affecting the rate of carbon-cobalt bond homolysis and re-formation (step 4, Scheme 5.1). To investigate this hypothesis further, SF UV-vis studies were undertaken using RTPR that was in the oxidized state after having carried out a single turnover. This pre-oxidized RTPR was mixed with AdoCbl and a reducing system (TR/TRR/NADPH or DTT) in the presence of ATP and dGTP or dGTP alone, and the rate of cob(II)alamin formation was measured.

When both ATP and dGTP are present, preliminary studies showed that \( \sim 0.04 \) eq. of cob(II)alamin are formed with a rate constant of \( 20 \text{ s}^{-1} \) after a lag phase of \( \sim 25 \text{ ms} \) (Fig. 5.10). One explanation for these results is that oxidized RTPR can bind AdoCbl in a mode that leads to slower and/or less thermodynamically favorable carbon-cobalt bond homolysis. Slow binding of AdoCbl to oxidized RTPR might explain the lag phase. These experimental results are thus consistent with a value for \( 20 \text{ s}^{-1} \) for \( k_{4} \), which would support the hypothesis that this rate constant dominates the rate of approach to equilibrium for the second kinetic phase (\( k_{\text{obs}} \sim 30 \text{ s}^{-1} \)). An alternative explanation for these results is that re-reduction of the enzyme
occurs at 20 s\(^{-1}\), and the cob(II)alamin formation represents reaction of reduced RTPR (step 2, Scheme 5.1). To help distinguish between these two alternatives, these experiments will have to be repeated using concentrations of dGTP and ATP identical to those used when RTPR and TR/TRR/NADPH were incubated together, and the reaction will have to be followed for a longer time to ensure that the true steady state is reached (i.e., cob(II)alamin formed is the same as when RTPR and TR/TRR/NADPH are initially in the same syringe).

Similar experiments were also carried out under exchange reaction conditions (i.e., in the absence of substrate) (Figs. 5.10 and 5.11). In this case, reaction of the oxidized enzyme with AdoCbl is negligible on the millisecond timescale, so any cob(II)alamin formed can be assumed to result from reduced enzyme. Thus, the observed rate of cob(II)alamin formation can be identified with the effective rate of RTPR reduction by the reducing system.

Regardless of the reducing system used, a lag time of \(~35\) ms is observed before cob(II)alamin formation begins. One possible explanation for this lag phase is that oxidized enzyme might need to undergo a conformational change before it can react with the reductant. Alternatively, the lag phase might represent TR or AdoCbl binding to oxidized RTPR. In earlier experiments, Tamao and Blakley observed no cob(II)alamin formation through 120 ms after mixing RTPR with dihydrolipoate and AdoCbl (Tamao and Blakley, 1973). It is possible that Tamao and Blakley's result is a manifestation of a longer lag phase than observed in these experiments. Clearly, the steady-state level of cob(II)alamin should be independent of the order of mixing, suggesting that cob(II)alamin formation would have been observed in the earlier study if longer times had been studied. The reason for the discrepancy in lag phases between our experiments and the experiments of Tamao and Blakley is unknown. It is possible that the oxidation of the enzyme in the course of turnover locks it into a different conformational state than would be observed in adventitiously oxidized enzyme, as used by Tamao and Blakley. Investigation of the dependence of the length of this lag phase on concentrations of reductant and AdoCbl might help determine the cause of the lag phase.
After the lag phase there is a slow formation of cob(II)alamin (k_{obs} = 4–5 s^{-1}) to the same steady state level observed with reduced enzyme. This kinetic phase is likely to represent re-reduction of the enzyme by the reducing system. If reduction of the enzyme is slow, but required for catalysis of carbon-cobalt bond homolysis, the rate of reduction will determine the rate of carbon-cobalt bond homolysis. It is interesting to note that the rate constant for cob(II)alamin formation under these conditions is similar to the steady-state turnover number (~2 s^{-1}). This is consistent with re-reduction of the enzyme being both required for rapid cob(II)alamin formation and at least partially rate-limiting in catalysis.

**Rapid Acid Quench Experiments Under Single Turnover Conditions: Fate of the 5'-Hydrogens and Rate of Single Turnover**

In order to obtain further information about the identity of the rate-limiting step in catalysis, the presteady state kinetics of ATP reduction were investigated using rapid acid quench techniques. These experiments support the hypothesis that nucleotide reduction is not the rate-limiting step in catalysis. The presteady state rate of turnover of ATP, at 55±10 s^{-1} (Fig. 5.13), is an order of magnitude greater than the rate constant for steady-state turnover (~2 s^{-1}). This result is remarkable, since it means that although the proposed mechanism for nucleotide reduction includes several steps expected to be thermodynamically uphill and/or slow, such as abstraction of the 3' hydrogen from the substrate by the thyl radical, this chemically difficult nucleotide reduction is not the slowest step in the overall reaction. This observation also confirms the prediction (derived from the isotope effect data) that turnover of ATP occurs with a rate constant of at least ~30 s^{-1}.

In the presteady state, 0.6–0.7 eq. of dATP are formed. This is consistent with the presteady state turnover corresponding to the first turnover, with concomitant oxidation of the active site cysteines C119 and C419. While the amount of product formed is less than 1 eq., this is in agreement with the amount of product observed in steady state single turnover experiments, in which ~0.75 eq. of product are formed by the C731/736S mutant, which cannot re-reduce the C119/C419 disulfide, and 1.5 eq. of product are formed by the wild-type enzyme (Booker et al., 1994). The observation of only a single turnover suggests that the second turnover, which requires re-reduction of the disulfide by C731 and C736, is slower than
the first, consistent with the hypothesis that disulfide re-reduction is rate-limiting in catalysis.

The observation that nucleotide reduction occurs faster than net carbon-cobalt bond re-formation is consistent with carbon-cobalt bond re-formation following the first turnover. To investigate this issue further, tritiated water release during turnover was measured. The ratio of tritiated water release to deoxynucleotide formation in the same experiment was calculated. If the carbon-cobalt bond must re-form after every turnover, each turnover will be accompanied by a transfer of hydrogen or tritium from 5´-dA to the thyl radical. However, due to the kinetic isotope effect and the statistical effect (because the extent of tritium labeling is so low, a 5´-dA molecule that has a tritium label in the methyl group will, on average, have only one tritium and two hydrogens), not every carbon-cobalt bond re-formation involving a tritiated AdoCbl molecule will result in tritiated water release. Assuming a selection effect against tritium abstraction of 10 and a statistical effect of 2 (Essenberg et al., 1971), a re-formation of the carbon-cobalt bond after every turnover should result in 1/20 of an equivalent of tritiated water released per deoxynucleotide produced. The observed ratio of 1/10 after 300 ms (Fig. 5.16) is thus consistent with carbon-cobalt bond re-formation occurring after each turnover.

However, according to Scheme 5.1, carbon-cobalt bond homolysis can also occur before turnover. In order to determine whether tritiated water release was occurring predominantly before or after turnover, the ratio of tritiated water release to product formation was measured as a function of time (Fig. 5.16). If most of the tritiated water detected were released prior to nucleotide reduction, this ratio would be expected to decrease with time, since the step responsible for most of the tritiated water release (k⁺₂, k₂ in Scheme 5.1) would essentially have come to equilibrium while product was still being formed. However, if most of the tritiated water were released after nucleotide reduction, the ratio would increase with time, since product formation would be essentially complete while the step responsible for most of the tritiated water release (k⁺₄, k₄ in Scheme 5.1) was still approaching equilibrium. The observation that this ratio increased with time is thus consistent with carbon-cobalt bond re-formation occurring after the first turnover. The observation of less 5´-dA than predicted based on SF UV-vis experiments suggests that some re-formation of the carbon-cobalt bond also
occurs as an artifact of quenching, as discussed below. However, the amount of tritiated water expected to be released (∼0.015 eq.) as a result of this artifact (based on a maximum 0.3 eq. of AdoCbl re-formed, at ∼20 ms, Fig. 5.18) is not significant for the purposes of this analysis.

This problem can also be analyzed in terms of the observed rate constant for tritium washout, obtained by fitting the tritium washout data to a single exponential (Fig. 5.15). Hydrogen/tritium abstraction by the thyl radical from 5′-dA is analogous to a branched pathway, since the thyl radical can abstract either hydrogen or tritium. Thus, as for a branched pathway, the observed rate constant for tritium washout represents not the rate constant for tritium abstraction alone, but rather the sum of rate constants for hydrogen abstraction and tritium abstraction (Espenson, 1981). Given the isotope effect and statistical effect against tritium abstraction, the rate constant for hydrogen abstraction is likely to be much larger than the rate constant for tritium abstraction. Therefore, perhaps counterintuitively, the observed rate constant for tritium washout will be dominated by the rate constant for re-abstraction of hydrogen, not tritium, by the thyl radical. The slower rate constant for tritium abstraction compared to hydrogen abstraction will manifest itself in the amount of tritium released.

Because the SF UV-vis experiments indicate that the rate of approach to equilibrium is much faster for step 2 than for step 4, the rate constant for tritiated water washout can be used to infer that most of the tritium washout observed occurs through step 4, rather than step 2. According to the analysis described above, the rate constant for tritium washout occurring through step 2 in Scheme 5.1 would be expected to be ∼10² s⁻¹, depending on what the reverse rate constant (k₂⁻) is under these conditions. The reverse rate constant when CTP and dATP are present is estimated to be ∼75 s⁻¹, and the reverse rate constant under the conditions used for the tritium washout experiment (ATP as substrate) is likely to be comparable, since kₖobs for the initial formation of cob(II)alamin is comparable for the two substrates (∼250 s⁻¹ for ATP, ∼150 s⁻¹ for CTP, based on kₖobs for the first kinetic phase). Similarly, the rate constant for tritium washout occurring through step 4 in Scheme 5.1 is no greater than 30 s⁻¹ for ATP (kₖobs for the second kinetic phase), depending on what the forward rate constant (k₄⁻) is. If, as discussed earlier for CTP turnover, product release is required to drive carbon-cobalt bond re-formation, k₄⁻ could in fact be much less than 30 s⁻¹. The measured
value of 10±2 s⁻¹ for the observed rate constant of tritium washout (Fig. 5.15) is thus more consistent with tritium washout occurring predominantly through step 4 (Scheme 5.1), rather than step 2. Since the amount of tritium released is consistent with one carbon-cobalt bond re-formation per turnover, and the rate constant for tritium release suggests that carbon-cobalt bond re-formation is much more likely to occur after turnover than before, the conclusion can be drawn that carbon-cobalt bond re-formation follows each turnover.

The observation that the rapid net carbon-cobalt bond homolysis (k⁺₂, k⁻₂ in Scheme 5.1) does not contribute the bulk of the tritiated water also implies that the commitment to catalysis of the thyl radical/cob(II)alamin intermediate is high. This result is in agreement with the relatively small V/K isotope effects observed on turnover with [3⁻²H]-NTPs (Stubbe et al, 1981, Ashley et al, 1986). Further investigation of the kinetics of cob(II)alamin formation and new investigations into presteady state tritium washout from [3⁻²H]-NTP might thus help put a lower limit on the rate of 3⁻ hydrogen abstraction from the substrate. Presteady state experiments investigating tritium washout from [3⁻²H]-NTPs might also help address this question.

**Possibility of Quenching Artifacts**

The possibility of quenching artifacts in this system deserves some comment. Experimentally, the most reproducible acid quench data in this system has come from products that are formed in an essentially irreversible fashion: deoxynucleotide product and tritiated water (since unlabeled water is present in tremendous excess). Reaction intermediates that are involved in reversible reactions, such as the thyl radical itself and 5'-dA appear to be subject to quenching artifacts. To examine intermediates formed reversibly in this system, alternative quenching agents must be investigated.

Artifacts of quenching have nonetheless led to an interesting result in this system. The observation of [¹⁴C] in the protein pellet, decreasing with a rate constant of 30 s⁻¹ (derived from a single exponential fit, Fig. 5.17), suggests that this [¹⁴C] labeling may measure the decline in the concentration of a catalytic intermediate. This rate constant is lower than that observed for turnover (55±10 s⁻¹, Fig. 5.13). However, since protein labeling is observed to increase rapidly, then decrease, at least two exponentials are required to
completely describe its kinetics. Because the scatter in the data at early time
points precludes precise measurement of the maximum $^{14}\text{C}$ labeling at
early times, it is not possible to obtain a precise double exponential fit. The
faster the rate of decline in $^{14}\text{C}$ labeling compared to the rate of the rapid
increase, the smaller the maximum $^{14}\text{C}$ labeling will be. Fitting the data to
a single exponential decrease assuming a starting value of 0.7 eq. may thus
underestimate the true value of the rate constant.

While the number of carbons in the label is not yet known, the
observed kinetics suggest that the species responsible for $^{14}\text{C}$ labeling of the
protein is generated rapidly, with at least 0.4 eq. formed within the first 5 ms,
assuming all the carbons in the substrate are maintained in the label. This
observation is consistent with the idea that an early step in nucleotide
reduction is much faster than re-formation of the carbon-cobalt bond $(k_2)$. If
$^{14}\text{C}$ labeling comes about through a substrate-based radical, the formation of
this radical must be essentially complete within the first ~10 ms of the
reaction. The kinetics of $^{14}\text{C}$ labeling of the protein are thus consistent with
the hypothesis that a high commitment to catalysis for the thiyI radical
formed in step 2.

*Rate of turnover in D$_2$O and with [5'-2H$_2$]-AdoCbl*

Preliminary studies on the steady-state rate of turnover in D$_2$O with
[5'-2H$_2$]-AdoCbl are also consistent with a high commitment to catalysis for
both bound AdoCbl and the thiyI radical intermediate in step 2 (Scheme 5.1).
Although the results (Fig. 5.19) are derived from a small number of initial
rates, a preliminary study of the [AdoCbl] dependence of the initial rate of
turnover suggests that there is no V/K deuterium isotope effect. This result
indicates that deuterium in the C408 thiol does not have a significant effect
on the partitioning between initial carbon-cobalt bond homolysis ($k_{+2}$) and
dissociation of AdoCbl from the enzyme, suggesting that the commitment to
catalysis for bound AdoCbl is high. In addition, it indicates that deuterium
in the cofactor does not have a significant effect on the partitioning of the
thiyI radical between re-formation of the carbon-cobalt bond ($k_2$) and
abstraction of the 3' hydrogen of the substrate, meaning that the
commitment to catalysis of the thiyI radical is high. Repetition and
continuation of these preliminary studies will be needed to test
these hypotheses.
SF UV-vis Studies on the Turnover Reaction: Order of Addition

Knowledge of the order of binding is important for the design of RFQ EPR experiments, as the order of mixing can affect the amount of intermediates that build up in the presteady state. However, the order of binding of substrate, effector, and AdoCbl to RTPR in the course of catalysis is still unknown. Since there are at least five species that must be bound, at least transiently, to the enzyme during catalysis (substrate, effector, product, AdoCbl, and reductant), and the product also serves as an allosteric effector, steady state kinetic experiments have so far proven difficult to interpret (Vitols et al., 1967), and design of appropriate steady state kinetic experiments for determining the order of binding would be difficult.

SF UV-vis experiments carried out in the absence of substrate (Chapter 3) indicate that AdoCbl binding to RTPR is rapid in the presence of allosteric effector, suggesting that the relative order of addition of effector and AdoCbl should not have a significant effect on the observed kinetics. In order to investigate the rate of substrate binding, the kinetics of cob(II)alamin formation observed when RTPR, dGTP, and ATP were mixed with AdoCbl were compared with the kinetics observed when RTPR and dGTP were mixed with ATP and AdoCbl (Fig. 5.20).

Incubation of RTPR and ATP in the same syringe before mixing increased both the observed rate of cob(II)alamin formation and the maximum amount of cob(II)alamin formed, consistent with binding of ATP being slow compared to carbon-cobalt bond homolysis. Thus, when RTPR is pre-incubated with ATP, cob(II)alamin can accumulate rapidly before slower cobalt-carbon bond re-formation occurs, leading to the observed burst (Fig. 5.20). However, when RTPR is mixed with ATP and AdoCbl simultaneously, binding of ATP partially limits the rate of cob(II)alamin formation (step 2, Scheme 5.1), making the \( k_{\text{obs}} \) for cob(II)alamin formation smaller. The decrease in \( k_{\text{obs}} \) for cob(II)alamin formation also makes the observed burst of cob(II)alamin formation smaller, since the net rate of carbon-cobalt bond homolysis is no longer much faster than the net rate carbon-cobalt bond re-formation. The decrease in \( k_{\text{obs}} \) for net re-formation of the carbon-cobalt bond is likely also to be due to substrate binding becoming partially rate-limiting. An investigation of the rate of cob(II)alamin formation as a function of [AdoCbl] may help clarify this issue.
In the experiments as performed, the higher final concentration (1 mM vs. 0.5 mM) of ATP in the experiment where RTPR and ATP were pre-incubated cannot be ruled out as a contributor to the differences in kinetics. Nonetheless, these results provide two guidelines for the design of RFQ EPR experiments. First, they suggest that for maximal formation of paramagnetic intermediates, RTPR should be incubated with substrate in the same syringe before mixing. This guideline may also be applicable to studies with mechanism-based inactivators. Second, they are a reminder that the enzyme should be saturated with the substrate (or inactivator) for most efficient processing of the substrate to an intermediate.

Attempts to Detect Substrate-Based Radicals formed by C419S RTPR

Since carbon-cobalt bond cleavage and substrate turnover are both rapid (rate constants ≥ 50 s⁻¹), large amounts of product can build up in the pre-steady state. This is a disadvantage for detecting substrate-based radicals with wt RTPR with the normal substrate, as these radicals would have a short lifetime. If reduction of intermediate radicals to form product could be hindered, however, the large rate constants for early steps in catalysis would be an advantage in detecting substrate-based radicals, as large concentrations of these radicals would build up.

One way to hinder the reduction of intermediate radicals is to mutate one of the active site cysteines that interacts with substrate to a serine (Booker et al., 1994). SF UV-vis studies indicated that a substrate-based radical might be formed in detectable concentrations when C419S is mixed with AdoCbl in the presence of dGTP and ATP. In SF UV-vis experiments, both the rate of cob(II)alamine formation and the maximum amount of cob(II)alamine formed are observed to be decreased for C419S relative to wt RTPR (∼10 s⁻¹ and ∼0.15 eq. vs. ∼200 s⁻¹ and 0.4-0.6 eq.) (Fig. 5.21). Presumably, this mutation affects the structure of the active site such that C408 is no longer in optimal position to participate in carbon-cobalt bond cleavage. These kinetics are consistent with the drastically reduced rates of tritium exchange from [5'-³H]-AdoCbl observed with this mutant (Chapter 3).

The observed rate constant for the first phase of cob(II)alamine formation in this reaction shows a temperature dependence and isotope effects (with [5'-²H₂]-AdoCbl) qualitatively similar to those observed with wt
RTPR. In addition, the maximum amount of cob(II)alamin formed is greater (by a factor of ~1.5) in the presence of [5'-2H2]-AdoCbl than in the presence of unlabeled AdoCbl. However, the rate of decline of cob(II)alamin does not exhibit a kinetic isotope effect, whereas a kinetic isotope effect ~2 is observed with wt RTPR. These observations suggest that while the formation of cob(II)alamin by C419S is mechanistically similar to that catalyzed by wt RTPR, the decline in A524 may be due to irreversible reaction with cysteine thiols, as observed with several mechanism-based inhibitors (D. Silva and C. Lawrence, unpublished results). This observation would be consistent with an irreversible reaction of a substrate-based radical at the active site preventing reformation of the carbon-cobalt bond, and rendering cob(II)alamin vulnerable to reaction with enzyme-bound thiols on the timescale of seconds.

RFQ EPR experiments show that at 250 ms, an intermediate is formed having an EPR spectrum with a prominent feature at g ~2 (Fig. 5.22). While background subtraction artifacts are a complicating factor for spin quantitation in this case, the radical has a spin concentration of ~0.1 eq. This EPR spectrum may represent a substrate-derived radical. However, the width of the feature at g ~2 (25 G from peak to trough) argues against the observed radical being coupled by exchange (Gerfen et al, 1996) or dipolar (Hamilton et al., 1972) interactions; such interactions would lead to much broader features (~100 G), even at a cobalt-organic radical distance of ~10 Å (Buettner and Coffman, 1977). On the other hand, features spaced ~30 G apart in the high field region of the spectrum are reminiscent of hyperfine features observed in the thyl radical intermediate (Chapter 2). These preliminary studies need to be repeated to determine whether further efforts are warranted. If so, RFQ EPR experiments could be carried out with C419S RTPR and [U-13C]-ATP to determine whether this EPR spectrum actually represents a substrate-based radical.

Attempts to Detect Substrate-Based Radicals formed by wt RTPR using Subliming TR, or wt RTPR with ara-ATP as Substrate

An attempt was also made to limit the delivery of reducing equivalents to substrate-based radical intermediates by using an excess of RTPR over TR. Under these conditions, delivery of external reducing equivalents would be slow after the first turnover. However, SF UV-vis and
RFQ EPR experiments carried out under these conditions gave results similar to those observed with larger amounts of TR. This is consistent with substrate binding and/or 3' hydrogen atom abstraction being inefficient for oxidized RTPR. The expected thyl radical is observed in the first turnover. After the first turnover, the steady state amount of cob(II)alamin is small enough that any new paramagnetic species formed might not be detectable. An alternate strategy might be to perform RFQ EPR experiments by mixing pre-oxidized RTPR with AdoCbl in the presence of substrate and allosteric effector. The amount of cob(II)alamin formed under these conditions is also small (~0.04 eq.), as discussed above. However, depending on the relative rate constants for the reaction, a sizable fraction of this might represent substrate-based radical intermediates. This would be consistent with the observation of an as-yet uncharacterized substrate-derived species when oxidized enzyme is incubated with substrate under turnover conditions (Booker et al., 1994).

The use of the substrate analog ara-ATP provided another potential means of observing substrate-based radicals. This compound is able to stimulate carbon-cobalt bond homolysis, although it is not reduced to a deoxynucleotide (Tamao and Blakley, 1973, Sando et al., 1975), presumably because its 2' hydroxyl group cannot interact with the active site residues necessary to protonate it and enhance its leaving group ability. SF UV-vis experiments were carried out to determine the concentration of cob(II)alamin-containing intermediates formed in the presteady state, in order to determine whether RFQ EPR studies would be practical. These experiments showed that ~0.1 eq. of cob(II)alamin was formed with a rate constant of 100 s^{-1} (Fig. 5.25). The second kinetic phase associated with turnover of the normal substrate is not observed in this case, presumably because the enzyme is never oxidized, so it does not assume the conformation leading to slow net re-formation of the carbon-cobalt bond. The observation of 0.1 eq. of cob(II)alamin in the steady state means that ~0.2 equivalents of unpaired spin will presumably be present. This would be enough to perform RFQ EPR experiments.

Whether these RFQ EPR experiments would be likely to trap a substrate based radical depends on the interesting and still unresolved question of whether RTPR acts to abstract the 3' hydrogen from ara-ATP. If 3' hydrogen atom abstraction requires energetic coupling to loss of the 2'
hydroxyl in order to proceed (Chapter 1), RTPR should not be able to catalyze 3' hydrogen atom abstraction with ara-ATP as a substrate. However, if deprotonation or development of partial negative charge at the 3' hydroxyl is sufficient to allow 3' hydrogen atom abstraction, ara-ATP could undergo 3' hydrogen atom abstraction. Further SF UV-vis and RFQ EPR studies with ara-ATP might not only allow trapping of a substrate-based radical but also provide insight into the mechanism of catalysis of 3' hydrogen abstraction.

*Effect of [13C]-ATP on the "Doublet Signal"*

Previous work pointed to the intermediate responsible for the "doublet" EPR spectrum as a possible substrate-based radical (Hamilton et al., 1972, Buettner and Coffman, 1977). To test this hypothesis, samples were prepared using both unlabeled and [U-13C]-ATP under conditions expected to generate the "doublet" spectrum (Fig. 5.26). While the spectrum observed differed significantly in several respects from the published spectrum for reasons that remain unclear, the spectra of isotopically labeled and unlabeled samples were essentially identical, indicating that the paramagnetic intermediate trapped under these conditions is not substrate-based.

The simulations of Buettner and Coffman (Buettner and Coffman, 1977) suggest that a species resembling the one observed is likely to be ~10 Å from cob(II)alamin. If C408 is 5.5-6Å from cob(II)alamin (Gerfen et al., 1996), C119 and C419 could be ~10 Å from cob(II)alamin. A disulfide radical anion derived from C119 and C419 and interacting with cob(II)alamin ~10 Å away might produce the observed signal. This hypothesis might be tested using [3-2H2-cys]-RTPR to determine whether the β hydrogen of a cysteine residue contributes to the observed hyperfine splitting. Thus far, however, there is no evidence that the doublet species is relevant to catalysis, so further exploration of this area may not be justified.

*AdoCbl* Dependence of the Rate of Turnover when [RTPR]>>[AdoCbl] and Preequilibrium Rate of NADPH Consumption

Comparison of the rates of the first turnover, tritium washout during the first turnover, and net re-formation of the carbon-cobalt bond provides strong evidence that the carbon-cobalt bond is re-formed after every turnover. This hypothesis suggests the possibility that AdoCbl may dissociate from RTPR after each turnover. A demonstration that AdoCbl
dissociates from RTPR after each turnover would serve as independent
evidence of carbon-cobalt bond re-formation after each turnover, since
dissociation of the cofactor cannot occur before carbon-cobalt bond re-
formation without destruction of the cofactor. Experiments on the steady
state kinetics of turnover when [RTPR] >> [AdoCbl] can address this question.
If AdoCbl does not dissociate from RTPR after turnover, the kinetic behavior
observed will be that of a holoenzyme complex of concentration equal to
[AdoCbl]. If AdoCbl does dissociate from RTPR after turnover, the
dissociation rate can become kinetically significant under these conditions,
and, in principle measurable. As described below, the kinetics observed
suggest that AdoCbl dissociates after each turnover.

Two unusual features of the kinetics of turnover (as measured by the
spectrophotometric coupled assay) when [RTPR] >> [AdoCbl] suggest that
AdoCbl dissociates after each turnover. First, the rate does not increase
linearly with [AdoCbl], as it would if [AdoCbl] were acting as a stoichiometric
reagent, but rather exhibits saturation behavior (Fig. 5.28). Second, the
turnover number (\( V_{\text{max}}/[A]_o \)) measured under these conditions is larger
(\( \sim 15-20 \text{ s}^{-1} \)) than the turnover number (\( V_{\text{max}}/[E]_o \)) measured when
[AdoCbl] >> [RTPR] (2 s\(^{-1} \)). When [RTPR] >> [AdoCbl], the system is behaving
as if there is more enzyme turning over substrate than there is AdoCbl, even
though AdoCbl is required for the reaction and present in limiting
quantities. This kinetic behavior is consistent with a mechanism in which
AdoCbl dissociates from oxidized RTPR after turnover and re-binds to a
second molecule of RTPR (in the reduced state), initiating a second turnover
faster than the first molecule of oxidized RTPR can be re-reduced.

Scheme 5.3 shows a highly simplified mechanism for substrate
turnover that takes into account both the possibility that AdoCbl dissociates
from RTPR after every turnover and the possibility that AdoCbl remains
bound to RTPR for multiple catalytic cycles. This mechanism was used to
obtain an estimate for what fraction of the total flux through the enzyme
goes through the pathway requiring dissociation of AdoCbl. In this
mechanism, \( k_{\text{on}}' \) and \( k_{\text{on}}'' \) represent the effective rate constants for binding
of substrate and effector or substrate alone, respectively. The rate constant \( k_1 \)
represents all steps that follow binding up to and including nucleotide
reduction. This includes carbon-cobalt bond homolysis and substrate
E = reduced enzyme
F = oxidized enzyme
A = AdoCbl
S = nucleotide substrate
P = deoxynucleotide product

Scheme 5.3
turnover. The rate constant $k_{\text{off}}$ represents the dissociation of AdoCbl from RTPR. The partitioning between the two alternate pathways is represented by $k_{\text{off}}/(k_2+k_{\text{off}})$. The rate constant $k_2$ represents re-reduction of the AdoCbl-bound form of the enzyme, while $k_2'$ represents re-reduction of free enzyme.

The mechanism in Scheme 5.3 can be used to analyze $V_{\text{max}}$ data. Allosteric effector is assumed to be bound throughout the catalytic cycle. It is also assumed that binding of substrate and AdoCbl is much faster than re-reduction of the enzyme ($k_{\text{on}}, k_{\text{on}'}' >> k_2$). This is reasonable, since SF UV-vis and rapid acid quench experiments suggest that turnover and the steps that precede it are much faster than the steps that follow it. While ATP binding is partially rate-limiting in cob(II)alamin formation, as described above, it is still much faster ($k_{\text{obs}}$ $\sim$ 70 s$^{-1}$) than the steady state turnover rate.

To derive an expression for the initial rate, the approximation is made that [RTPR] $\gg$ [AdoCbl] $\gg$ $K_m$, AdoCbl. This approximation is warranted, as [RTPR] = 10 $\mu$M, [AdoCbl] = 1 $\mu$M, and $K_m$, AdoCbl = 0.2 $\mu$M. Thus, in Scheme 5.3, [ESA] + [FA] + [EA] $=$ [A]$_0$. Because the rate is measured spectrophotometrically, the observed rate is given by $v = k_2[\text{FA}] + k_2'[\text{F}]$. By applying the steady-state approximation for intermediates, expressions for [FA] and [F] in terms of [A]$_0$ can be obtained. Using these expressions, the assumption that $k_{\text{on}}, k_{\text{on}'}' >> k_2$, the expression

$$V_{\text{max}} = \frac{k_1(k_2 + k_{\text{off}})[A]_0}{k_1 + k_2 + k_{\text{off}}}$$

Eq. 5.2

is obtained. Assuming disulfide re-reduction (or a conformational change associated with it) to be rate-limiting in steady state turnover, the value for $k_2$ can be taken as the value for $k_{\text{cat}}$ when [AdoCbl] $\gg$ [RTPR], which is 2 s$^{-1}$. Rapid acid quench and SF UV-vis studies suggest that 55 s$^{-1}$ is a reasonable estimate for $k_1$, since $k_1$ includes both carbon-cobalt bond homolysis ($k_{\text{obs}}$ $\sim$ 250 s$^{-1}$) and substrate turnover ($k_{\text{obs}}$ = 55±10 s$^{-1}$). A lower limit for $k_{\text{off}}$ of 17–30 s$^{-1}$ can thus be calculated from the experimental values for [A]$_0$ and $V_{\text{max}}$. This corresponds to a partitioning of at least 85–95% of the total flux through the pathway in which AdoCbl dissociates after the first turnover.
This analysis is in accord with the length of the lag in NADPH consumption measured when [AdoCbl]>>[RTPR]. This lag phase is ~125 ms (Fig. 5.29), which is consistent with a k_{off} of ~10 s^{-1} if dissociation is the slowest step occurring before disulfide re-reduction. Quantitative interpretation of the length of the lag phase is complex, however, as K_d for AdoCbl may be greater than K_m. Thus, while all the AdoCbl may be bound to the enzyme in the steady state, since [AdoCbl]>>K_m, if K_d ~ 20 μM, all the AdoCbl will not be bound to the enzyme in the presteady state. In this case, the rate of presteady state steps will not be at a maximum, although the steady state rate is saturated. This may account for the longer lag time (~200 ms) observed when [AdoCbl] is 2 μM (Fig. 5.31). A rigorous quantitative interpretation of the presteady state kinetics of NADPH consumption would require examination of the length of the lag phase as a function of [AdoCbl].

The experiments on NADPH consumption when [RTPR]>>[AdoCbl] thus serves as independent evidence that carbon-cobalt bond re-formation follows each turnover. The lower limit of 85% for the average probability of dissociation of AdoCbl after turnover is also a lower limit for the average probability of carbon-cobalt bond re-formation after turnover. The most parsimonious hypothesis is that carbon-cobalt bond re-formation and AdoCbl dissociation take place, on average, after each turnover.

These experiments also furnish independent support for the hypothesis that nucleotide reduction is not rate-limiting in catalysis. Eq. 5.2 shows that the observed rate constant for steady state turnover when [RTPR]>>[AdoCbl] provides a lower limit for k_1, the rate constant that includes nucleotide reduction. That is, as k_1 approaches 0, the expression for V_{max} reduces to V_{max} = k_1[A]_o. These experiments thus put a lower limit of 15–20 s^{-1} on the single turnover rate, consistent with rapid acid quench experiments that directly measure the rate of turnover as 55±10 s^{-1} when [AdoCbl] and [RTPR] are ~50 μM.

The ability of RTPR to release AdoCbl after a single turnover may also have implications for physiology. These experiments suggest that when AdoCbl is only present in low quantities in the cell, the rate of nucleotide reduction can be maximized by increasing the concentration of RTPR, so that each molecule of AdoCbl can initiate catalysis on multiple molecules of RTPR. This may explain the seemingly paradoxical observation of Goulian and Beck (Goulian and Beck, 1966) that depriving L. leichmannii of vitamin
B_{12} de-represses RTPR biosynthesis. If RTPR bound AdoCbl over the course of multiple turnovers, there would be no advantage to synthesizing RTPR in excess over AdoCbl, as only the holoenzyme would be active. Of course, this assumes that the rate-limiting step in catalysis is the same under physiological conditions. The presence of the endogenous reducing system may make re-reduction of RTPR faster in vivo than observed with the *E. coli* reducing system used in vitro. The kinetics observed in vitro nonetheless suggest that the unusually high expression of ribonucleotide reductase in *L. leichmannii* is a way for the organism to make most efficient use of a scarce nutrient.
References


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