Cloning, Sequencing, Expression, and Characterization of the Adenosylcobalamin-Dependent Ribonucleotide Reductase from *Lactobacillus leichmannii*

by

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To my grandmother, Cleona Price,

with love and appreciation

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ABSTRACT

The ribonucleotide reductases (RNRs) catalyze the rate limiting step in DNA biosynthesis, the conversion of ribonucleotides to 2'-deoxyribonucleotides. Four classes of RNRs are currently acknowledged, with each class differing in cofactor requirement. The two best-characterized reductases to date are the differic iron center-tyrosyl radical-dependent reductase isolated from *Escherichia coli* (RDPR), and the adenosylcobalamin-dependent reductase isolated from *Lactobacillus leichmannii* (RTPR). Although these two enzymes differ in the cofactor used to effect nucleotide reduction, numerous studies in our laboratory have suggested that their mechanisms of catalysis are astonishingly similar. In the work reported herein, the RNR from *L. leichmannii* was cloned, and its DNA and protein sequences determined. In addition, the protein was hyperexpressed in *E. coli* under the control of the *tac* promoter. The enzyme has been purified to ≥95% homogeneity, yielding ~90 mg of protein from 2.5 g of the recombinant *E. coli*. Although the amino acid sequence of RTPR displays no statistical homology with that of RDPR, a thorough review of the sequence by eye resulted in the discovery of 4 cysteines which lay in sequence motifs similar to those of cysteines previously found to be important in catalysis in RDPR. An additional cysteine was implicated from early biochemical studies as being important in catalysis. These five cysteines, as well as a control cysteine, were mutated to serines, and the effect of the resulting mutant proteins on nucleotide reduction was analyzed. Two of the mutant proteins were unable to catalyze nucleotide reduction, but produced similar products to the wild-type enzyme when incubated with the mechanism-based inhibitor 2'-chloro-2'-deoxyuridine 5'-triphosphate. One mutant protein produced no product at all. The mutated cysteines from these proteins were assigned to the active site of RTPR. Two additional cysteines were inactive in the presence of the *in vivo* reductant, the protein thioredoxin, but catalyzed nucleotide reduction at the same rate as wild-type RTPR when DTT, a small organic dithiol, was used as the reductant. These cysteines were thus assigned the role of a redox shuttle. Their function in the cell is to deliver reducing equivalents from thioredoxin into the active site disulfide formed concomitant with nucleotide reduction.

Thesis Supervisor: Professor JoAnne Stubbe
Title: John C. Sheehan Professor of Chemistry and Biology

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was a classic in and of himself, and an exceptional scientist. His studies on the *E. coli* enzyme paved the way for the mutagenesis studies on the *L. leichmannii* enzyme.

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Ave in Chelsea is an experience that I'll never forget. I wish him a speedy recovery, and continued success in finishing his studies here at MIT.

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Abbreviations

aa	amino acid
AdoCbl	5'-deoxyadenosylcobalamin
$B_{12}r$	cob(II)alamin
bp	base pair
Bq	becquerel
BSA	bovine serum albumin
cDNA	complementary DNA
Ci	curie
CIP	alkaline phosphatase from calf intestine
dA	5'-deoxyadenosine
Da	Daltons
dC	deoxycytidine
DE-52	diethylaminoethyl cellulose from Whatman
ds	double-stranded
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EPR	electron paramagnetic resonance
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HPLC	high pressure liquid chromatography
IPTG	isopropyl β-D-thiogalactoside
kb	kilo base pairs
K_m	Michaelis constant
Lac	lactose
LB	Luria-Bertani broth
LCM	*Lactobacillus* carrying media

mRNA	messenger RNA
MW	molecular weight
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NMR	nuclear magnetic resonance
OAc	acetate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethanesulfonyl fluoride
PNK	T4 polynucleotide kinase
PVDF	poly[(vinylidene difluoride)]
RNase H	ribonuclease H
RDPR	ribonucleoside diphosphate reductase from *E. coli*
RTPR	ribonucleoside triphosphate reductase from *L. leichmannii*
SDS	sodium dodecylsulfate
TAE	tris-acetate/EDTA
TBE	tris-borate/EDTA
TE	tris-EDTA, pH 8
TEA	triethylamine
TEAB	triethylammonium bicarbonate
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
V_{max}	maximal velocity
Wt	wild-type
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

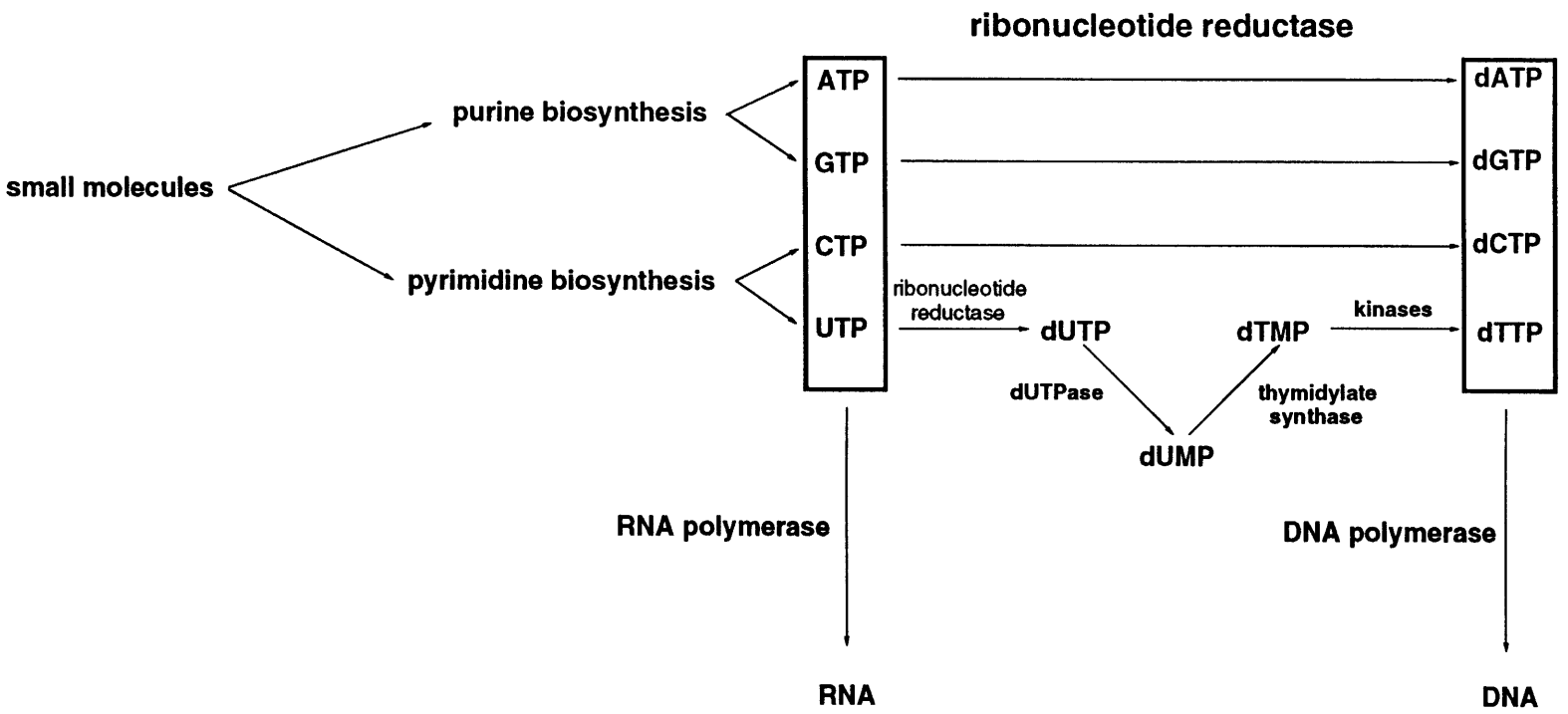
Chapter 1:

The Ribonucleotide Reductases: Radical Enzymes Employing Radically Differenct Cofactors.

The *de novo* biosynthesis of DNA is a complex process involving several metabolic pathways and many different enzymes (Figure 1.1). One well known enzyme in this overall process is DNA polymerase. Using the parent strands as templates, this enzyme constructs the nascent DNA polymer from four monomer units, dATP, dTTP, dCTP, and dGTP, with the concomitant release of pyrophosphate (PPi) upon the addition of each monomer. The action of this enzyme is understandably dependent upon the presence of the monomer units, or deoxyribonucleoside triphosphates (dNTPs); however, the fidelity of this polymerization reaction, and hence the unflawed transmittance of genetic information during cell division, is dependent not only upon the presence of the four dNTPs, but upon a well-regulated balance of each (Reichard, 1988). Several enzymes participate in the fine tuning of the cellular pools of dNTPs; however, in all self sufficient organisms, as well as some viruses and phages, nature has invented one and only one mechanism for the formation and regulation of the dNTPs necessary for DNA biosynthesis. The enzyme that governs these processes is ribonucleotide reductase.

The ribonucleotide reductases (RNRs) constitute a unique class of metalloenzymes which catalyze the reduction, at a single active site, of all four ribonucleotides to the corresponding 2'-deoxyribonucleotides (Thelander & Reichard, 1979; Lammers & Follmann, 1983; Stubbe, 1990b). This transformation is the first committed step in DNA biosynthesis, and as a consequence of being at such a critical juncture in metabolism, RNRs are under very rigid regulation. Indeed, several studies have shown that RNRs can be controlled at the transcriptional level (Tuggle & Fuchs, 1986), the translational level (Noronha et al., 1972; Standart et al., 1985), at the level of cofactor synthesis and destruction (Barlow et al., 1983), and also by an astonishing network of allosteric interactions

Figure 1.1: Pathway for the *de novo* biosynthesis of DNA in *L. leichmannii*.


ribonucleotide reductase
small molecules
purine biosynthesis
pyrimidine biosynthesis
ATP
GTP
CTP
UTP
dATP
dGTP
dCTP
dTTP
ribonucleotide reductase
dUTP
dTMP
kinases
dUTPase
thymidylate synthase
dUMP
RNA polymerase
DNA polymerase
RNA
DNA

(Singh et al., 1977; Eriksson & Sjöberg, 1989; Reichard, 1993b). Because of their key role in producing the dNTPs for DNA biosynthesis, as well as regulating the only pathway that the cell has for making these dNTPs, it might be presumed that the reductases are extremely conserved. Surprisingly however, in contrast to most other enzymes which govern key metabolic processes, the RNRs are not conserved at all. This lack of homology applies to the level of the quarternary structure of the enzymes, the cofactors employed in effecting substrate turnover, and as will be shown in Chapter 2, in primary sequence (Stubbe, 1990a; Booker & Stubbe, 1993; Reichard, 1993b).

The study of ribonucleotide reductases has developed into quite an expansive area of biological science. Many scientists are interested in the enzyme from an evolutionary standpoint, as it is presumed to be the major player in the transformation from an RNA-based world to a DNA-based world (Benner et al., 1989). There are those who are interested in the enzyme from a therapeutic standpoint, as a target for antineoplastic and antiviral agents (Stubbe, 1990b). Still, some researchers are interested in RNR as a model for allosteric regulation while others want to understand its role in the regulation of the cell cycle. Lastly, there are those scientists who are driven by its mode of catalysis. Although the net transformation is ostensibly simple - the replacement of a hydroxyl group with a hydrogen - the mechanism by which this is carried out is deceivingly complicated (Ashley & Stubbe, 1985; Stubbe, 1990a). This introduction seeks to provide an overview of the ribonucleotide reductase class of enzymes. However, because of the broad scope of the subject, it will focus primarily on the structural and mechanistic aspects of the enzymes, describing the initial characterization of ribonucleotide reductase activity, the classes of RNR, and the mechanistic similarities of the two RNRs that have been extensively studied.

Characterization of Ribonucleotide Reductase Activity

Studies in the early 1950s by Hammarsten, Reichard, and Saluste, suggested that ribonucleotides were the precursors to deoxyribonucleotides (Hammarsten et al., 1950). Using whole rats in combination with ^{15}N labeled cytidine and uridine, these researchers showed that the isolated DNA contained significant amounts of labeled cytosine and uridine. Earlier experiments by Bendich, Getler, and Brown, revealed that the free base cytosine could not be used for the synthesis of DNA (Bendich et al., 1949), and studies by Irwin Rose showed that cytidine labeled in both the base and sugar did not change in specific activity upon its incorporation into DNA (Rose & Schweigert, 1953). The results of these investigations in combination with experiments that demonstrated that RNA is not the direct precursor to DNA (Abrams, 1950), led to the advancement of the hypothesis that deoxyribonucleotides were synthesized directly from ribonucleotide precursors, and not from a free base and a deoxyribose sugar. Relatively soon after, several laboratories were able to show evidence in cell free extracts from several sources that deoxycytidine phosphates could be formed from cytidine 5'-phosphate (Abrams et al., 1960; Moore & Hurlbert, 1960; Reichard, 1961). The most extensive characterization of this enzymatic activity came from studies in Sweden in the laboratory of Professor Peter Reichard. Working in *E. coli*, he isolated two protein fractions which were able to convert cytidine monophosphate (CMP) to deoxycytidine diphosphate (dCDP) (Reichard, 1962). Fraction A was ideintified to be CMP kinase, a previously characterized enzyme which phosphorylates CMP to cytidine diphosphate (CDP). Fraction B, which could be fractionated further into two proteins, B1 and B2, required the presence of fraction A for activity when CMP

was used as substrate, but turnover could be achieved in the absence of fraction A when CDP was used as the substrate.

Studies of nucleotide metabolism in the bacterium *Lactobacillus leichmannii* suggested that a cofactor is required for deoxynucleotide production. Acting on results from E.E. Snell's laboratory at the University of Wisconsin, which showed that *L. leichmannii* needed vitamin B_{12} for growth in the absence of deoxyribonucleosides (Kitay et al., 1950), R.L. Blakley and H.A. Barker (1964) demonstrated that cell-free extracts of this bacterium synthesized deoxyribosides from the corresponding riboside only in the presence of vitamin B_{12}. Subsequent studies by Blakley (1965) as well as Beck and Hardy (1965) led to the identificaion of 5'-deoxyadenosylcobalamin, a derivative of vitamin B_{12}, as the active cofactor in the *L. leichmannii* reductase. Meanwhile, experiments in the laboratory of P. Reichard demonstrated rather convincingly that the reductase isolated from *E. coli* was not cobamide dependent (Moore & Reichard, 1963). In fact, the small subunit, B2, was shown to contain two atoms of non-heme iron per mol of protein, the removal of which resulted in loss of enzyme activity (Brown et al., 1969). No labile sulfide was detected upon removal of the irons, suggesting that the iron was not present as an Fe/S cluster. The UV-vis spectrum of protein B2 displayed a very broad feature at 360 nm, a very steep shoulder at 325 nm, and a very sharp peak at 410 nm. The removal of iron from protein B2 resulted in the loss of these features; the reconstituion of protein B2 with Fe^{2+} resulted in the reappearance of these features (Brown et al., 1969). Subsequent electron spin resonance (EPR) studies of protein B2 suggested that it also contained an organic free radical, the presence of which was dependent upon the formation of the diiron center (Ehrenberg & Reichard, 1972). In seminal experiments, EPR spectroscopy was used in combination with protein B2 that had been expressed in the presence of specifically deuterated amino acids to show that this organic

radical resided on a tyrosine residue (Sjöberg et al., 1977). This was the first example of a stable protein radical that played an essential role in an enzymatic reaction.

As uncommon as it is for enzymes that play key roles in metabolism to employ more than one cofactor, nature has devised at least three, and perhaps four different classes of RNRs, each employing a unique cofactor for catalysis. As will be discussed subsequently, a ribonucleotide reductase has been isolated from *E. coli* grown under anaerobic conditions, that is distinct from the *E. coli* reductase discussed above (Fontecave et al., 1989; Eliasson et al., 1990; Eliasson et al., 1992). In addition, a RNR isolated from *Brevibacterium ammoniagenes* appears to require manganese and not iron for catalytic activity (Willing et al., 1988b; Willing et al., 1988a).

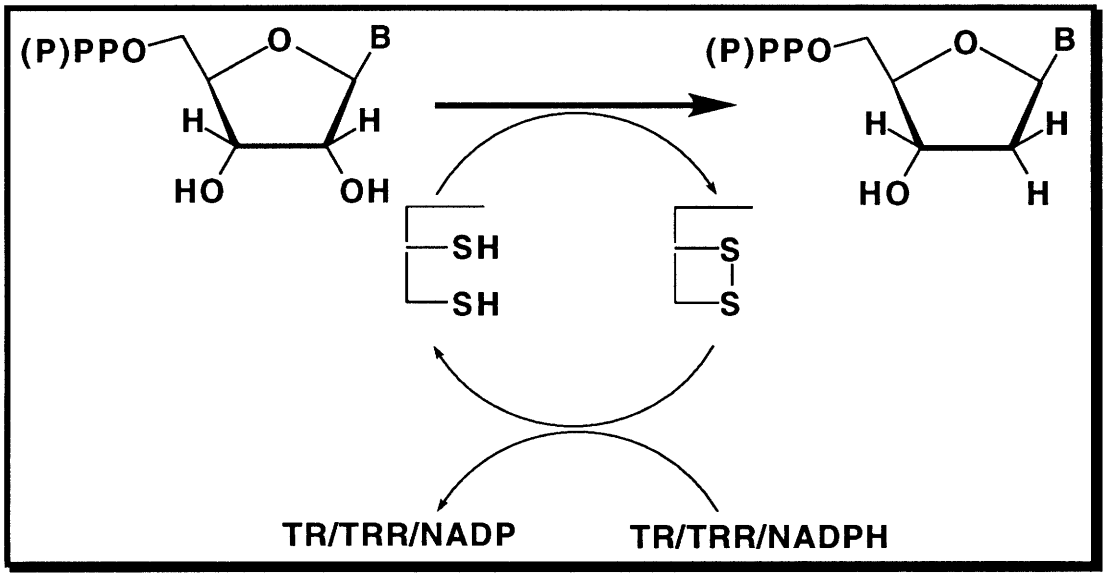
Classes of Ribonucleotide Reductase

E. coli Ribonucleotide Reductase

The RNR isolated from *E. coli* (EC 1.17.4.1) is by far the best characterized of all the reductases - especially with respect to its structure and modes of regulation. Although this is partly due to the novelty of this enzyme harboring a stable organic radical, the fact that it is the prototype for reductases isolated from viruses as well as mammalian systems has also intensified its study for medicinal reasons (Stubbe, 1990b). This enzyme acts on nucleoside substrates that are diphosphorylated, giving rise to the name ribonucleoside diphosphate reductase, or simply RDPR. The newly-generated dNDPs are then phosphorylated to the corresponding dNTP by nucleoside diphosphate kinase before being incorporated into DNA via an appropriate polymerase. As mentioned previously, RDPR is composed of two subunits, B1 and B2, which are now universally known as R1 and R2. In the holoenzyme both of these subunits are

dimeric, yielding a putative overall tetrameric $\alpha_2\beta_2$ quarternary structure (Thelander & Reichard, 1979; Eriksson & Sjöberg, 1989; Stubbe, 1990b). The R1 subunit is the larger of the two, having a monomeric molecular weight of 86 kDa. It contains the binding site for NDP substrates, as well as binding sites for NTP and dNTP allosteric effectors. Also, the R1 subunit contains catalytically important cysteine residues which become oxidized concomitant with substrate reduction (Thelander, 1974). In order to achieve multiple turnovers, the resulting disulfide bond must be rereduced. This can be achieved *in vitro* with small dithiols such as dithiothreitol (DTT) or dihydrolipoic acid (DHL), albeit at concentrations around 25-30 mM. *In vivo* however, these cysteines are reduced by a low molecular weight (12 kDa) protein, thioredoxin (TR), which derives its reducing equivalents from thioredoxin reductase (TRR). TRR is a flavin-containing protein which in turn obtains its reducing equivalents from the oxidation of NADPH. This process is summarized in Scheme 1.1. The

Scheme 1.1: The reaction catalyzed by ribonucleotide reductase



viability of *E. coli* mutants deficient in thioredoxin activity led to the discovery of glutaredoxin as a hydrogen donor for RDPR (Holmgren, 1985). Glutaredoxin derives its reducing equivalents ultimately from NADPH as well, via a system coupled to glutathione reductase and glutathione.

The R1 subunit contains two different binding sites which regulate the activity and substrate specificity of the enzymes (Eriksson & Sjöberg, 1989). The high affinity site - named for its strong affinity for the allosteric effectors as compared to the low affinity site - interacts with various NTPs and dNTPs to determine which particular NDP will be turned over. The method of actual allosteric regulation is complex, however a simple scenario is described below. When ATP (or dATP at low concentrations) is bound the enzyme reduces CDP and UDP. When dGTP is bound, the enzyme reduces ADP; and when dTTP is bound, GDP is reduced. The low affinity site binds either ATP or dATP. When ATP is bound to this site, the activity of the enzyme is enhanced. When dATP is bound to this site, the enzyme is turned off. These interactions act in concert to maintain a balanced level of the deoxynucleotides needed for DNA biosynthesis.

Although the R1 subunit of the *E. coli* reductase appears to be the business end of the enzyme, catalysis cannot occur without the second subunit, R2. The R2 subunit of the *E. coli* reductase is indeed a novel protein. Early spectroscopic studies suggested that the protein contains two high-spin Fe(III) atoms that are antiferromagnetically-coupled through a μ-oxo bridge (Bunker et al., 1987; Scarrow et al., 1987; Sjöberg et al., 1987; Backes et al., 1989). This fully-assembled iron center is a necessary requirement for the maintenance of the tyrosyl radical, which in turn is required for catalysis. This tyrosyl radical has a half-life that is on the order of hours at room temperature, and years at -80°C. Based on an alignment of sequences of the R2 protein from several diiron-requiring

reductases, as well as the use of the mutant protein Y122F-R2, the tyrosyl radical was assigned to tyrosine 122 of the *E. coli* RDPR (Sjöberg et al., 1977). Very recent work by Nordlund and Eklund has resulted in the solving of the 3-dimensional structure of the R2 protein, and the diiron cofactor (Scheme 1.2) (Nordlund et al., 1990; Nordlund & Eklund, 1993). The two proteins of R2 fit together in an arrangement which makes the dimeric protein appear heart-shaped. Each monomeric subunit, contains 1 iron center and potentially 1 tyrosyl radical, which are both situated deep within each protein. The tyrosyl radical is 5.3 Å from the nearest Fe(III) atom and 10 Å away from the nearest surface of the R2 protein. Each Fe(III) atom is attached to the protein via histidine and carboxylate ligands, and in addition to the μ-oxo bridge, the carboxylate from glutamate 115 also bridges the two irons which are 3.3 Å apart (Scheme 1.2).

Lactobacillus leichmannii Ribonucleotide Reductase

Although the enzyme isolated from *E. coli* is the best characterized reductase to date, a resurgence in interest of the enzyme isolated from *L. leichmannii* is taking place. This is no doubt due in large part to the fact that the *L. leichmannii* reductase (EC 1.17.4.2) is structurally the simplest of all of the known reductases, as well as the desire among scientists to explore the function and catalytic capabilities of nature's only proven organometallic cofactor, 5'-deoxyadenosylcobalamin (AdoCbl). This cofactor is composed of a cobalt-containing corrin ring, a dimethylbenzimidazole group, and a 5'-deoxyadenosine moiety coordinated to the cobalt via a unique Co(III)-carbon bond (Figure 1.2). The cobalt is held in place by four coordinating nitrogen atoms provided by the corrin macrocycle. The nitrogen of the dimethylbenzimidazole group provides the proximal ligand to the octahedral Co(III) atom, while the distal ligand is provided by the 5' carbon of 5'-deoxyadenosine.

Scheme 1.2: X-ray structure of the diferric iron center-tyrosyl radical cofactor of the R2 subunit of RDPR. Adapted from Nordlund *et al.* (1990).

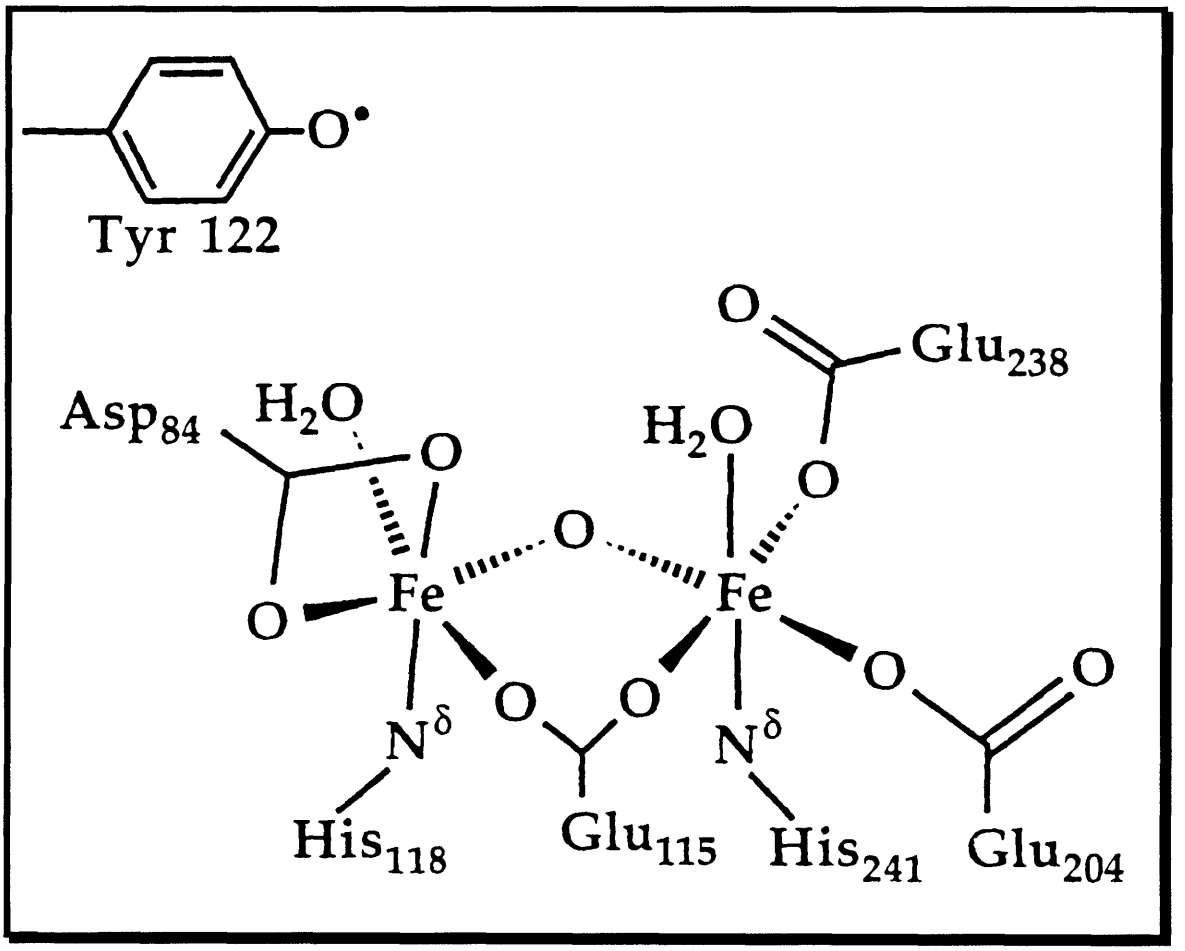
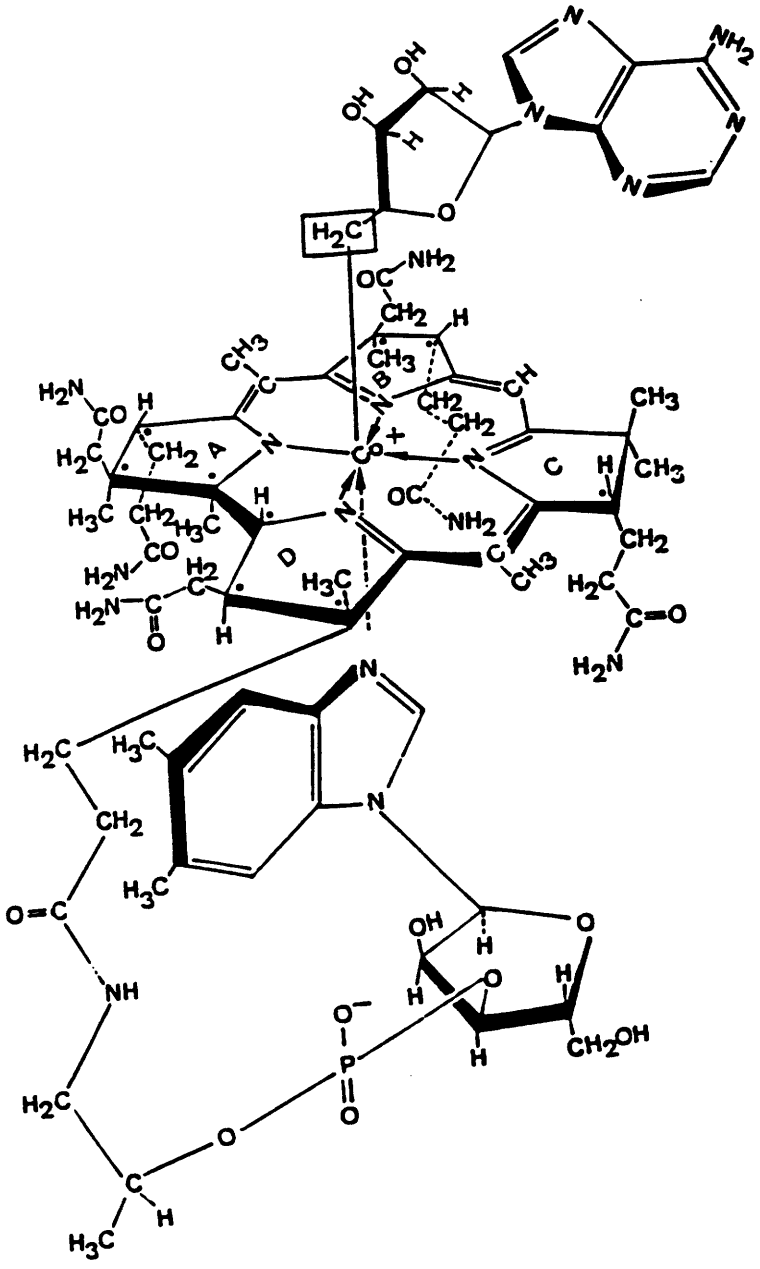


Figure 1.2: Structure of coenzyme B_{12}. The 5'-methylene carbon of 5'-deoxyadenosine is surrounded by a rectangle, as it is the carbon which forms the unique organometallic bond in the cofactor. The four pyrroline rings are labeled A, B, C, and D, and the chiral centers of the corrin macrocycle are denoted with asterisks. Figure adapted from **Vitamin B_{12}** (Zagalak & Friedrich, 1979).



The reductase from *L. leichmannii* catalyzes the reduction of ribonucleotides that are triphosphorylated, giving rise to the name ribonucleoside triphosphate reductase (RTPR). This enzyme is a single polypeptide of M_r = 82,000 (Panagou et al., 1972; Booker & Stubbe, 1993). Analogously to the *E. coli* reductase, RTPR is also allosterically regulated (Beck, 1967). Moreover, the pattern of allosteric regulation is similar to that of the *E. coli* reductase; CTP reduction is stimulated by dATP, UTP reduction by dCTP, ATP reduction by dGTP, and GTP reduction by dTTP (Beck, 1967). This elaborate array of allosteric regulation can be abrogated in the presence of various cations and anions (Jacobsen & Huennekens, 1969). When acetate is present as the anion, the activating effect of cations is in the order $Na^+ < K^+ < Rb^+ < Cs^+ < NH_4 << Li^+$. In the case of NaOAc, the maximum activating effect is obtained with a concentration of 1 M. Also analogously to the *E. coli* reductase, RTPR contains cysteines which become oxidized concomitant with substrate reduction. Artificial reductants such as DTT and DHL can be used to rereduce the active site disulfide so that multiple turnovers can occur. *In vivo*, however, *L. leichmannii* contains a TR/TRR/NADPH reducing system which can re-reduce the active site disulfide (Orr & Vitols, 1966). The *E. coli* TR is fully capable of supplying reducing equivalents to RTPR, and displays a K_m for substrate turnover of 4 μM (Blakley, 1978). The TR from *L. leichmanni* displays a K_m that is only slightly lower (Blakley, 1978). Because the *E. coli* TR and TRR have been cloned and overexpressed, they are routinely used as the reductant for RTPR (Lunn et al., 1984; Russel & Model, 1985).

RTPR catalyzes two other reactions in addition to deoxynucleotide production. The first reaction is the equilibration with solvent of the 5' methylene hydrogens of the cofactor (Abeles & Beck, 1967; Hogenkamp et al., 1968). In a reaction requiring NTPs or dNTPs, and reductant, RTPR will catalyze

the washout of tritium from [5'-^{3}H]AdoCbl to solvent. Likewise, in a reaction performed under similar conditions but in ^3H_2O, RTPR will catalyze the washin of tritium into unlabeled cofactor. When this reaction was allowed to proceed to equilibrium, a maximum of 1.4 atoms of tritium per molecule of AdoCbl was found to be incorporated (Hogenkamp et al., 1968). In the reverse direction, [5'-^{3}H]AdoCbl labeled by chemical means was found to transfer all of its tritium to solvent. These results provide strong evidence that this exchange reaction proceeds through an intermediate in which the two 5' methylene hydrogens are equivalent. This intermediate is suspected to be cob(II)alamin and 5'-deoxyadenosine. The exchange reaction is the focus of Chapter 5, and a detailed history and analysis of it will be presented therein.

Evidence which is highly suggestive of the cob(II)alamin/5'-deoxyadenosine intermediate comes from the second reaction that is catalyzed by RTPR. This reaction is the slow degradation of the cofactor to cob(II)alamin and 5'-deoxyadenosine (Yamada et al., 1971). As in the exchange reaction, this slow decomposition of the cofactor is also dependent upon the presence of reductant and an NTP or dNTP. The products of this reaction bind tightly to the enzyme in a mutally cooperative fashion, and display K_ds of 37 μM [cob(II)alamin] and 14 μM (5'-deoxyadenosine) (Yamada et al., 1971).

Although the decomposition of the cofactor is too slow to be on the catalytic pathway, stopped flow spectrophotometric studies by Tamao and Blakley (1973) revealed that cob(II)alamin is also produced in a very rapid reaction with a first order rate constant of 38-46s^{-1}. This rapid reaction is also contingent upon the presence of a dithiol and an NTP or dNTP, but unlike the slow reaction, this rapid reaction is fully reversible, and upon cooling to 5°C the UV-vis spectrum indicative of cob(II)alamin disappears. Confirmation that this species is indeed cob(II)alamin was established by Orme-Johnson *et al.* (1974)

using rapid freeze-quench EPR. The paramagnetic species formed was produced with a rate constant similar to that observed in the spectrophotometric experiments of Tamao and Blakley. The EPR spectrum differed from that of free cob(II)alamin, and later studies suggested that it was consistent with a cob(II)alamin species interacting with an organic radical (Hamilton et al., 1972).

All of the AdoCbl-dependent reductases are not monomeric enzymes. Tsai and Hogenkamp (1980) have isolated an AdoCbl-dependent reductase from *Corynebacterium nephridii* which is dimeric, and which uses nucleoside diphosphorylated substrates. This enzyme is also allosterically regulated; however, the the pattern of regulation is very complex. In general, as in the enzyme from *E. coli*, it appears that dNTPs act as effectors (Tsai & Hogenkamp, 1980). The AdoCbl-dependent reductases appear to be restricted to prokaryotes, with the exception of the enzyme isolated from Euglenophyta (Gleason & Frick, 1980).

Ribonucleotide Reductase from Anaerobically-grown Escherichia coli

The ability of *E. coli* to grow in the absence of oxygen necessitates that they be able to anaerobically synthesize the dNTPs necessary for DNA biosynthesis. Genetic experiments suggested that *E. coli* possessed a separate reductase for anaerobic growth (Jamison & Adler, 1987; Hantke, 1988). This reductase, (which constitutes the third class of RNRs) was subsequently isolated in the laboratory of P. Reichard, and found to be a ribonucleoside triphosphate reductase. It contains what appears to be a [4Fe-4S] cluster (Mulliez et al., 1993), and requires S-adenosylmethionine (AdoMet) and other low molecular weight factors (including K^+ and HCO_2^-) for catalytic activity (Eliasson et al., 1990; Eliasson et al., 1992). The gene for this reductase has been cloned, and its primary sequence

determined. The protein appears to be monomeric, and from the its primary sequence, the molecular weight was determined to be 80.1 kDa (Sun et al., 1993).

The pattern of allosteric regulation of this enzyme is simlar to those discussed above. Adenosine triphosphate promotes the reduction of CTP and UTP, dTTP promotes the reduction of GTP, and dGTP promotes the reduction of ATP. As in the *E. coli* RDPR, dATP is a general inhibitor of the enzyme (Reichard, 1993a).

Although very little sequence homology exists between this reductase and either of the subunits of the *E. coli* RDPR, the C terminus of the anaerobic reductase contains a sequence (RVCGY) which is very similar to a sequence (RVSGY) from the enzyme pyruvate formate-lyase (pfl). In pfl, a radical on glycine-734 in the conserved sequence shown above has been shown to be generated in the presence of AdoMet and 5-deazariboflavin (Wagner et al., 1992). In the presence of oxygen, the radical is destroyed, and the protein is cleaved into two fragments. The role of the glycyl residue in catalysis has not been clearly defined in either protein, however oxygen similarly inactivates the anaerobic ribonucleotide reductase.

Brevibacterium ammoniagenes Ribonucleotide Reductase

The enzyme isolated from *B. ammoniagenes* remains still relatively uncharacterized. This has oftentimes resulted in its exclusion as a class of RNRs (Reichard, 1993b). Early studies of *B. ammoniagenes* and *Micrococcus luteus* by several labs showed that manganese deficiency in these organisms resulted in unbalanced growth, filamentous morphology, and an arrest of DNA synthesis, by not RNA synthesis nor protein synthesis. The addition of Mn(II) to these cells restored DNA synthesis as well as the normal pattern of growth exhibited by

these organisms. Further studies showed that the abrogation of DNA synthesis caused by a deficiency in Mn was due to an inhibition of ribonucleotide reduction and not DNA replication (Schimpff-Weiland et al., 1981). A Mn-dependent reductase from *Brevibacterium ammoniagenes* was subsequently isolated (Willing et al., 1988b). This enzyme contains two subunits, a large monomeric subunit of M_r = 80,000, and a smaller dimeric subunit of M_r = 50,000 per monomer, giving rise to an overall $\alpha\beta_2$ quarternary structure (Willing et al., 1988b). The growth of *B. ammoniagenes* in the presence of $^{54}MnCl_2$ and the subsequent isolation of the reductase, in combination with non-denaturing/denaturing gel electrophoresis, showed that ^{54}Mn migrates with the smaller subunit of the reductase (Willing et al., 1988b).

As in the *L. leichmannii* and aerobic *E. coli* reductases, a small protein, thioredoxin, supplies the reducing equivalents necessary for multiple turnover in the *B. ammoniagenes* reductase. The *E. coli* TR will also serve in the same capacity (Willing et al., 1988a). The Mn-dependent enzyme is a ribonucleoside diphosphate reductase, and a study of its nucletide specificity revealed a similar pattern of allosteric regulation to that of the *E. coli* RDPR and the *L. leichmanni* enzyme. The reduction of CDP (and to a lesser extent UDP) is stimulated by dATP, ADP by dGTP, and GDP by dTTP (Willing et al., 1988a).

Although evidence for an organic radical is not presently available in this reductase, several lines of reasoning might suggest that this enzyme is similar to the *E. coli* RDPR. Firstly, the enzyme is inhibited by hydroxyurea, a compound known to inhibit the *E. coli* RDPR presumably by scavenging the tyrosyl radical (Willing et al., 1988b). Secondly, the R2 subunit of the *E. coli* RDPR has been reconstituted with Mn(II), and the resulting structure characterized by spectroscopic and crystallographic studies (Atta et al., 1992). Although this protein does not contain a tyrosyl radical and is therefore not active, two Mn(II)

ions occupy the iron-binding sites of the protein, and are bridged by E115 and E238, with no oxygen bridge present. Thirdly, the UV-vis spectrum of the *B. ammoniagenes* RDPR is similar to model compounds that contain two Mn(III) atoms coupled through a μ-oxo bridge (Sheats et al., 1987). Whether this enzyme contains a tyrosyl radical or perhaps some other organic radical awaits its further characterization.

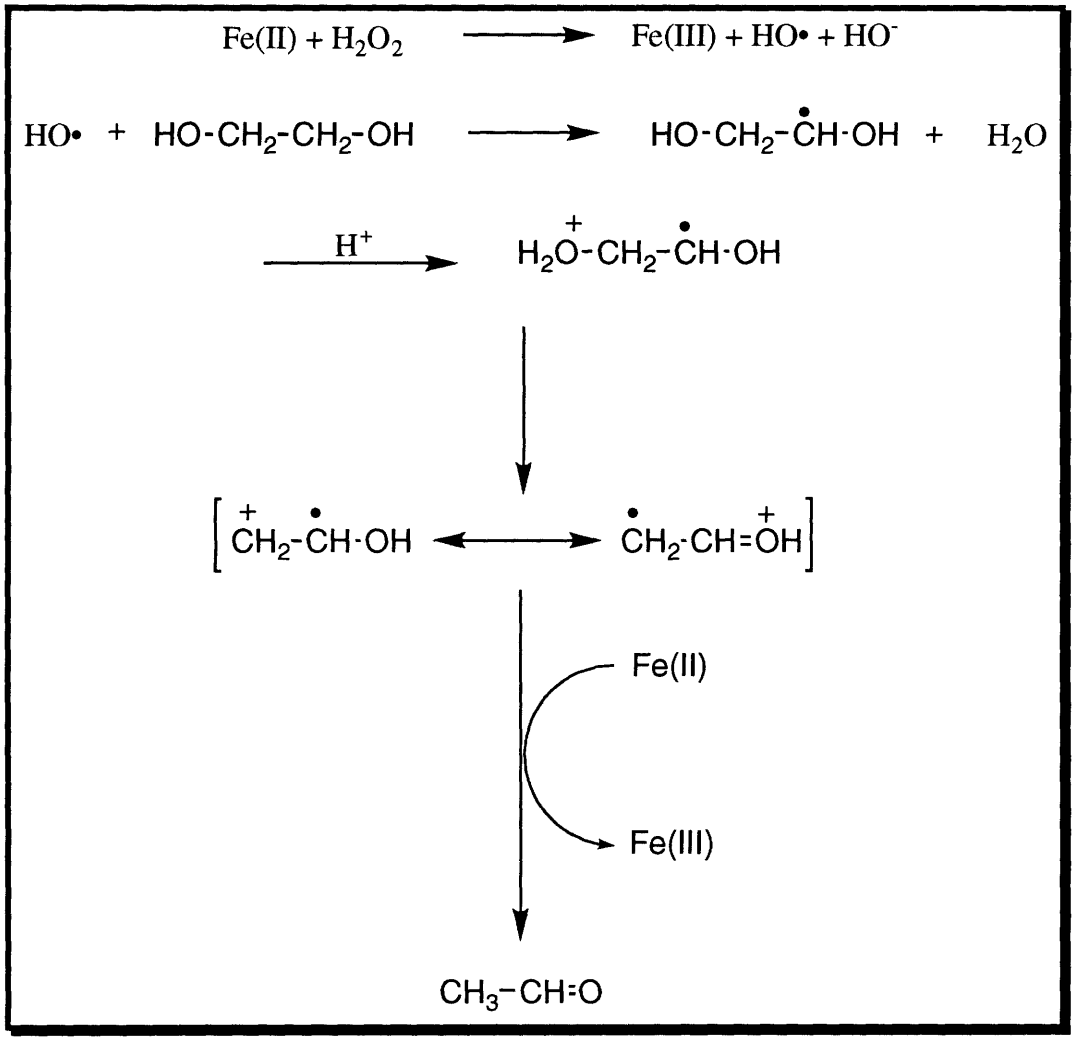
The Mechanism of Ribonucleotide Reduction

Models for the Mechanism of Ribonucleotide Reduction

Despite the difference in quarternary structure as well as cofactor requirement, studies on the *L. leichmannii* and *E. coli* reductases have suggested that these two ostensibly different enzymes may function by similar mechanisms of catalysis (Stubbe, 1990b). This hypothesis stems from the fact that (1) in both cases the hydrogen that replaces the 2' hydroxyl group is derived from solvent with retention of configuration (2) both enzymes couple substrate reduction to the oxidation of two cysteine residues on the protein to a disulfide (3) in both enzymes the disulfide bond formed concomitant with substrate reduction can be rereduced by a TR/TRR/NADPH reducing network, with the *E. coli* TR being able to function with the *L. leichmannii* enzyme.

Although the presence of a tyrosyl radical in the *E. coli* RDPR suggested to some that radicals might be involved in catalysis, at what stage they were involved and in what capacity was a mystery. Studies by Walling and Johnson (Walling & Johnson, 1975), as well as Gilbert *et al.* (Gilbert et al., 1972) on the mechanism of Fenton's reagent [Fe(II)/H_2O_2] provided an initial clue as to how this reaction might proceed (Scheme 1.3). When Fenton's reagent is reacted with glycols or halohydrins, the primary products are aldehydes, although other

Scheme 1.3: Proposed mechanism for the reaction of Fenton's reagent with 1,2-ethanediol.

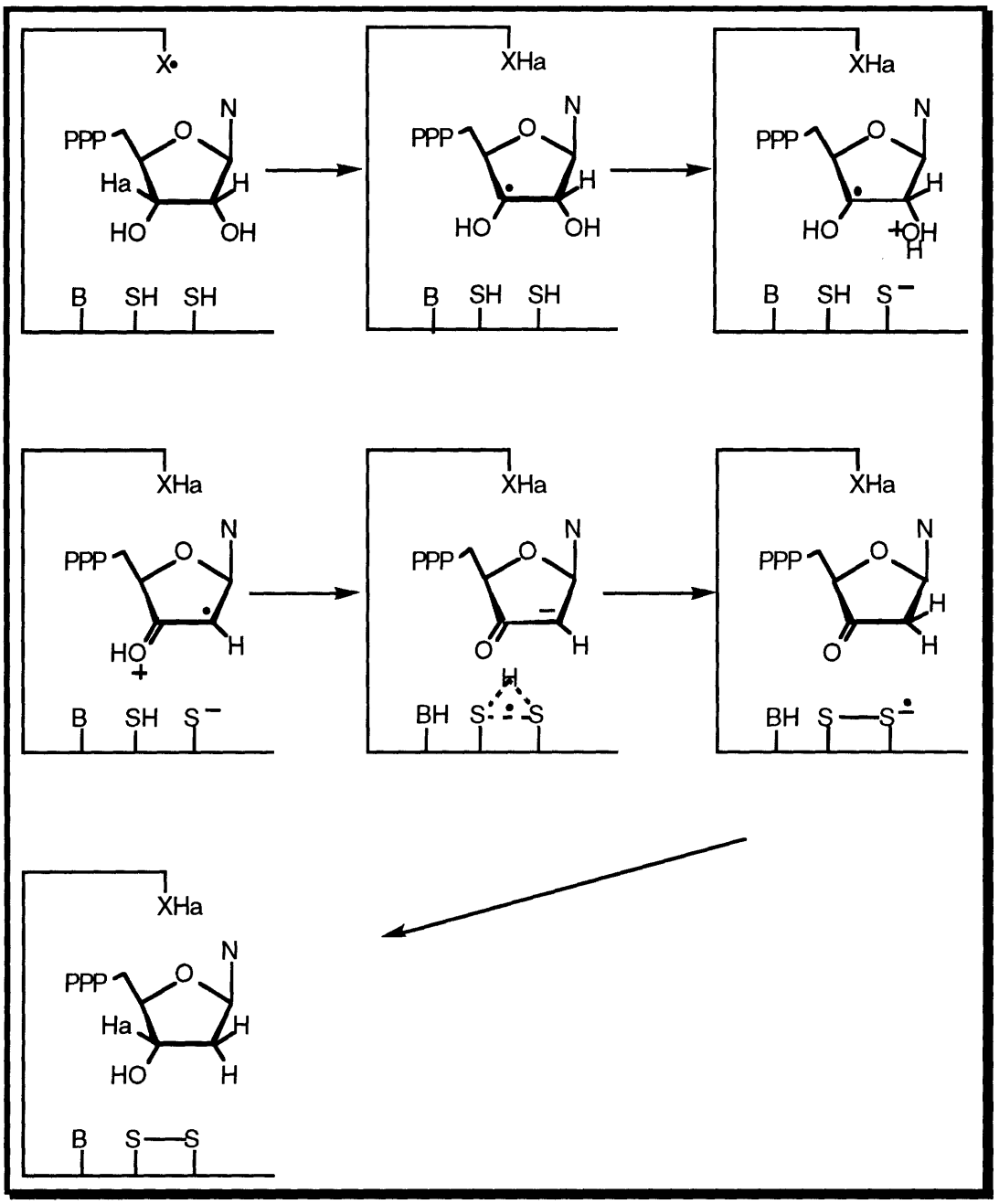


products are generated due to side reactions. The reaction requires acid catalysis, and is initiated by the reduction of H_2O_2 by Fe(II) to yield HO•,HO^-, and Fe(III). The hydroxyl radical abstracts a hydrogen atom from substrate, which upon protonation of the β-hydroxyl, yields H_2O and a radical-cation species which can be drawn in the shown resonance forms. A subsequent $1e^-$ reduction yields an

enolate which subsequently affords the aldehyde upon tautomerization. This resulting product is $2e^-$ oxidized from the expected product of ribonucleotide reduction. Since both the *L. leichmannii* and *E. coli* reductases were known to couple substrate turnover to the oxidation of two protein cysteines to a disulfide bond, the above model proved to be an attractive working hypothesis for the reaction catalyzed by ribonucleotide reductase. Based on this model, X• (some oxidizing species) plays the role of the hydroxyl radical created by Fenton's reagent. The first step in the reaction is the abstraction of a hydrogen atom from the 3' position of the substrate. The 2' hydroxyl group is protonated - ultimately by one of the redox-active cysteines on the protein - and the loss of a molecule of H_2O affords a radical-cation intermediate, as proposed in the mechanism of Fenton's reagent with 1,2-ethanethiol. This radical-cation intermediate is proposed to undergo two stepwise $1e^-$ reductions by the redox-active cysteines, the first of which affords a disulfide radical anion and a 3'-keto deoxynucleotide upon the addition of a proton. The second $1e^-$ reduction is followed by the return of the initial hydrogen atom (Ha) abstracted back to the 3' position of the deoxynucleotide product (Scheme 1.4).

The postulated mechanism for ribonucleotide reduction (Scheme 1.4) makes several predictions that have been experimentally tested. The first prediction is that the 3' C-H bond of the substrate is cleaved. This was tested using substrates which were specifically tritiated at the 3' position of the nucleotide. When [3'-^{3}H]UD(T)P or [3'-^{3}H]AD(T)P is used as substrate with RDPR (RTPR), small V/K isotope effects are observed on the reduction of these radiolabeled nucleotides (Stubbe et al., 1981; Stubbe et al., 1983; Ashley et al., 1986). In the case of RTPR, these isotope effects are essentially invarient with pH (1.6-1.8, pH 6.1-8.3, for UTP; 1.9-2.1 pH 8.3-5.5, for ATP); however, in the case of RDPR the isotope effects vary from 1.4 to 1.9 (pH 8.6-6.6) for the reduction of

Scheme 1.4: Working hypothesis for the mechanism of ribonucleotide reduction.



ADP, and 2.8 to 4.7 (pH 8.4-6.6) for the reduction of UDP. In addition very small but reproducible amounts (up to 1%) of ^3H_2O are produced in a time dependent

fashion upon the incubation of these radiolabeled substrates with both of the above RNRs. This result provides additional evidence that the above isotope effects are indeed primary effects rather than secondary effects, unambiguously establishing that the cleavage of the 3' C-H bond is required for nucleotide reduction.

The second prediction that this model makes, is that at the end of nucleotide reduction the hydrogen atom originally abstracted from the 3' position of the substrate is returned to the 3' position of the product. This prediction was investigated using nuclear magnetic resonance (NMR) techniques in combination with substrates that were specifically deuterated at the 3' postion. When substrate reduction is carried out with [3'-^{2}H]UD(T)P, the NMR spectrum of the isolated product shows that it is completely deuterated (Ashley & Stubbe, 1985).

Although none of the intermediates shown in Scheme 1.4 have been seen during normal turnover, each step has precedent in the chemical literature [For a recent review see Stubbe (1990)]. As discussed above, all steps leading to the radical cation intermediate are supported by the chemistry of Fenton's reagent with 1,2-ethanediol. Evidence supporting the 3'-keto intermediate comes from work with the substrate analog 2'-chloro-2'-deoxyuridine 5'-di(tri)phosphate, as well as studies with several RDPR protein analogs, and will be presented subsequently. Furthermore, there is precendent for the reduction by e^{-} transfer of a formylmethyl radical by DTT at high pH (Stubbe, 1990b). This reduction would afford a disulfide-radical anion and the 3'-keto intermediate as shown in Scheme 1.2.

The Nature of X•

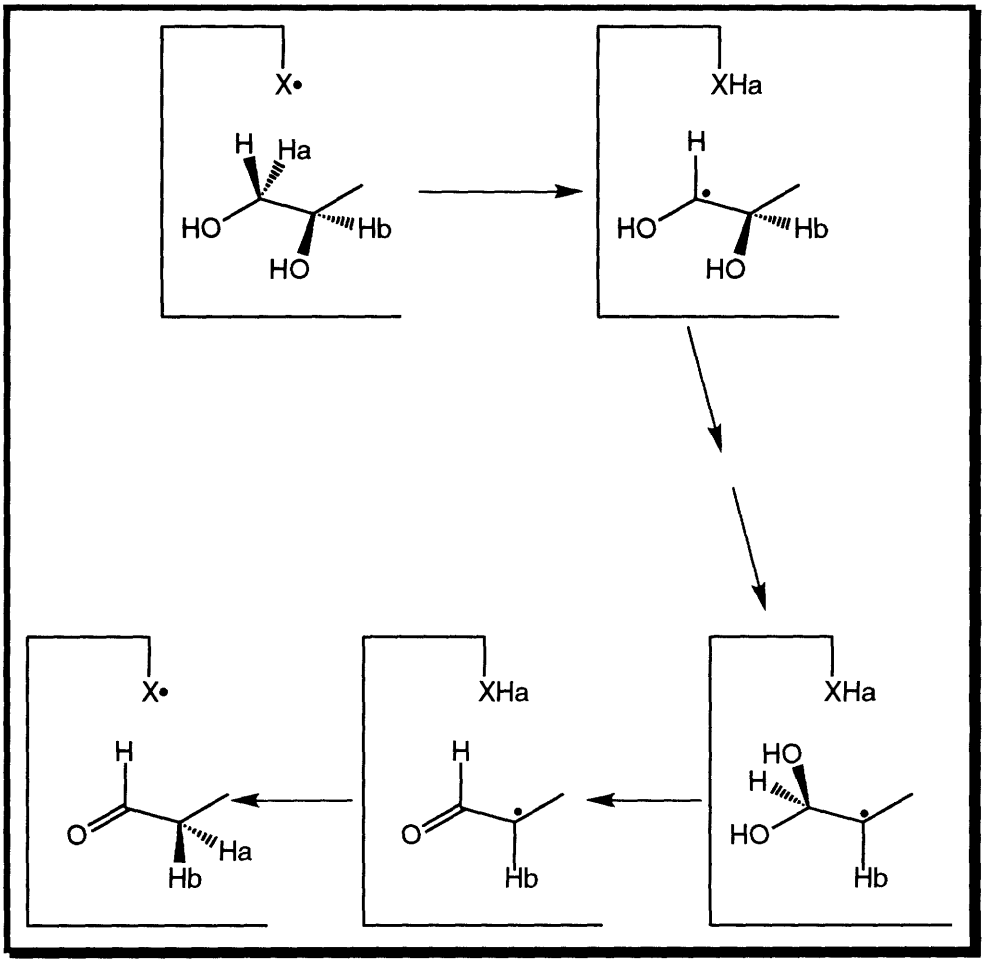
The mechanism in Scheme 1.4 presents a reasonable working hypothesis for the reaction catalyzed by the ribonucleotide reductases. The burning question thus becomes the nature of X•. From the standpoint of the *E. coli* enzyme it appears that X• might be the tyrosyl radical on the R2 subunit. Recent crystallographic studies by Nordlund and Eklund (1993) (1990) strongly suggest that this is indeed not the case. As discussed above, the tyrosyl radical is buried deep within the R2 subunit approximately 10 Å from the surface of the protein, and is therefore not capable of mediating 3' hydrogen atom abstraction unless gross conformational changes occur. This corroborates the long-held hypothesis by Stubbe and coworkers that the function of the R2 subunit is to generate a radical on the R1 subunit (the subunit which binds substrates and allosteric effectors, and which contains the redox-active cysteines) by long-range e^-/H^+ transfer (Stubbe, 1990b). It is then the radical on the R1 subunit which initiates catalysis by abstracting the 3' hydrogen of the substrate. Evidence that suggests that the transient radical formed on protein R1 is a thiyl radical will be provided below.

That radical intermediates might be involved in the AdoCbl-dependent reductase has huge precedent in the chemical literature [For a review see Dolphin (1982)]. AdoCbl participates in a class of enzymes that carry out a rearrangement between a hydrogen atom on one carbon, and some functional group that is located on an adjacent carbon. The enzyme dioldehydrase is a prototype for this class, and its reaction is shown in Scheme 1.5. The tremendous amount of evidence in support of this scheme has been reviewed by Babior and Krower (1979). The reaction is initiated upon the homolytic cleavage of the cofactor and the abstraction of a hydrogen atom from substrate. The resulting substrate radical rearranges (by a mechanism that is unclear to date) to give the

product radical, which re-abstracts a hydrogen atom from XHa to give a geminal diol. The geminal diol stereospecifically loses a molecule of H_2O in a reaction which is catalyzed by the enzyme to afford the product aldehyde. Seminal studies carried out in the Abeles laboratory on dioldehydrase showed that AdoCbl functions as an intermediate hydrogen atom carrier. In the event that the substrate [1,1-^{3}H]DL-propanediol is incubated with the enzyme, tritium is found to be located in the cofactor. If [5'-^{3}H]AdoCbl is incubated with enzyme and substrate, tritium is found to be located in the product (Abeles & Zagalak, 1966; Frey et al., 1967). These studies show that AdoCbl can act as a hydrogen carrier in this reaction; however, the degree to which it functions in this capacity is uncertain. Indeed, studies have shown that $k_H/k_T = 125$ for the transfer of tritium from cofactor to product (Essenberg et al., 1971). Cleland has proposed that this anomalously high isotope effect could be explained by a pool of hydrogen atoms on the enzyme with which the migrating hydrogen can equilibrate, or by an alternate pathway (perhaps a protein radical?) with which the hydrogen can be transferred (Cleland, 1982). In the event that the transfer occurs through a protein residue 90% of the time, and through 5'-deoxyadenosine 10% of the time, the true isotope effect (12.5) would be in the realm of what is normally observed for tritium.

These studies with dioldehydrase present a starting point to ask whether AdoCbl functions in the same manner in the reaction catalyzed by RTPR. That radical intermediates might be involved in the reaction catalyzed by RTPR was evidenced by early biophysical studies on the enzyme. As previously mentioned stopped flow UV-vis studies by Tamao and Blakley (1973) as well as rapid freeze-quench EPR studies by Orme-Johnson, Beinert, and Blakley (1974) showed that the *L. leichmannii* RNR catalyzes the homolytic cleavage of the Co-carbon bond of AdoCbl with a first order rate constant of 38-46 s^{-1} in the presence of dGTP and

Scheme 1.5: Proposed mechanism for the enzyme dioldehydrase.



reductant. The turnover number for this reductase is 1-3 s^{-1}, suggesting that the homolytic cleavage is kinetically competent to be on the reaction pathway. The products of this homolytic cleavage of AdoCbl are cob(II)alamin, and presumably a 5'-deoxyadenosyl radical. This rapid production of cob(II)alamin could also be observed in the presence of substrate; however, in a slow process, the concentration of cob(II)alamin declines until a steady state level is reached. Interestingly, in the presence of [5'-$^2H_2$]AdoCbl a small primary isotope effect of 2.2 was observed on the second slow process (Tamao & Blakley, 1973). Efforts to

observe the 5'-deoxyadenosyl radical, using [5'-$^{2}H_2$]AdoCbl and looking for changes in the EPR spectrum were, however, unsuccessful (Orme-Johnson et al., 1974).

If the 5'-deoxyadenosyl radical is involved in initiating catalysis by abstracting the 3' hydrogen of the substrate, then tritium might be observed in the cofactor upon incubation with substrates that are specifically tritiated at the 3' position. When RTPR was incubated with [5'-^{3}H]AdoCbl, tritium was found in H_2O, and not in the resulting product (Beck et al., 1966; Abeles & Beck, 1967). Likewise, when the reaction was carried out in $^{3}H_2O$ in the presence of unlabeled AdoCbl, the cofactor was found to contain tritium (Abeles & Beck, 1967). This equilibrium between solvent and cofactor is addressed in detail in Chapter 5. Studies carried out in the Stubbe laboratory showed that no tritium is transferred to AdoCbl or H_2O under single turnover (pre-reduced enzyme in the absence of reductant) or multiple turnover (in the presence of DTT) conditions when RTPR is incubated with [3'-^{3}H]ATP (Ashley et al., 1986). More than 99% of the tritium is accounted for in the substrate and product. If AdoCbl were assuming a role analogous to its role in the AdoCbl-rearrangement reactions, tritium should be observed in the cofactor or in H_2O, with the relative ratios depending on the on/off rate of the cofactor, and the rate of tritium exchange between the solvent and the cofactor.

As proposed for the *E. coli* RDPR, the radical initially generated in RTPR is not the radical that is reponsible for initiating catalysis by abstracting the 3' hydrogen atom of the substrate. The sole function of the cofactor (R2 in RDPR, AdoCbl in RTPR) in both cases is to generate a protein radical which is subsequently responsible for initiating catalysis. In the case of RTPR, evidence will be given in this thesis (Chapter 4) that this transient radical is formed on a cysteine residue.

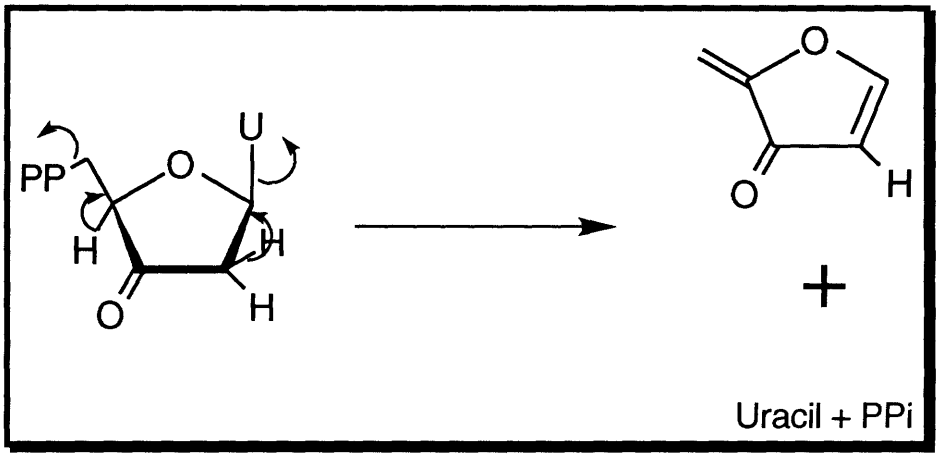
Interaction of Ribonucleotide Reductase with 2'-Chloro-2'-deoxyuridine 5'-di(tri)phosphate

The mechanism of ribonucleotide reduction has also been investigated with the aid of substrate analogs or mechanism-based inhibitors. Of the many substrate analogs tested with RDPR or RTPR, 2'-chloro-2'-deoxyuridine 5'-di(tri)phosphate is the best characterized, and has provided a wealth of information concerning the catalytic capabilities of both of these enzymes. In 1976, Thelander *et al.* (1976) reported that 2'-chloro-2'-dCDP and 2'-chloro-2'-dUDP irreversibly inactivated the R1 subunit of RDPR. Although the R2 subunit was not inactivated, it was necessary for the inactivation of the R1 subunit. They also demonstrated that upon inactivation of RDPR, cytosine, Cl^-, and 2-deoxyribose 5-diphosphate was produced. In addition, they reported that a chromophore at 320 nm formed slowly subsequent to inactivation, and was also associated with the R1 subunit.

The inactivation of RDPR was reinvestigated by Stubbe and Kozarich, (1980) (Stubbe & Kozarich, 1980), and revealed that PPi and not 2-deoxyribose 5-diphosphate was produced. Similar studies with the *L. leichmannii* reductase showed a commonality with the *E. coli* RDPR in the reaction with 2'-chloro-2'-dUTP. Incubation of the inhibitor with RTPR resulted in the production of PPPi, Cl^-, uracil, and the 320 nm chromophore (Harris et al., 1984). However, unlike the case of the *E. coli* enzyme the cofactor (AdoCbl) was destroyed during the inactivation reaction, yielding cob(II)alamin when the reaction was carried out under anaerobic conditions. This observation suggests a pathway that can be rationalized by a mechanism similar to that of the normal reduction process, but not requiring protonation of the leaving group. In the event that a $2e^-$ reduction is prevented a 3'-keto species could result if the

hydrogen atom initially abstracted from the 3' position is placed at the 2' position. As shown in Scheme 1.6, this intermediate could collapse to form PPi, uracil, and a furanone species.

Scheme 1.6: Postulated intermediate leading to inactivation of RDPR when incubated with 2'-chloro-2'-dUTP.



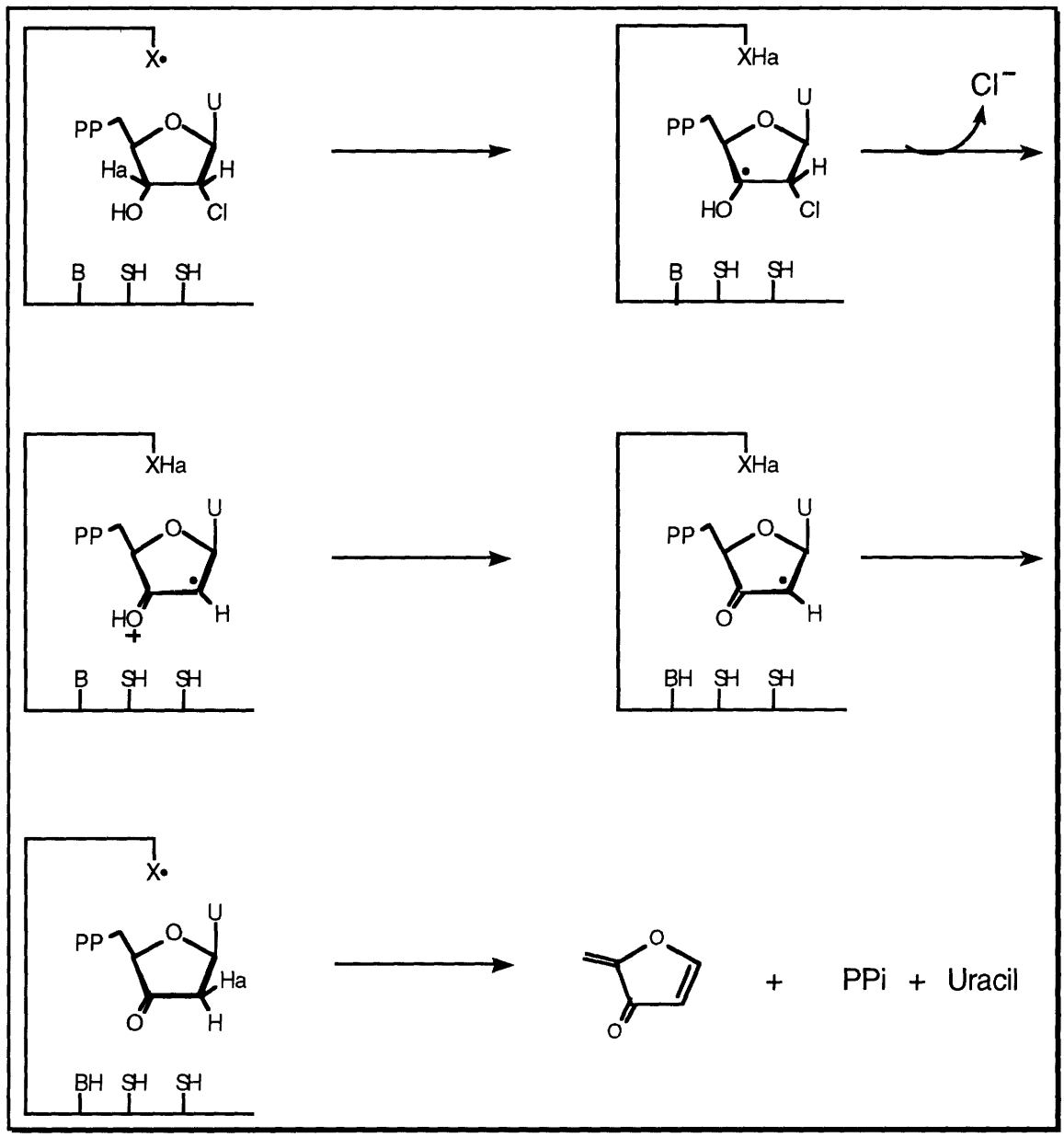
Based on this hypothesis, a mechanism was proposed to explain the inactivation of RDPR by 2'-chloro-2'-dUTP (Scheme 1.7). The reaction is initiated in the same fashion as turnover with the normal substrate with the abstraction of the 3' hydrogen atom by X•. However, as determined by model studies (Walling and Johnson, 1975), Cl^- can leave without the aid of general acid assistance. The active site cysteines are subsequently left in the protonated state, which slows down the reduction of the radical-cation intermediate by several orders of magnitude (Stubbe, 1990b) The substrate radical at the 2' position of the nucleotide is then quenched by the abstraction from XH of the hydrogen initially present at the 3' position. As shown in Scheme 1.6, the resulting 3'-keto species collapses to form PPi, uracil, and the reactive furanone species which can

inactivate the protein by alkylating some critical residue within the active site.

The mechanism shown in Scheme 1.7 makes several predictions which have been tested experimentally. The first prediction is that the 3' C-H bond of the inhibitor is cleaved. This was investigated with the aid of isotopically-labeled inhibitors, specifically tritiated at the 3' position. When either reductase is incubated with [3'-^{3}H]-2'-chloro-dUD(T)P and the TR/TRR/NADPH reducing system, tritium is shown to be transferred to solvent in a time dependent fashion (Harris et al., 1984). In the case of RDPR, the amount of tritium reaches as high as 4.7 equiv of ^3H_2O per equiv of inactivated R1. In the presence of DTT, as much as 75% of the total tritium present in the substrate is recovered in the solvent. The reasons for the higher amounts in the presence of DTT will become apparent subsequently. These studies present unambiguous evidence for the cleavage of the 3' C-H bond of the inhibitor.

The second prediction that the above mechanism makes is that a 3'-keto-2'-deoxynucleotide should be produced. In the case of RDPR, this intermediate was effectively trapped when the inactivation reaction was run in the presence of 100 mM $NaBH_4$. In addition, incubation of RDPR with [3'-^{3}H]-2'-chloro-dUDP in the presence of $NaBH_4$ resulted in the tritium being transferred to H_2O as discussed above, or to the β-face (*pro* S position) of the 2' position of the 3'-keto intermediate. In the case of RTPR, trapping experiments were more complicated. When [3'-^{3}H]-2'-chloro-dUTP was incubated with RTPR in the presence of $NaBH_4$, 85% of the tritium was found to be transferred to the solvent, while 15% was found to be located in the *pro* S position of the trapped nucleotide. A closer inspection of this reaction using [2'-^{3}H]-2'-chloro-dUTP showed an interesting difference between RDPR and RTPR upon inactivation with the "chloro" inhibitor. In RTPR, the tritium at the 2' position is found to undergo inversion of configuration 70% of the time, while in RDPR, this

Scheme 1.7: Proposed mechanism for the inactivation of RDPR by 2'-chloro-2'-dUDP.



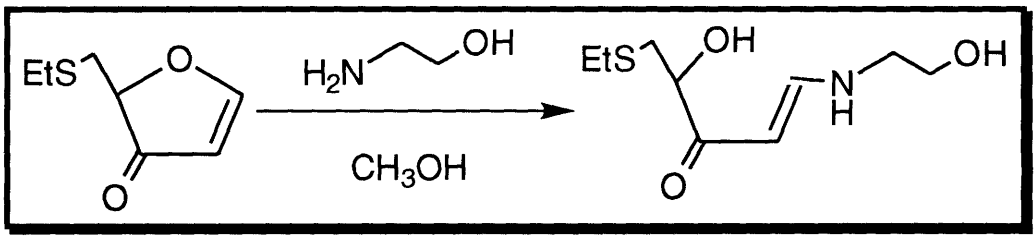
inversion takes place 100% of the time (Stubbe, 1990b). This study provides a compelling hypothesis for why the cofactor is destroyed in the case of RTPR, but not RDPR. Turning again to Scheme 1.5, if reduction to give the 3'-keto species occurs by re-abstraction of a hydrogen atom from XH, then inversion of configuration at the 2' position would be expected, as well as regeneration of X•. However, if reduction were to occur from the bottom face (perhaps by one of the redox-active cysteines) then retention of configuration would be expected. In addition, X• would not be regenerated, resulting in destruction of the cofactor. The results with RTPR suggest that 30% of the time the reduction takes place from the bottom face of the nucleotide. This is in agreement with the observation that AdoCbl is destroyed during the inactivation reaction, yielding cob(II)alamin and 5'-deoxyadenosine.

A third prediction that this mechanism makes is that a 2-methylene-3(2H)-furanone species is formed which alkylates and inactivates the enzyme. That a convalent adduct is formed upon inactivation of RDPR or RTPR with ClUDP or ClUTP was investigated with [5'-^{3}H]-2'-chloro-2'-dUD(T)P. In both reductases label is found to be associated with the protein. The number of equiv of label per equiv of reductase varies from 1-5, and depends on the ratio of inhibitor to enzyme (Harris et al., 1984).

Evidence for the intermediacy of a 2-methylene-3(2H)-furanone comes from trapping experiments. DTT and other exogenous thiols protect the enzyme from inactivation, suggesting that they might be trapping a reactive intermediate. When the inactivation reaction is carried out with RTPR or RDPR in the presence of ethanethiol, the resulting adduct is extractable into $CHCl_3$. NMR and infrared (IR) spectra of this extractable material are identical to spectra of an ethanethiol adduct of the furanone species synthesized by chemical methods. Further studies using the ethanethiol-trapped furanone species shed light on the 320 nm

absorbance that grows in subsequent to inactivation. As shown in Scheme 1.8, if the ethanethiol-adduct of the furanone species (which has a λ_{max} = 260 nm) is treated with ethanolamine, the resulting species has an absorbance at λ_{max} = 316 nm. These results suggest that inactivation is due to nucleophilic addition onto the exocyclic methylene of the furanone species. In a slower step, the ε-amino group of a lysine does a 1,4 conjugate addition, which is followed by ring opening and enamine formation (Ashley et al., 1988).

Scheme 1.8: Model study for the formation of the species giving rise to the chromophore having a λ_{max} of ~320 nm.



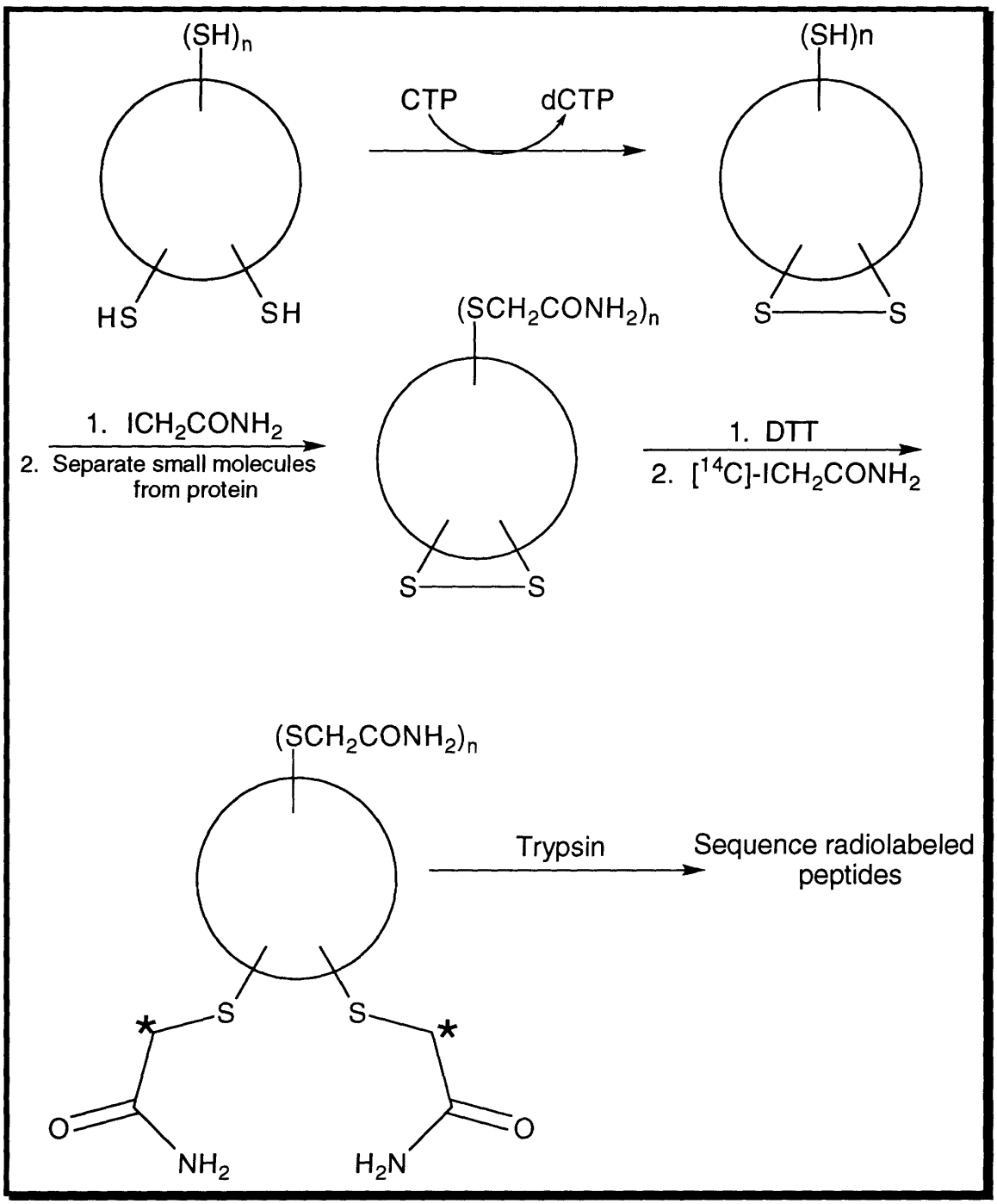
In summary, the compound 2'-chloro-2'-deoxyuridine 5'-di(tri)phosphate has provided an intricate look at the mechanism of ribonucleotide reduction, and has supplied more evidence to support the hypothesis that although these two enzymes use different cofactors, they operate by similar mechanisms of catalysis. As shown unambigously in studies with this substrate analog, both enzymes cleave the 3' C-H bond, and return the hydrogen to the 2' position of the molecule. Both enzymes release PPi (PPPi), base, and a highly reactive furanone species which is reponsible for inactivating the enzyme and which gives rise to the absorbance at 320 nm. Lastly, RTPR gives rise to cofactor destruction. This in combination with the mechanism for inactivation as shown in Scheme 1.7,

corroborates the finding that the hydrogen at the 2' position of the 3'-keto intermediate undergoes retention of configuration 30% of the time. The fact that the tyrosyl radical of RDPR is not quenched during the inactivation, supports the hypothesis that reduction to the 3'-keto species occurs from the top face of the molecule via XH.

Attempts to Locate the Redox-active Cysteines of RNR

Given the similarities in mechanism between the *E. coli* RDPR and the *L. leichmannii* RTPR, efforts were undertaken by the Stubbe laboratory to locate the active sites of both of these reductases and determine whether these enzymes are homologous with repect to their active site sequences (Lin et al., 1987). The strategy for this experiment is outlined in Scheme 1.9. Each of the enzymes was first incubated with DTT in order to ensure that the redox-active cysteines were in the reduced state. The DTT was subsequently removed from the protein fraction by chromatography on a size exclusion column. The protein was then treated with substrate in the absence of reductant in what is termed a single-turnover experiment. Upon completion, the redox-active cysteines are present as a disulfide bond, and are therefore resistant to alkylation by iodoacetamide. The protein was subsequently denatured and treated with unlabeled iodoacetamide, and then separated from the excess iodoacetamide by size-exclusion chromatography. The redox-active cysteines were then re-reduced by subsequent treatment with DTT, and then alkylated in the same fashion as above with [^{14}C]iodoacetamide. The protein was then cleaved by treatment with trypsin, and the radiolabeled peptides were isolated by HPLC and submitted for sequencing by Edman degradation. A control experiment was also run in the absence of substrate, which would identify disulfide bonds in both proteins that

Scheme 1.9: Strategy for locating the cysteines which are oxidized concomitant with substrate reduction.

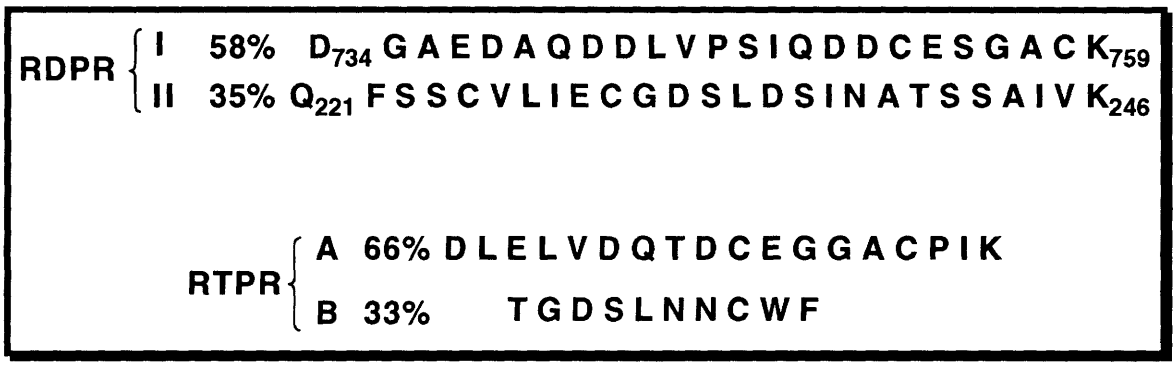


were not formed concomitant with substrate reduction. In the case of RDPR, 3 equiv of dCDP are produced upon interaction of the pre-reduced enzyme with CDP, and 5 equiv of [^{14}C]iodoacetamide are found to be covalently attached to the R1 subunit. In the case of RTPR, 1 equiv of dCTP is produced upon interaction of the pre-reduced enzyme with CTP, and 2 equiv of [^{14}C]iodoacetamide are found to be covalently attached to the protein. The results of the peptide-mapping studies are shown in Scheme 1.10 (Lin et al., 1987). In the studies carried out with RTPR, analysis of the peptides shows that all of the radioactivity can be accounted for in two different peptides, but among three different cysteines. The peptide containing two cysteines, or 66% of the radioactivity, has a significant degree of homology with peptide I isolated from the *E. coli* enzyme. In addition, peptide I is known to be located at the extreme C terminus of the R1 subunit by analysis of its gene sequence, and shares homology with several other non-heme iron-dependent reductases (Stubbe, 1990b). As will be apparent in the subsequent chapter, the homologous peptide in the *L. leichmannii* enzyme is also located at the extreme C terminus of the protein. Although the remaining peptide in each enzyme shares no homology with the other, this experiment suggests that the labeled cysteine is close enough within three-dimensional space to the C-terminal cysteines to allow it to undergo disulfide interchange with them. The function of this cysteine in the *E. coli* reductase will be discussed in the subsequent section, while the function of this cysteine in the *L. leichmannii* reductase will be discussed in Chapter 4.

Investigation of the Mechanism of Ribonucleotide Reduction with Protein Analogs

Protein analogs or site-directed mutants have recently been used to probe the mechanism of the *E. coli* ribonucleotide reductase, and assign more definitive roles to the cysteines isolated in active-site labeling studies, as well as others in

Scheme 1.10: Isolated peptides from active-site labeling studies.



the R1 subunit (Aberg et al., 1989; Mao et al., 1989; Mao et al., 1992a; Mao et al., 1992b; Mao et al., 1992). Five cysteines were targeted for mutagenesis. Cysteines 754 and 759 were identified in active-site labeling studies discussed above, and are located at the C-terminal end of the R1 subunit. Their sequence context shares significant homology with other R1 subunits of the non-heme iron-requiring reductases, as well as a peptide isolated from the *L. leichmannii* reductase. Each cysteine was mutated to a serine, and the two single mutant proteins as well as the double mutant protein were incubated with the normal substrate (CDP) under varying conditions. In the presence of the TR/TRR/NADPH reducing system, each of these mutants catalyzed the reduction of CTP at a rate that was ~3% that of the wild-type (wt) enzyme. In the presence of DTT however, the rate of CTP reduction was calculated to be on the order of the wt enzyme with each of the single mutants, and ~4 times greater than that of wt enzyme with the double mutant. These studies suggested that the function of these two cysteines is to deliver reducing equivalents from TR into

the active-site disulfide bond in order that multiple turnovers can be achieved. Corroborating this hypothesis are the single-turnover experiments carried out with these mutants. When pre-reduced wt-RDPR is treated with substrate in the absence of external reductant, 2.6 equiv of dCTP per R1 dimer are observed. Each single mutant as well as the double mutant produces only 1.2 equiv, suggesting that one set of redox-active cysteines has been altered. Marring these results as well as the subsequent ones to be discussed, is the significant level of wt contamination in each of the mutant preparations. This is due to the fact that ribonucleotide reductase is needed for cell growth and viability; therefore, it is not possible to inactivate the chromosomal gene for the enzyme.

Cysteine 225 was also located on the basis of the above biochemical studies, and mutated to a serine. When the resulting mutant protein is incubated with the normal substrate, a very unusual sequence of events occurs. As seen above with the mechanism-based inhibitor 2'-chloro-2'-dUTP, the substrate is converted to cytosine, and PPi. In addition, a chromophore at 320 nm is observed to be associated with the R1 subunit, suggesting that the furanone species is produced. Unlike with the substrate analog 2'-chloro-2'-dUDP, destruction of the tyrosyl radical is observed. Lastly, the protein cleaves itself into two pieces. Given the many similarities of this protein analog with the substrate analog, this cysteine was proposed to be one of the active site cysteines directly involved in substrate reduction. The similarity in the products produced upon incubation of this mutant protein with the normal substrate, suggests that in the event that substrate reduction is uncoupled from 3' C-H bond cleavage the substrate is turned into a mechanism-based inhibitor of the enzyme.

Cysteine 462 was targeted by analysis of its sequence with other reductases in this class. The corresponding C→S mutant protein results in a phenotype that is very similar to that of C225, with the exception that the protein

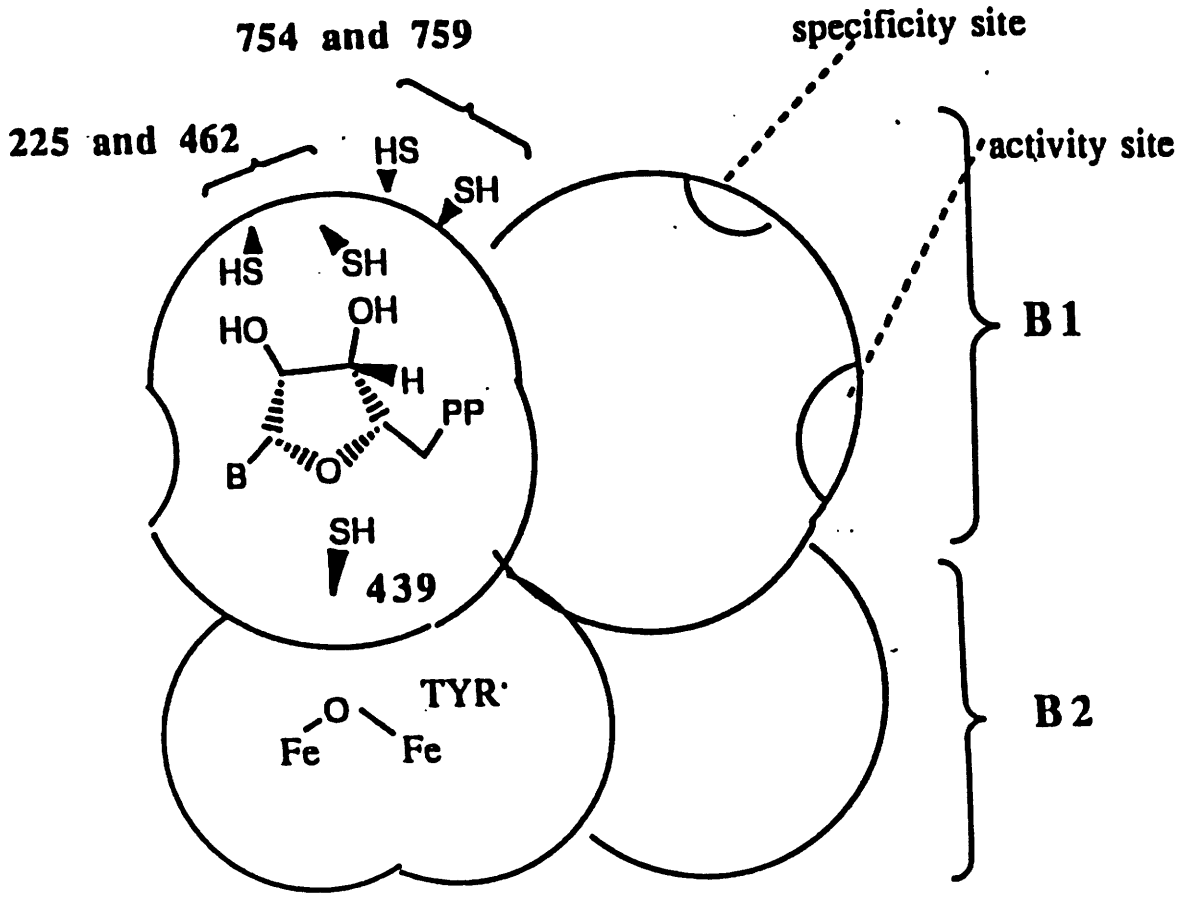
is not cleaved in half. This result suggests that this cysteine is also at the active site of RDPR, and supplies reducing equivalents directly to the substrate.

Lastly, C439 was identified by its homology with other cysteines in this class of RNR. Under all conditions, the resulting C→S mutant protein has no activity for the reduction of CDP to dCDP. In addition, no cytosine or any other product is produced. This suggested that this cysteine might be the X• which is responsible for initiating catalysis by abstracting the 3' C-H bond of the substrate. Several experiments were carried out to show that the inactivity of this protein is not due to a structural alteration. Its circular dichroism spectrum is identical to that of the wt enzyme. In addition it is capable of binding substrate and effector with approximately the same K_ds as the wt enzyme.

These studies suggest a model in which five cysteines are required for nucleotide reduction (Figure 1.3). Two cysteines shuttle electrons into the active site disulfide from the TR/TRR/NADPH reducing system. Two cysteines are at the active site and function to directly reduce the substrate. Lastly, cysteine 439 is proposed to be X•, the amino acid reponsible for initiating catalysis by abstracting the 3' hydrogen atom of the substrate. As discussed above, the presence of contaminating wt enzyme prevents the unambiguous establishment of this hypothesis. Given the similarities in mechanism between the *E. coli* enzyme and the *L. leichmannii* enzyme, it is reasonable to hypothesize that if the above hypothesis is correct, then RTPR might also involve five cysteines in catalysis as well. More importantly, the ability to express the *L. leichmannii* enzyme in *E. coli* would preclude the problems of contaminating wt enzyme since different cofactors as well as substrates are employed by the two enzymes. This thesis describes the cloning, sequencing, and expression of the *Lactobacillus leichmannii* ribonucleoside triphosphate reductase, and the characterization of five cysteines on the protein believed to be involved in

substrate reduction. In addition it reports our reinvestigation of the AdoCbl-dependent exchange reaction as a model for the generation of X•.

Figure 1.3: Postulated role of 5 cysteines involved in nucleotide reduction in the RDPR from *E. coli*. Cysteines 754 and 759 shuttle reducing equivalents from TR into the active site disulfide. Cysteines 462 and 225 are at the active site, and function to directly reduce the substrate. Cysteine 419 is the postulated X•.


754 and 759
specificity site
225 and 462
activity site
HS
SH
HS
SH
HO
OH
H
PP
B
O
SH
439
B1
TYR·
O
Fe
Fe
B2

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Chapter 2:

Isolation and Sequencing of the Gene for Ribonucleoside Triphosphate Reductase from *Lactobacillus leichmannii*

The cloning of a gene refers to isolating a segment of DNA which encodes the particular protein of interest, and placing it inside of a host (usually *E. coli* or yeast) extrachromosomally such that the DNA can replicate autonomously with respect to the replication of the host's own genome. This process usually entails inserting the DNA into a self-replicating vehicle called a vector. The most commonly used vectors are derivatives of naturally occurring plasmids (which usually confer to a host the ability to grow in the presence of certain antibiotics), or derivatives of certain bacteriophages which infect bacteria but do not lyse them. The ability to clone DNA is without doubt the result of the exploitation of a vast assortment of cellular enzymes that allow DNA to be modified in many ways. This is manifested in the ability to cut DNA at specific locations based on nucleotide sequence, join DNA fragments, and add or remove terminal phosphate groups at will. The cloning and expression in high yield of these DNA modifying enzymes have allowed them to be extensively purified in large quantities. This has in turn enabled the cutting and pasting of DNA to be a routine procedure for molecular biologists. This routine procedure of moving defined sequences of DNA to and from various vectors is termed subcloning.

The rate-limiting step in cloning a gene from an organsim in which it is present in only a single copy per genome is defining its location. This can seem to be quite a daunting task given that a simple bacterium such as *E. coli* contains ~$4x10^6$ base pairs (bp) in its single chromosome. Eukaryotic genomes tend to be more complex, with yeast having ~$1.6x10^7$ bp, Drosophila having ~$1.2x10^8$ bp, and mammals having ~$3x10^9$ bp per haploid genome (Sambrook et al., 1989). With the invention of several innovative techniques within the last 30 years, this process is by no means insurmountable. In fact, because of the mind-boggling progress that molecular biology has made within the last 3 decades, it is fair to say that almost any gene for which a protein product can be isolated can be

cloned today. This notion is supported by the recent isolation of some of the "Holy Grails" of molecular biology such as the genes for cystic fibrosis and Huntingtons disease (Riordan et al., 1989; Goldberg et al., 1993).

Before a gene can be cloned by any technique, there first must be a source of DNA beyond the organism's chromosome that can be easily managed and manipulated. This source is called a gene library. A gene library is an assortment of DNA fragments from an organism's genome which is maintained and propagated in a host as a vectoral insert. The most common way to create genomic libraries is by cloning fragments of an organism's genome into a suitable vector. These fragments are usually generated from partial digests of the genomic DNA with a restriction enzyme that cuts relatively frequently (about once every 256 nucleotides), such as *Sau*3A. Partial digests ensure that relatively large fragments (~6-20 kb) can be obtained that are a series of overlapping sequences of the organism's genome. Equation 2.1 relates the size of the insert with the minimum number of clones that must be generated for a library to have a certain probability (usually 95%) of containing the entire gene of interest.

$$N = \frac{\ln (1-P)}{\ln (1-f)} \qquad \textbf{Eq. 2.1}$$

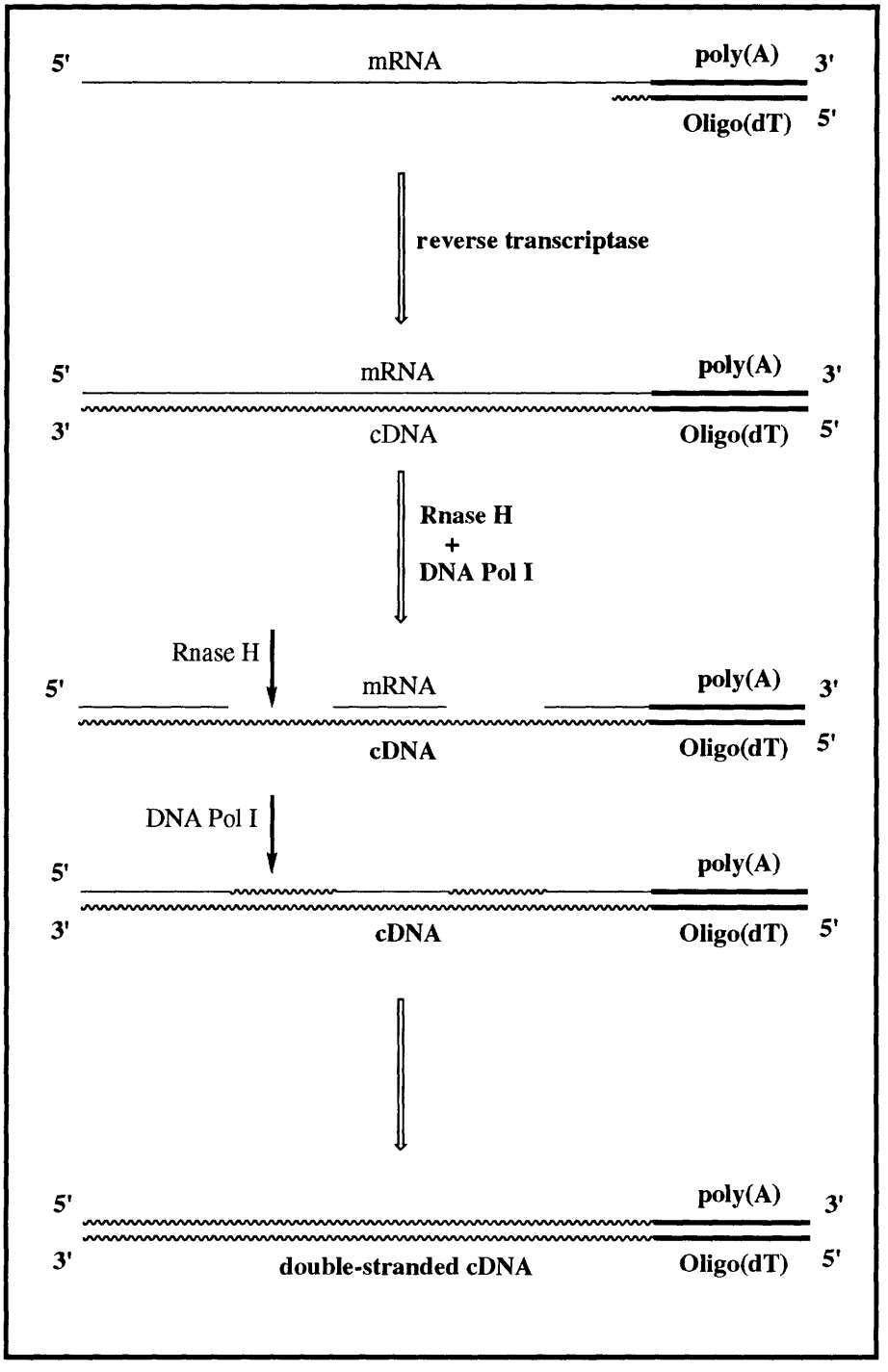
The variable **P** represents the probability that **N** clones will contain any given gene in a library. The variable **f** represents the average insert size in base pairs divided by the total size of the organism's genome.

Because eukaryotes often contain many introns in their genomes, libraries from these organisms are usually made in a different manner. Molecular biologists have learned to exploit the fact that messenger RNA (mRNA) is the direct template from which proteins are synthesized, and therefore contains no

intervening sequences. Upon isolating mRNA from an organism, a DNA copy of the mRNA can be made with the aid of an enzyme called reverse transcriptase in combination with the enzymes ribonuclease H (RNase H) and DNA polymerase I (Figure 2.1). This complementary DNA, or cDNA, can then be ligated into a suitable vector to give a gene library containing the coding sequences for proteins. Also included is upstream information such as the gene's promoter and ribosome binding site, and downstream information such as termination signals for RNA polymerase. Although this procedure is more tedious, oftentimes growth conditions of the organism from which the gene will be isolated can be altered in order to increase the production of the specific protein's mRNA. This in turn decreases the number of clones that a library must contain in order that the gene of interest be present at the 95% confidence level.

There are three basic techniques employed to screen a library, or locate a clone of interest. The first and most common method is based on the ability of oligonucleotides to hybridize to complementary sequences of single-stranded DNA (Wallace et al., 1979; Wallace et al., 1981). Clones from a library are plated on agar-containing petri dishes and allowed to grow. Colonies that appear are then transferred to a solid support such as nitrocellulose or nylon by a technique termed replica plating (Grunstein & Hogness, 1975; Sambrook et al., 1989). Two copies of the master plate are made, and the newly generated colonies are then lysed, and their DNA denatured by treatment with NaOH. Oligonucleotides constructed from known sequence information are then tagged with a label (usually ^{32}P), and allowed to hybridize with the membranes containing the denatured DNA from colonies in the library. After proper washing, colonies containing complementary sequences to the labeled oligonucleotides can be visualized upon autoradiographic exposure (Figure 2.2) The corresponding colony on the master plate giving rise to a positive hybridization signal is

Figure 2.1: Generation of cDNA from isolated messenger RNA. An oligo(dT) primer is allowed to hybridize to the poly(A) tail of isolated cellular mRNA. The enzyme reverse transcriptase is then used to make a DNA copy of the mRNA. The RNA strand of the DNA-RNA hybrid is digested with the enzyme RNase H, while the concomitant use of DNA polymerase I fills in the gaps with the appropriate deoxynucleotides using the RNA fragments as primers. Upon completion a double-stranded DNA fragment is generated which contains almost the full length of the original mRNA. Sequences at the extreme 5′ terminus of the RNA are not converted into DNA due to the lack of a primer in this region for DNA pol I.


5'
mRNA
poly(A)
3'
Oligo(dT)
5'
reverse transcriptase
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mRNA
poly(A)
3'
3'
cDNA
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5'
Rnase H
+
DNA Pol I
Rnase H
5'
mRNA
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cDNA
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DNA Pol I
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poly(A)
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poly(A)
3'
3'
double-stranded cDNA
Oligo(dT)
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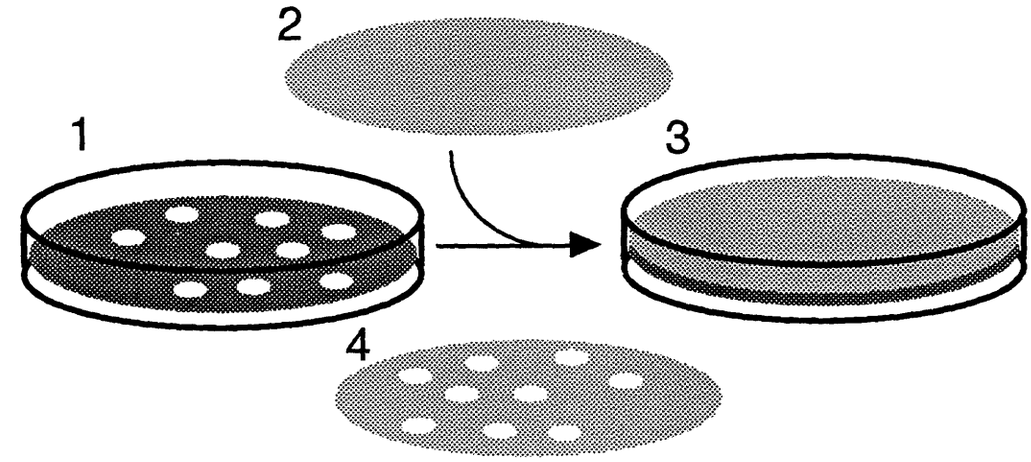
located, and its plasmid DNA isolated and sequenced.

The second technique used to identify genes in a library is termed cloning by complementation or phenotypic expression. This technique is based on a clone's ability to confer a distinct and easily identifiable phenotype to a host. Often this phenotype is the host's ability to grow in the absence of certain compounds or nutrients. This method of cloning is exemplefied by the cloning of the gene for β-galactosidase from *Lactobacillus bulgaricus* (Schmidt et al., 1989). Briefly, genomic DNA from *L. bulgaricus* was isolated, digested, and fractionated by electrophoresis on a polyacrylamide gel. Fragments ranging in size from 2-15 kb were ligated into a vector and transformed into *E. coli*. The colonies were plated on media containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). Clones containing the entire gene for β-galactosidase expressed the functional protein which cleaved the chromogenic substrate, turning the colony a blue color. These blue colonies were selected, and their corresponding plasmid DNA was isolated and their inserts sequenced.

The last method used to identify genes in a library is based on the ability of an antibody to recognize antigens produced by specific recombinants in the library (Young & Davis, 1983; Helfman & Hughes, 1987). Specifically, cDNA libraries are constructed and fused to a portion of the gene for the prokaryotic protein β-galactosidase. This fusion stabilizes foreign proteins which tend to be degraded by cellular proteases. Clones are titered on agar plates, and transferred to nitrocellulose as described for cloning a gene by hybridization. The colonies are lysed and then incubated with an antibody that was raised against the protein product for which the gene is being sought. After proper washing steps,

Figure 2.2: Isolation of a gene by hybridization to a complementary probe. A nitrocellulose filter **2** is pressed onto an agar plate (shown in **3**) containing the clones from a gene library of the organism of interest **1**. The clones are transferred to the nitrocellulose filter, generating a replica **4** of the master plate **1**. After the clones are lysed on the filter, the filter is placed inside of a pouch **5** containing the radiolabeled hybridization primer. The filter is removed, and after appropriate washing steps, subjected to autoradiography **6**. Positive clones are identified **7**, and the corresponding colonies from the master plate are isolated **8** and grown in liquid culture. The plasmid DNA corresponding to the positive colonies is isolated and sequenced.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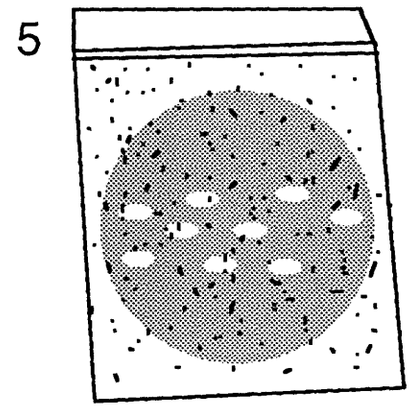
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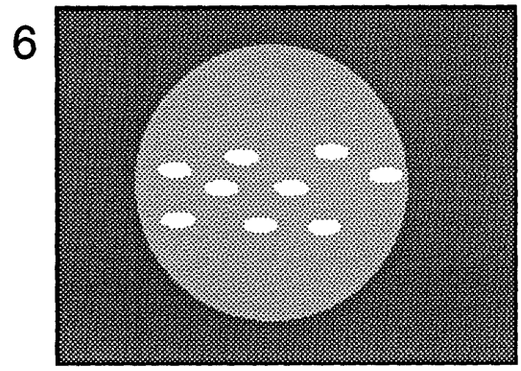
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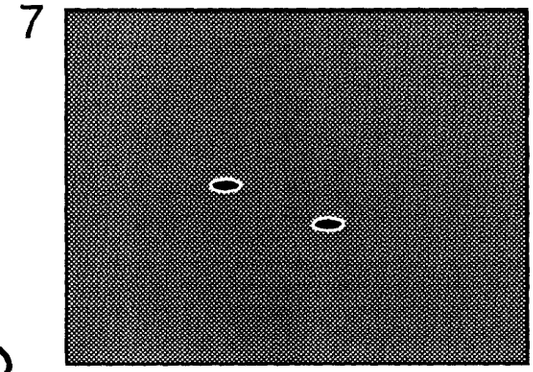
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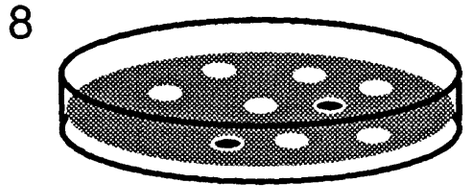

8

the membrane-antibody-antigen complex is allowed to react with an anti-IgG-alkaline phosphatase conjugate. Alkaline phosphatase-specific color reagents are then added, and positive clones turn a dark purple color. Other methods exist for detecting antibody-antigen complexes, however the alkaline phosphatase protocol tends to give more reliable results (Mierendorf et al., 1987).

With the above information in hand, a strategy for cloning the *L. leichmannii* ribonucleotide reductase was formulated. Given the degree of protein sequence information from previous active site labeling studies as well as labeling studies designed to locate cysteine residues involved in catalysis (Lin et al., 1987), it was decided that screening by hybridization would be the appropriate choice. The second technique, cloning by complementation, suffers from the difficulty in obtaining *E. coli* or yeast mutants deficient in the gene for ribonucleotide reductase; and screening with antibodies suffers from inherent background problems in antibody detection, and in the need to make cDNA libraries fused to β-galactosidase. This chapter describes the detailed methods used to isolate and sequence the gene for ribonucleotide reductase isolated from *L. leichmannii*.

Materials and Methods

Materials

Immobilon poly[vinylidene difluoride] (PVDF) membranes (0.45 μm pore) were purchased from Millipore. Nitrocellulose membranes (BA-85, 0.45 μm pore) were purchased from Schleicher & Schuell. Trypticase was purchased from BBL Microbiology Systems (Cockeysville, MD). Yeast extract, tryptose, and tween-80 were from Difco Laboratories (Detroit, MI). Polyethylene glycol (PEG) 20M was from Baxter Scientific Products (McGaw Park, IL). Lysozyme (specific activity, 63,000 units/mg), ampicillin, N-laurylsarcosine, mutanolysin (specific

activity, 6600 units/mg), ethidium bromide, Ponceau S, ficoll 400, gelatin, polyvinylpyrrolidone, bovine serum albumin (BSA) (fraction V), denatured salmon sperm DNA, and cysteine were from Sigma. [γ-^{32}P]ATP (6000 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. Nick columns were from Pharmacia. Sequenase was purchased from United States Biochemical Co. The dsDNA Cycle Sequencing system, T4 DNA ligase, agarose (Ultra Pure), and competent DH5α and HB101 cells were from GIBCO/BRL. AmpliTaq DNA polymerase was from Perkin-Elmer / Cetus. Centricons were purchased from Amicon, and restriction endonucleases, DNA molecular size markers (Lambda DNA-*Hin*dIII Digest), and T4 polynucleotide kinase were purchased from New England Biolabs. Phosphoramidites and other reagents required for DNA biosynthesis were supplied by Cruachem. All other reagents were of the highest possible grade.

Peptide Mapping

RTPR (0.5 mg; specific activity, 1.3 units/mg) was subjected to SDS / PAGE in a 3 mm-thick 10% (2.6% crosslinking) gel matrix. The gel was removed and soaked in transfer buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11 / 10% methanol] for 30 min, assembled in a Hoeffer TE 70 SemiPhor semi-dry transfer unit, and blotted to a nitrocellulose membrane (which had been wetted with water and soaked in the aforementioned transfer buffer) at 100 mA (constant current) for 3 h. The nitrocellulose membrane was removed and stained for 90 s in 0.2% Ponceau S / 1% acetic acid, and destained for 90 s in 1% acetic acid. The protein band corresponding to RTPR was excised with a razor blade, rinsed with water, and submitted for peptide mapping at the Harvard University microchemistry facility.

Synthesis and Purification of Oligomers

Primers **1, 2, I-1,** and **I-2** (Table 2.1) were synthesized on a Biosearch 8600 DNA synthesizer using standard solid phase phosphoramidite chemistry (Beaucage & Caruthers, 1981; Schott, 1985). The oligomers were cleaved from the solid support upon treatment with concentrated NH_4OH, and then heated overnight at 55°C in concentrated NH_4OH to remove the protecting groups on the exocyclic amines of deoxyadenosine, deoxycytidine, and deoxyguanosine. The oligomers were dried *in vacuo*, and then redissolved in H_2O. Approximately 1/3 of the oligomer was loaded onto a 15-20% acrylamide (0.8% Bis-acrylamide) / 7M urea gel, and subjected to electrophoresis using Tris-borate / EDTA buffer (9 mM Tris base, 9 mM boric acid, and 0.2 mM EDTA). The oligomers were visualized against a fluorescent TLC plate by UV shadowing (Biosystems, 1987), and the slowest migrating band was excised with a sterile razor blade and placed in a 2 mL plastic vial. A 1 mL aliquot of TE buffer [10 mM Tris-HCl (pH 8.0) / 1 mM EDTA] was added, and the acrylamide gel fragment was crushed with a glass stirring rod. The solution was then agitated overnight at 37°C to elute the oligomer. The acrylamide was pelleted by centrifugation, and the supernatant was removed and filtered through a membrane (0.45 μm Millex GV, Millipore) which had been prewetted in TE. The filter was washed further with a 1 mL aliquot of TE, and the two fractions were combined and loaded onto a Sep-Pak cartridge (Millipore) that had been pretreated with 10 mL of CH_3CN followed by 10 mL of H_2O. The Sep-Pak was washed with 10 mL of water, and the oligomer was eluted with a 50% solution of CH_3CN in 50 mM triethylamine-acetate buffer [(TEA)OAc], pH 7.6. The oligomer was dried *in vacuo* and redissolved in water to the approriate concentration. Oligomer concentrations were estimated using a computer program based on the algorithm of Cantor and Warshaw (Cantor & Warshaw,

1970; Fasman, 1975). Total yields, based on the OD_{260} loaded onto the gel and the OD_{260} obtained after purification, were typically 40%.

Table 2.1: Synthesized oligonucleotide primers used in cloning.

Primer	Sequence
1	5'd[CGC-GGA-TCC-GC(C/G/T/A)-GA(G/A)-TT(C/T)-AT(C/T/A)-GA(C/T)-(C/A)G]3'
2	5'd[GCG-GAA-TTC-(G/A)CA-(G/A)TC-(C/G/T/A)GT-(C/T)TG-(G/A)TC]3'
I-1	5'd[GAI-ITI-GAI-ITI-GTI-GAI-CAI-ACI-GAI-TGI-GAI-GGI-GGI-GCI-TGI-CCI-ATI-AA]3'
I-2	5'd[GCI-GAI-TTI-ATI-GAI-(C/A)GI-GTI-AAI-GCI-(T/A)(C/G)I-GTI-AAI-CCI-CA]3'

Isolation of High Molecular Weight Genomic DNA

Chromosomal DNA was isolated from *L. leichmannii* (ATCC 7830) grown in *Lactobacillus* carrying medium (Table 2.2) (Efthymiou & Hansen, 1962) supplemented with 1% dextrose and 20 mM D,L-threonine. One liter of *L. leichmannii* was grown at 37°C (without aeration) to an OD_{600} of ~7. The cells were pelleted by centrifugation, washed in 0.02 M Tris-HCl (pH 8.2), and then resuspended in 25 mL of the same buffer. Lysozyme (730 mg in 20 mL of the above buffer), PEG [M_r, 20,000; 50 mL of a 24% (wt / vol) solution], and mutanolysin (0.5 mg in 0.5 mL of the above buffer) were added to the resuspended cells, and the resulting mixture was incubated for 1.5 h at 37°C. The mixture was then centrifuged at 5000xg for 20 min. The supernatant was

discarded and the pellet was resuspended in 40 mL of TE (10 mM Tris-HCl, pH 8.0 / 1mM EDTA). A 5 mL aliquot of 10% (wt / vol) N-laurylsarcosine was

Table 2.2: Recipe for 1 L of *Lactobacillus* carrying medium.

Ingredient	Amount
Trypticase	10 g
Yeast extract	5 g
Tryptose	3 g
Dibasic potassium phosphate	3 g
Monobasic potassium phosphate	3 g
Ammonium citrate	2 g
Tween-80	1 g
Sodium acetate	1 g
Cysteine	0.2 g
Salt solution*	5 mL

*100 mL of salt solution contains 0.68 g ferrous sulfate (heptahydrate), 2.4 g manganous sulfate (monohydrate), and 11.5 g magnesium sulfate (heptahydrate)

added, and the resulting solution was incubated with agitation at 65°C for 15 min. Forty grams of CsCl and 4 mL of ethidium bromide at 10 mg/mL were added to the supernatant, and the solution was spun in a clinical centrifuge for 5-10 min. The resulting solution was underneath a layer of precipitate, and was removed with a syringe fitted with a 15 gauge needle and placed into Beckman polyallomer quick-seal ultracentrifuge tubes. The samples were spun in a Beckman ultracentrifuge (model L-8) for 44 h at 48,000 rpm and 20°C. Genomic DNA banded in the middle of the ultracentrifuge tube, and was removed with an

18 gauge needle. The ethidium bromide was removed by repeated extractions with n-butanol. The volume of the resulting solution was doubled by addition of TE, and the DNA was precipitated with 2 volumes of ethanol equilibrated at room temperature. The DNA was hooked with a bent Pasteur pipet, and subjected to a gentle stream of air to remove the residual ethanol. The DNA was then dissolved in TE at a concentration of 1.2 mg/mL, determined by the relationship: 1 A_{260} = 50 μg/mL of double-stranded DNA.

Southern Transfers

Approximately 10-20 μg of *L. leichmannii* genomic DNA was digested with *Eco*R1 (20 U), *Hin*dIII (20 U), *Pst*I (40 U), or *Bam*HI (30 U), in a volume of 100-200 μL for 2 h. Another equal aliquot of the appropriate enzyme was added, and the digestion was allowed to continue for 3 h. The reaction was terminated with the addition of EDTA to a final concentration of 10 mM. The DNA was precipitated overnight at -20°C with 3M $(NH_4)OAc$ and 2 volumes of absolute ethanol. The DNA was dried *in vacuo* and redissolved in 20 μL of H_2O. A 5 μL aliquot of gel loading buffer (20 mL contains 3 g ficoll 400, 40 mg bromphenol blue, 40 mg xylene cyanol, and 50 mM EDTA) was added and the samples were subjected to electrophoresis in a 1% agarose matrix at 20 V for 12 h. The gel was removed and the DNA was transferred to a Genescreen Plus hybridization membrane (Dupont, New England Nuclear) by standard molecular biolgical techniques (Ausubel et al., 1987) according to the orginal procedure of Southern (1975). The acid depurination step was omitted as it was found to be unnecessary for the efficient transfer of the DNA. Upon completion, the hybridization membrane was removed and immersed in a solution of 0.4 N NaOH for 1 min, and then neutralized in a solution of 0.2 M Tris-HCl (pH 7.5) /

2xSSC (0.3 M NaCl / 0.03 M NaCitrate). The membrane was removed and allowed to dry on a sheet of Whatmann 3MM paper at ambient temperature.

Radiolabeling of Oligomers

Oligonucleotides used for hybridization were 5′ end-labeled with T4 polynucleotide kinase (PNK). The reaction consisted of 10-30 pmols of the oligomer, 250 μCi [γ-^{32}P]ATP (6000 Ci/mmol), 4 μL of 10x PNK buffer [0.5 M Tris-HCl (pH 7.6) / 0.1 M $MgCl_2$ / 50 mM dithiothreitol / 1 mM spermidine / 1 mM EDTA (pH 8)], and 30-50 U of PNK. The final volume was adjusted to 40 μL with H_2O, and the reaction was incubated at 37°C for 45-60 min. The radiolabeled oligomer was separated from unreacted [γ-^{32}P]ATP using a Nensorb cartridge (Dupont / New England Nuclear) with the following modifications of the manufacturer's specifications. After loading the reaction mixture onto the Nensorb cartridge, the cartridge was washed with 50 mL of buffer A (100 mM Tris-HCl (pH 7.7) / 10 mM triethylamine / 1 mM EDTA), followed by 3 mL of H_2O. The oligomer was then eluted with a 1 mL aliquot of a 50% methanol / 50% buffer A solution. It was frozen in liquid nitrogen, dried *in vacuo*, and redissolved in 500 μL of H_2O. A 1 μL aliquot was subjected to scintillation counting on a Packard 1500 liquid scintillation analyzer using 8 mL of S¢INT-AF scintillation cocktail (Packard). A lower limit for the specific activity of the labeled oligomer was calculated from the amount of oligomer used in the labeling reaction, and was typically 3-15x10^8 cpm/μg.

Screening of L. leichmannii Genomic DNA

Filters bearing Southern transfers were placed inside of heat sealable pouches (Kapak Co.), and prehybridized at 63-65°C for 10-15 h in 10 mL of 6xSSC / 50 mM sodium phosphate (pH 6.8) / 5x Denhardt's solution (1x contains

ficoll, polyvinylpyrrolidone, and BSA, each at concentrations of 0.2 mg/mL) / 200 μg/mL denatured salmon sperm DNA / 1% sodium dodecyl sulfate (SDS) / and 2 mM EDTA (pH 8). After prehybridization the solution was removed and replaced with 10 mL of the same solution containing labeled probe at a concentration of 2-20x10^6 cpm/mL. The filters were incubated with the probe for 36 h at 37-50°C. After hybridization, the filters were removed and washed twice in 100 mL of 2xSSC at room temperature. They were again washed twice for 30 min each time in 500 mL of 2xSSC / 1% SDS at 50°C, and then twice at room temperature in 100 mL of 0.1xSSC. The membranes were blotted dry of excess liquid, secured in Saran Wrap, and exposed to a phosphorimager plate (Molecular Dynamics) for 12 h.

Isolation of 6.6 kb HindIII Fragments and Construction of a Subgenomic Library

L. leichmannii genomic DNA (200 μg) was digested with *Hin*dIII (100 U) for 4-6 h in a total volume of 200 μL. The reaction was quenched by the addition of EDTA (pH 8) to a final concentration of 12 mM, and divided into 10 equal aliquots. Each sample was loaded into a separate well of a 1% agarose gel, and the DNA was subjected to electrophoresis for 12 h at 30 V using Tris-acetate EDTA (TAE) buffer. The gel was stained for 30 min in a 0.5 μg/mL solution of ethidium bromide. The gel was visualized with a hand-held UV lamp, and the region of the gel which contained DNA fragments that migrated with the 6.6-kb molecular size marker (*Hin*dIII cut λ DNA) was excised. This agarose plug was placed inside of dialysis tubing (3000 M.W. cutoff) and TAE buffer was added to just cover the plug. The dialysis bag was sealed and placed into a horizontal electrophoresis chamber, and the assembly was subjected to 60 V for 4 h. Upon completion, the leads from the power supply were reversed, and a potential difference of 60 V was applied for 2 min. The dialysis bag was removed from the

electrophoresis chamber and massaged to prevent DNA from adhering to its walls. The buffer was removed with a Pasteur pipet and extracted twice with a 1:1 buffer-saturated solution of phenol / chloroform / isoamyl alcohol (25:24:1). The DNA fragments were precipitated with ethanol (0.3 M NaOAc) and dried *in vacuo*. The DNA was redissolved in 100 μL of H_2O and then desalted on a Pharmacia Nick column. A UV spectrum was recorded to check the concentration and purity of the DNA fragments. The DNA solution was then lyophilized to dryness and adjusted to the appropriate concentration with TE.

The Polymerase Chain Reaction (PCR)

The PCR mixture contained in a total volume of 100 μL: 0.5-1.5 μg of genomic DNA, 58 pmol of primer **1**, 29 pmol of primer **2**, all four deoxynucleoside triphosphates (dNTPs) (each at 0.2 mM), and 10 μL of 10x PCR buffer (500 mM KCl / 100 mM Tris-HCl pH 8.3 / 15 mM $MgCl_2$ / 0.1% gelatin). The mixture was overlaid with 100 μL of paraffin oil and heated at 94°C for 5 min. *Taq* polymerase (2.5 U) was added under the oil layer, and 35 cycles of the following program were run: 1 min at 94°C, 30 s at 37°C, 15 s at 50°C, and 2 min at 72°C. Upon completion, the paraffin oil was extracted with 150 μL of $CHCl_3$, and the DNA was precipitated with ethanol. The DNA was dissolved in water and desalted by several dilutions and centrifugations in a Centricon 100. To complete any unfinished sequences generated during the amplification, the PCR mixture was treated with Sequenase in the following manner. The reaction contained (in a final volume of 500 μL) all four dNTPs (each at 0.1 mM), 1x *Hin*dIII restriction buffer (50 mM NaCl / 10 mM Tris-HCl, pH 7.9 / 10 mM $MgCl_2$ / 1 mM DTT), 10 U of Sequenase, and the PCR product. The reaction was incubated at 30°C for 30 min and stopped by the addition of EDTA to a final concentration of 10 mM. The DNA was purified by electrophoresis in a 1%

agarose gel, and the 2.1-kb piece was isolated by electroelution into dialysis tubing as described for isolating the 6.6-kb *Hin*dIII fragments. The DNA was concentrated and exchanged into TE using a Centricon 100, and its final concentration was determined using the relationship that 1 A_{260} unit corresponds to 50 μg/mL of double-stranded DNA.

Cloning and Sequencing of the Fragment Isolated by PCR

The blunt-ended PCR fragment was ligated into pUC19 that had been digested with *Sma*I. The reaction mixture contained (in a final volume of 10 μL) 100 ng of pUC19, 288 ng of the blunt-ended PCR fragment, 1 μL of 10x ligase buffer [500 mM Tris-HCl (pH 7.8) / 100 mM $MgCl_2$ / 200 mM DTT / 10 mM ATP / BSA (500 mg/mL)], and 7 Weiss units of T4 DNA ligase. The reaction mixture was incubated overnight at 16°C and then diluted to 200 μL with TE buffer. A 5 μL aliquot was used to transform competent *E. coli* DH5α. The transformation reaction was plated on SOC agar (Sambrook et al., 1989) containing X-Gal, and recombinants were identified by their white phenotype. Plasmid DNA was isolated from overnight cultures of several of the white colonies using a Qiagen plasmid Mini I kit according to the manufacturer's specifications, and screened for inserts by appropriate restricition digestion and agarose gel electrophoresis .

DNA sequencing was carried out using the dsDNA Cycle Sequencing system. Oligonucleotide primers used for DNA sequencing (Tables 2.3 and 2.4) were obtained from the MIT Biopolymers Laboratory, or Oligos Etc. of Wilsonville, OR. The universal primer was used to initiate the sequencing process. All subsequent primers (21-25 bases long) were designed using the new sequence data from each successive round of sequencing. Approximately 200-300 ng of either the cloned or uncloned PCR fragment and 1.5-5 μg of genomic

DNA were used for each sequencing reaction mixture. The sequence of both strands of the PCR fragment was determined.

Table 2.3: Forward oligonucleotide primers used in sequencing RTPR.

Primer	First base of RTPR added	Primer Sequence
Universal (forward)	1	5'd(CCC-AGT-CAC-GAC-GTT-GTA-AAA-CG)3'
SPF1	276	5'd(AGC-GCT-CTA-CAA-GCT-GAT-CTA-CGG)3'
SPF2	544	5'd(GCT-TCT-CAG-TTG-CCA-GAT-CCA-ACA)3'
SPF3	NONE	5'd(GAT-GCT-GAC-AGC-ATC-TAC-TAC-CGC)3'
SPF4	603	5'd(GAA-TCC-TAC-GAC-GCT-TCC-GTC-AAG)3'
SPF5	691	5'd(GAT-ACC-CGG-GAG-GCT-GGG-TTT-TGG)3'
SPF6	877	5'd(ATG-CCC-TTG-ATC-TCC-ATG-CTG-CTG)3'
SPF7	1035	5'd(AAG-CAG-GAC-CAA-GAG-AAG-CTG)3'
SPF8	NONE	5'd(GCG-GAA-GGG-ACC-AAC-CCT-GCG)3'
SPF9	1202	5'd(CTA-CCA-GGC-TGG-AAT-TGA-CGG)3'
SPF10	1383	5'd(CAA-GCG-GGT-AAC-CTT-CAG-TCC)3'
SPF11	1494	5'd(GTT-ACC-GGC-TTC-AAG-GAT-GAC)3'
SPF12	1647	5'd(CAA-TCA-AGC-ACA-CCA-CGG-TCA-AG)3'
SPF13	1837	5'd(GCG-TGG-AAT-TCC-CGA-TCA-AGG)3'
SPF14	2006	5'd(GGT-TGA-ATC-CTT-GCT-CCG-CCA-GTA-CC)3'
SPF15	2145	5'd(CCG-GCA-ACG-TGG-AGG-AAG-TCT-TCA-G)3'

Table 2.4: Reverse oligonucleotide primers used in sequencing RTPR.

Primer	First base of RTPR added	Primer Sequence
SPR1	184	5'd(GCT-CTG-CTT-CAG-TTC-CAG-ACT-TGG)3'
SPR2	351	5'd(CAT-ACT-TTT-GCG-GAC-GGA-TGG-CCA)3'
SPR3	483	5'd(GAT-CTG-GCA-ACT-GAG-AAG-CCA-ACC)3'
SPR4	607	5'd(GCG-GTA-GTA-GAT-GCT-GTC-AGC-ATC)3'
SPR5	809	5'd(CAG-CAG-CAT-GGA-GAT-CAA-GGG)3'
SPR6	1056	5'd(AGC-CGC-TGA-AGG-CTG-AAT-CAA-CTG)3'
SPR7	1249	5'd(GAA-GAC-TTC-AAA-GAG-GTT-GCA-AGG)3'
SPR8	1432	5'd(TTG-CCC-AGT-CTG-GTC-AAA-AGC-CAG)3'
SPR9	1643	5'd(CCA-ACT-TGG-CCA-CCG-TGC-CTG-ATG)3'
SPR10	1844	5'd(AGC-TGA-GGC-AAA-GTT-AGG-GTT-GTC)3'
SPR11	2026	5'd(CCA-ACT-TGG-CCA-CCG-TGC-CTG-ATG)3'
Universal (Reverse)	2220	5'd(AGC-GGA-TAA-CAA-TTT-CAC-ACA-GGA)3'

Results

Peptide Mapping

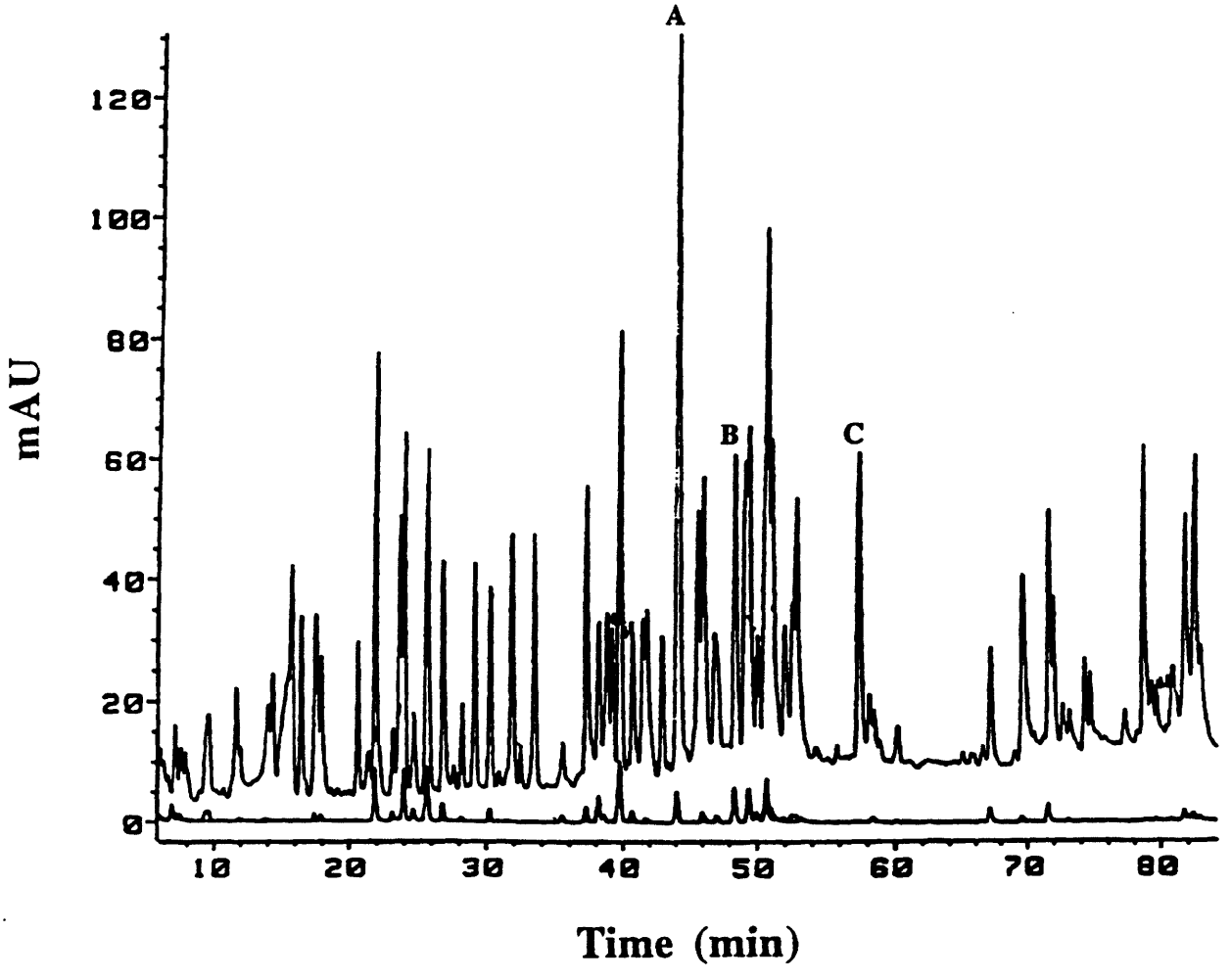
Protein sequence information is required to clone a gene using hybridization techniques. Several methods are generally employed to obtain relevant protein sequence information. One method is to selectively alkylate an active site residue with a mechanism-based inhibitor, photoaffinity label, or side chain-specific reagent. As described in Chapter 1, this was indeed done for RTPR (Lin et al., 1987). By exploiting the reversible disulfide formation during turnover, cysteines which become oxidized concomitant with substrate reduction were selectively alkylated with [^{14}C]iodoacetamide. The protein was then cleaved with trypsin, and the peptides containing the radiolabeled cysteines were isolated. The sequence of these peptides was then determined by automated Edman degradation.

A second method involves performing Edman degradation on the intact protein, and gives sequence information at the N terminus of the protein. Although this is less labor intensive than obtaining sequence information from the active site, the absence of a radioactive label necessitates that very pure protein be isolated to ensure that the N-terminal sequence gained is from the correct protein. In the previously described method the presence of a label gained through a mechanism-based inactivator, photoaffinity label, or side chain-specific reagent, provides confidence that the peptide sequence obtained is from the relevant protein. Another drawback of performing Edman degradation on an intact protein is that many proteins are blocked at the N terminus with N-formylmethionine. This blocked amino acid makes the protein resistant to N-terminal sequencing. Lastly, this procedure only gives information about one region of the protein.

Recent advances in protein and peptide microsequencing have resulted in the ability to gain sequence information from many regions of a protein with a much lower probability of sequencing contaminating proteins (Aebersold et al., 1987; Lane et al., 1991). In this technique, the appropriate protein is first subjected to SDS / PAGE to separate it from other proteins of different sizes. The gel is then blotted to a PVDF or nitrocellulose membrane, and the membrane is stained with a protein specific dye. The band corresponding to the appropriate protein is excised, and the protein is digested with the protease, trypsin, while still on the solid support. This treatment produces small peptides which have a lower affinity for the membrane, and as a result elute into the buffer during digestion. These peptides are then resolved by HPLC, and several well-separated fragments are then subjected to Edman degradation. The major advantage in this procedure is that a large amount of internal sequence information can be gained. This greatly facilitates not only the cloning of a particular gene, but also aids in maintaining the proper reading frame when sequencing the gene.

This last procedure was used to gain sequence information from RTPR. The protein was purified to near-homogeneity, and then subjected to SDS / PAGE. It was electroblotted onto a PVDF membrane or a nitrocellulose membrane. The N-terminal sequence of RTPR was determined by automated Edman degradation from the protein on the PVDF membrane. Internal sequence information, as described, was obtained by digesting the nitrocellulose-bound protein with trypsin, separating the eluted peptide fragments by HPLC, and sequencing several of the fragments by automated Edman degradation. Figure 2.3 is the peptide map of RTPR generated by trypsin digestion. Peptides **A**, **B**, and **C** were isolated and their sequences determined. In all cases each isolated peak gave very small amounts of secondary and / or tertiary sequences

Figure 2.3: Peptide map of RTPR generated by trypsin digestion. RTPR was blotted to a nitrocellulose membrane as described, and submitted to the Harvard University Microchemistry Facility. It was digested with trypsin while still on the membrane, and the eluted fragments were separted by HPLC. Peaks **A**, **B**, and **C** were isolated and sequenced by Edman degradation.


A
B
C
120
100
80
60
40
20
0
mAU
10
20
30
40
50
60
70
80
Time (min)

from similarly-migrating peptides. These extra sequences, in cases where the amino acids were reported with a high confidence level, were an added bonus and extended the amount of peptide sequence information. Table 2.5 lists the sequence information obtained from peptide mapping, N-terminal sequencing, and the active site labeling studies (Lin et al., 1987) previously described in Chapter 1.

Table 2.5: Peptides of *L. leichmannii* RTPR.

Method	Peptide Sequence
N-terminal sequencing	SEEISLSAEFIDRVKASVKPH
Active site labeling studies	TGDSLNNCWF
Active site labeling studies	DLELVDQTDCEGGACPIK
Peptide mapping studies (peak B)	RVTFSPYDWEISR
Peptide mapping studies (peak A)	VVTGFKDDFDPETH(E)AIKVPVYDKR
Peptide mapping studies (peak C)	SQEITGNVEEVFSQLDSDVK
Peptide mapping studies (peak C)	LILDLS_I(R)PY
Peptide mapping studies (peak A)	(L)GAV_ _DELVQDAD(W)IYI(R)

Parentheses indicate residues which are possible, but of low confidence

Design and Construction of Hybridization Probes and PCR Primers for the Polymerase Chain Reaction (PCR)

Although isolating a gene may be somewhat akin to "searching for a needle in a haystack," as described in the introduction to this chapter, the work of Wallace and Wood (Wallace & Miyada, 1987; Wood, 1987) has resulted in a very

elegant method of locating a gene based on making hybridization probes complementary to sequences within it. Since these probes are generated from peptide sequences, however, one obstacle becomes apparent. The degeneracy of the genetic code requires that each amino acid in a peptide be represented by all of its synonyms. This usually necessitates that a hybridization probe be actually a mixture of all of the possible nucleic acid combinations that can encode a particular peptide. Since the probability that a particular sequence will occur more than once within a genome depends (to a first approximation) on the length of the sequence, a hybridization probe must be at least 15-16 nucleotides in length to confer the degree of specificity needed to isolate one particular gene from thousands of others. This calculation is shown in Scheme 2.1, where $3x10^9$ bp is the size of a mammalian genome, 4 is the number of different deoxynucleic acid bases, and x is the length of an oligonucleotide.

Scheme 2.1: Calculation showing the minimum length of an oligonucleotide necessary for its sequence to occur only one time in a mammalian genome.

$$4^x = 3x10^9$$

$$x\ (\log 4) = \log 3x10^9$$

$$x\ (0.6021) = 9.477$$

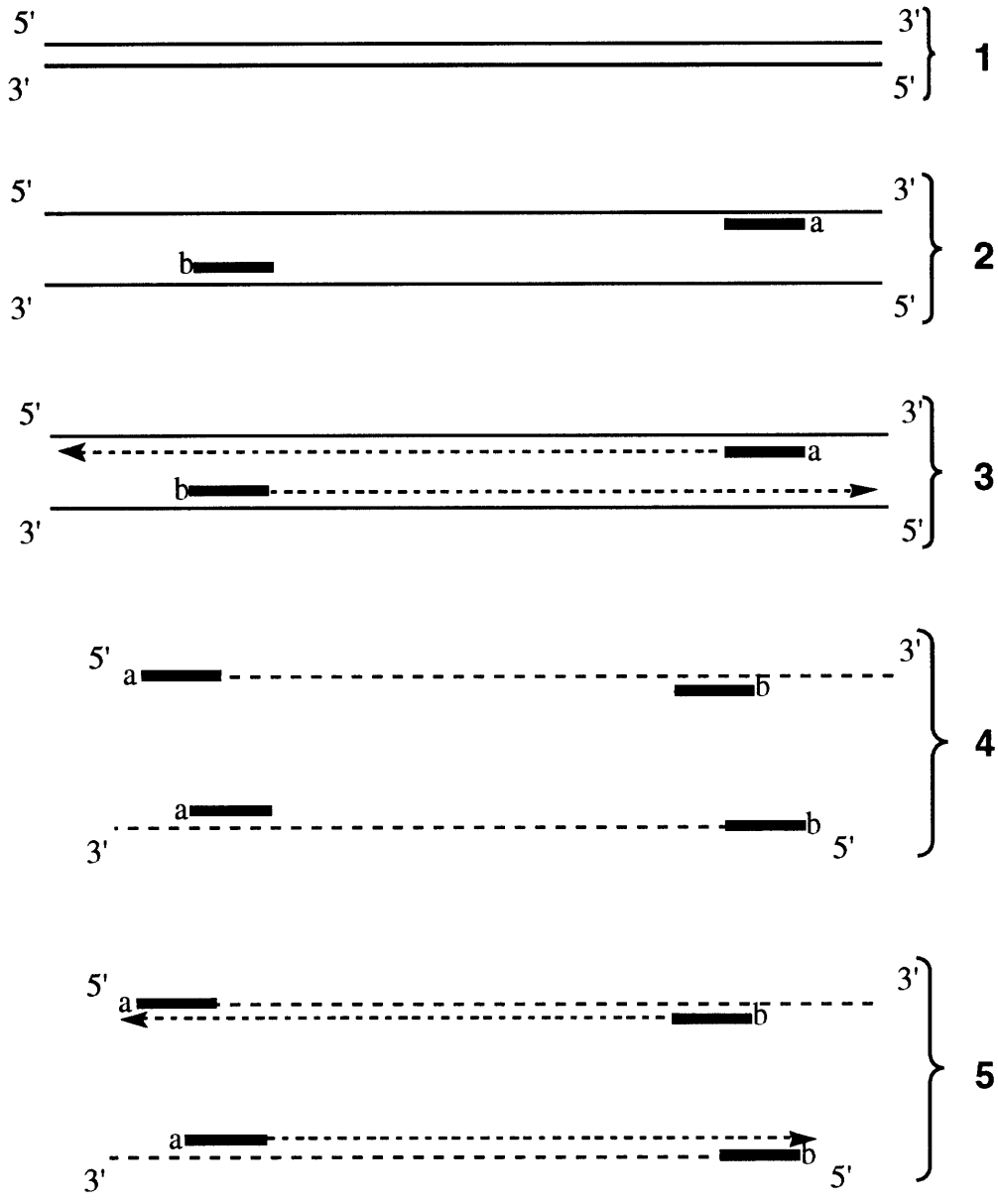
$$x = 15.7$$

Contrarily, added length also increases the degeneracy of a hybridization probe; the degree of which depends on the amino acid sequence. One method

used to reduce the complexity of a probe is to choose peptide sequences with stretches of amino acids having low or no degeneracy. This is certainly not always possible, especially when limited peptide sequence information is available. A second method used to overcome this problem is to substitute deoxyinosine at positions of degeneracy (Ohtsuka et al., 1985; Takahashi et al., 1985). Inosine has the unique ability to form hydrogen bonds with the other four nucleic acids (Martin & Castro, 1985). Although this substitution does not confer specificity, these probes are usually designed to be quite long, with specificity being determined by the other non-degenerate base pairings.

Recently a new method based on the polymerase chain reaction (PCR) has gained popularity as a tool for coping with the degeneracy of oligonucleotide probes. The polymerase chain reaction is a technique which allows stretches of DNA to be amplified several million fold (Bloch, 1991). This technique is based on the ability of DNA polymerase I to elongate primers that are hybridized to regions of genomic DNA. The procedure is usually performed under conditions in which the primers are in vast excess over the template DNA. After each cycle, the temperature of the reaction is raised and the newly synthesized strand dissociates from the template DNA. Two new templates are thus generated for each parental template, resulting in an exponential amplification of the target sequence. Subsequent cooling of the reaction allows a new set of primers to anneal, permitting the process to be repeated (Figure 2.4). The isolation of the thermostable polymerase from the bacterium *Thermus aquaticus* has allowed PCR to be automated, and indeed the procedure is now almost always carried out in thermocyclers. Before the isolation of *Taq* polymerase, it was necessary to add DNA polymerase I at the beginning of each cycle.

Figure 2.4: Amplification of a target sequence by PCR. Double-stranded DNA **(1)** is subjected to a heat denaturation step to melt the two strands. Subsequent cooling allows primers (made such that polymerization takes place towards the opposing primer) to hybridize to complementary sequences on the DNA target molecule **(2)**. The temperature is raised, and the primers are extended with *Taq* polymerase until the end of the template is reached, or the polymerase falls off **(3)**. The newly-generated double stranded DNA is again melted by heat, and a new set of primers are allowed to anneal **(4)**. After extension, a second set of double-stranded DNA is generated **(5)**. This procedure is usually repeated for thirty cycles, resulting in an exponential amplification of the region of the DNA between the two primers.

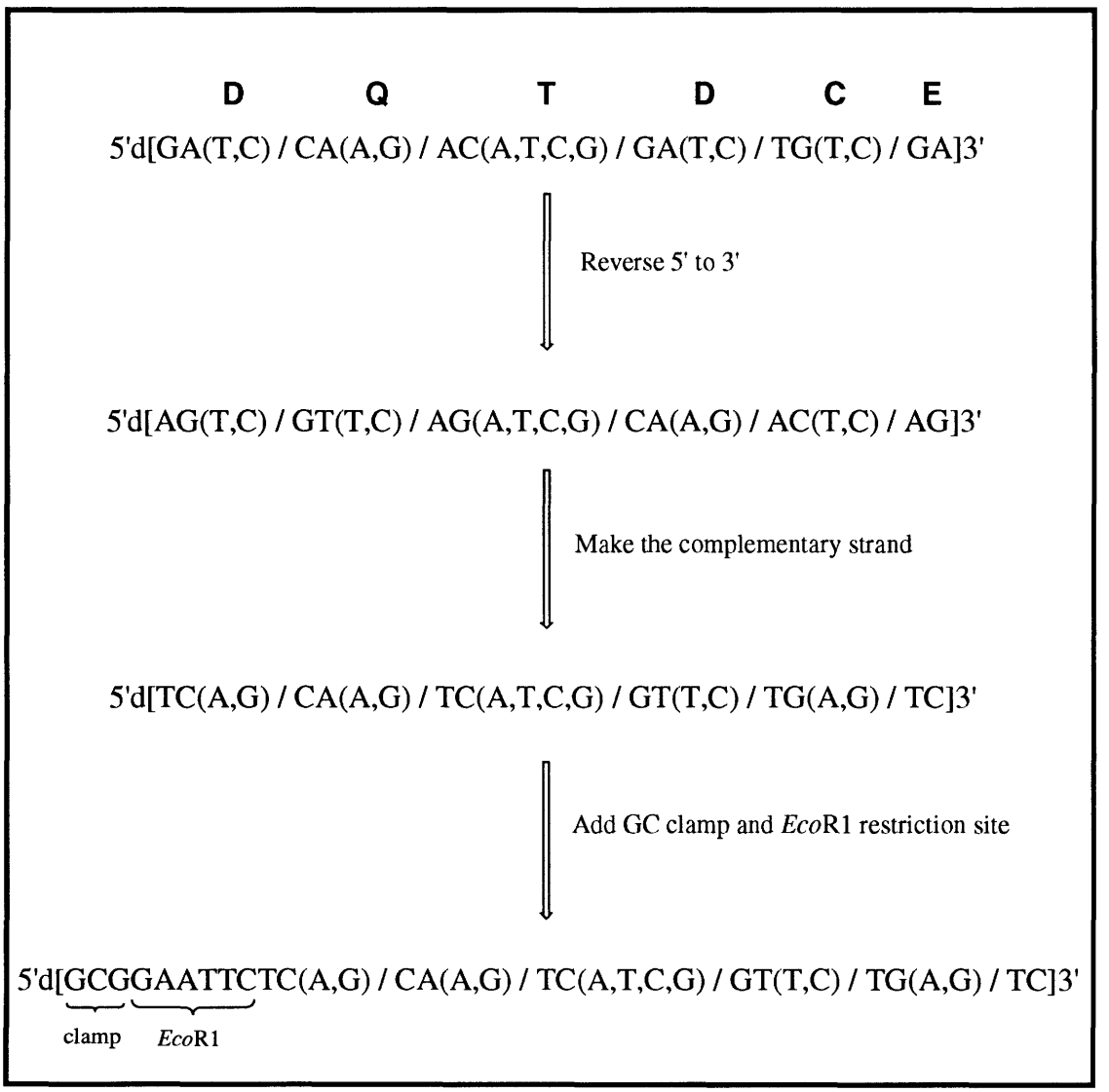

5'
3'
3'
5'
1
5'
3'
a
b
3'
5'
2
5'
3'
a
b
3'
5'
3
5'
a
3'
b
3'
5'
4
5'
a
3'
b
3'
5'
5

PCR has proved to be an invaluable tool for many involved in gene cloning. Using sequence information gained from the various aforementioned techniques, degenerate primers (~15-21 bp in length) to be used for PCR are synthesized by standard techniques. The general strategy is to find conditions (by modulating variables such as annealing times and temperatures, as well as the various concentrations of the components in the reaction mixture) such that only the correct primer anneals to the correct target sequence. This newly-amplified DNA can be sequenced, and then subsequently used as a hybridization probe to screen a gene library. An exact-match probe facilitates the screening of a library because very stringent conditions can be used in the hybridization and washing processes. This in turn greatly reduces the number of false positives, or clones that have primers annealed incorrectly to portions of their DNA. Because of the ease with which PCR can be performed, as well as the ability to do many reactions (each with varying concentrations of the reaction components) at once, with enough effort conditions can usually be found that result in the amplification of the desired target sequence.

From the N-terminal sequence of RTPR, amino acids 8-13 (AEFIDR) were chosen from which to construct a 192-fold degenerate PCR primer. A *Bam*HI restriction site was engineered at the 5' end of the primer to facilitate cloning any PCR product generated. As well, a 3 base GC clamp was added to aid the restriction endonuclease in cleaving at the extreme terminus of a DNA fragment. Also from the N-terminal sequence (AEFIDRVKASVKPH), a 41 base hybridization probe containing inosine at degenerate positions in the third base of each codon was constructed. From the peptide sequence gained from active site labeling studies (DQTDCE), a second PCR primer was constructed. This primer was 64 fold degenerate and contained in addition to a 3 base GC clamp, an *Eco*R1 restriction site at its 5' end. Because the two PCR primers must anneal

to opposite strands of the DNA in such a way that polymerizaton proceeds towards the opposing primer, the sequence of primer **2** is actually the complement of what its peptide sequence would predict, and its nucleic acid sequence is reversed 5' to 3' (Scheme 2.2). A deoxyinosine-containing probe was

Scheme 2.2: Design of reverse PCR primer.



also constructed from the active site labeling peptide (DLELVDQTDCEGGACPIK). It is 53 bases long, and deoxyinosine is placed at every position of degeneracy rather than just ones occuring in the wobble position. Both PCR primers and both deoxyinosine probes were synthesized on a Biosearch 8600 DNA synthesizer. The deoxyinosine-containing primers were purified by PAGE before use. This was necessary because the stepwise nature of the synthesis of oligomers results in failure sequences, the extent of which depends to a large degree on the length of the oligomer. Shortened sequences in combination with the non specific inosine nucleotide at one third of all bases in the primer could lead to unacceptable background levels upon hybridization.

Initial Attempts to Clone the L. leichmannii Ribonucleotide Reductase

Early attempts to clone RTPR were centered around an *L. leichmannii* gene library supplied by Dr. Steven Short of the Wellcome Research Laboratories in Research Triangle Park, N.C. The genomic DNA library consisted of 23 independent libraries termed zoos. Each zoo was constructed by cloning fragments from *Sau*3A partial digests of genomic DNA into the vector pUC13. The vector had been pretreated with the enzyme calf-intestinal alkaline phosphatase (CIP) to remove the 5′ terminal phosphates. This treatment prevents the vector from recircularizing; however, foreign DNA fragments can be successfully inserted if their ends are compatible and if they contain 5′ terminal phosphates. Although the resulting chimera contains 2 nicks, (one on each strand) it can be transformed into a suitable host without a significant loss in efficiency.

Each zoo was transformed into *E. coli* DH5α, and plasmid DNA was isolated by the boiling miniprep method (Ausubel et al., 1987). Large amounts of exceptionally clean plasmid DNA were isolated by CsCl / ethidium bromide

equilibrium centrifugation, subsequent to lysis by the boiling method (Ausubel et al., 1987). DNA from each zoo was subjected to restriction digestion with the endonucleases *Eco*R1, *Xba*I, and *Hin*dIII, and then analyzed by electrophoresis in a 0.7-1% agarose gel. Figure 2.5 is a photograph of a restriction analysis of zoos 21, 22, and 23, and represents how the overwhelming majority of the other zoos appear when they are treated in an analogous manner. What stands out is the large band which is present when the plasmid DNA is untreated, or when it is digested with either of the aforementioned restriction endonucleases. Since closed-circular and supercoiled DNA migrate differently than linear DNA, this band migrates at ~3 kb when the DNA is linearized, and 2 kb when the DNA is uncut. The presence of this fragment in both digested and undigested DNA, as well as its difference in migration rate upon digestion, suggests that this fragment is pUC13 containing no insert. Since pUC13 was treated with CIP to avoid generating clones lacking inserts, it appears that many recombinant plasmids lose their inserts at some point between their construction and their isolation subsequent to transformation into *E. coli* DH5α.

Several of the zoos were subjected to restriction digestion and Southern hybridization with the deoxyinosine-containing probes as described in the experimental section for the screening of *L. leichmannii* genomic DNA. As seen in Figure 2.6, two zoos (14, and 22), gave very distinct hybridization signals when probed with primer **I-1**. In addition, zoo 22 gave a hybridization signal when probed with primer **I-2** (data not shown). Attention was thus focused on zoo 22 with the desire to find specific clones containing sequences complementary to the deoxyinosine-containing probes. *E. coli* (DH5α) bacteria containing the zoo 22 clones were grown and plated on agar-containing media. Colonies were transferred to nylon membranes, and prepared for colony hybridizations by standard procedures (Ausubel et al., 1987; Sambrook et al., 1989). Approximately

Figure 2.5: Restriction analysis and gel electrophoresis of zoos 21, 22, and 23 (ethidium bromide stained). Lane 1 is undigested DNA from zoo 21. Lane 2 is DNA from zoo 21 digested with *Eco*RI. Lane 3 is undigested DNA from zoo 22. Lane 4 is DNA from zoo 22 digested with *Eco*RI. Lane 5 is undigested DNA from zoo 23. Lane 6 is zoo 23 digested with *Eco*RI. Lane 7 contains the molecular size markers (*Hin*dIII digest of λ DNA).

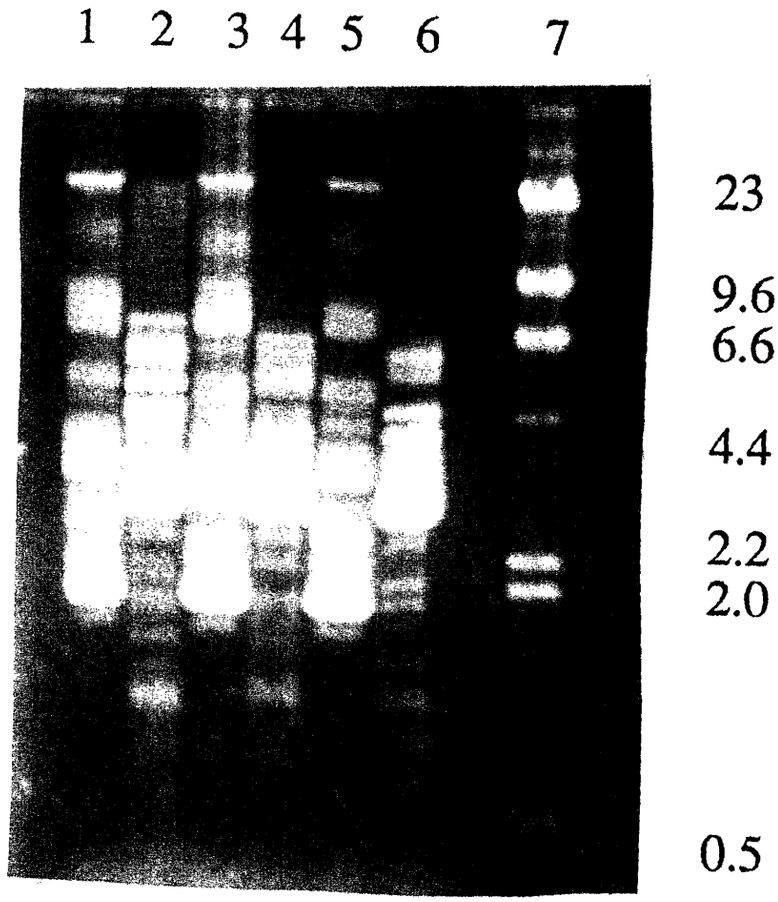
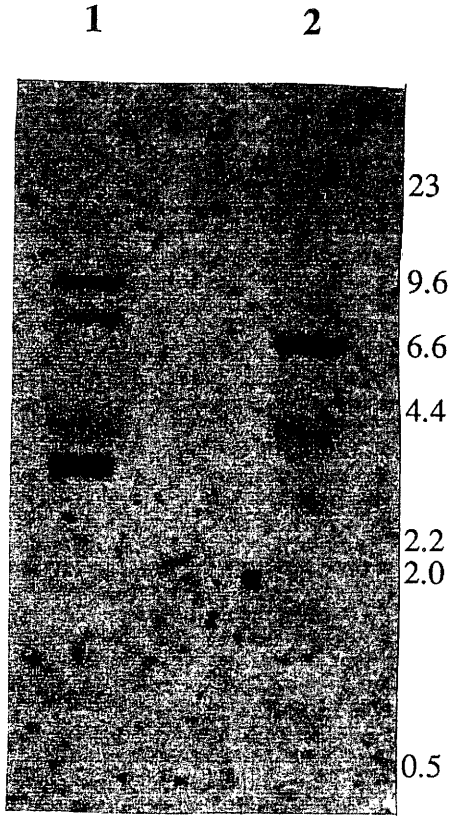

1 2 3 4 5 6 7
23
9.6
6.6
4.4
2.2
2.0
0.5

Figure 2.6: Phosphorimage of Southern transfer and hybridization with primer **I-1**. Lane 1 is zoo 14 digested with *Eco*RI. Lane 2 is zoo 22 digested with *Eco*RI. The numbers at the perimeter of the phosphorimage indicate the approximate positions of the molecular size markers (*Hin*dIII digest of λ DNA).


1
2
23
9.6
6.6
4.4
2.2
2.0
0.5

1500 colonies were screened with primer **I-2**, but no true positive clones were obtained.

Since agarose gel analysis of zoo 22 suggested that 30-50% of the clones contained no sizeable insert, an attempt was made to enrich the relevant DNA in this zoo by removing the region of hybridization and transforming it into a bacterial host. Uncut plasmid DNA from zoo 22 was subjected to Southern hybridization with primer **I-2**, and the region of the agarose gel which migrated similarly to the hybridization signal was removed. The DNA was purified and transformed into competent *E. coli* HB101. Colony hybridizations were performed as previously mentioned; however, again no true positive clones were obtained. Miniprep analysis of several of the transformation mixtures showed two bands. A thin band which migrated as a 5-kb piece of linear DNA, and a huge band which migrated as a 3-kb piece of linear DNA were present. The thin band did not hybridize to primer **I-2**. This experiment with zoo 22 suggested again that native *L. leichmannii* DNA is unstable in this particular construct.

Isolation of High Molecular Weight Genomic DNA from L. leichmannii

Many gram-positive bacteria, especially *Lactobacillae,* are resistant to most common methods of lysing the bacterial cell wall. However, stable spheroplasts of a variety of gram-positive bacteria can be prepared using lysozyme in a low ionic strength buffer, and PEG as an osmotic stabilizer (Chassy, 1976). These spheroplasts can then be lysed by treatment with a detergent such as SDS. High molecular weight genomic DNA from *L. leichmannii* was isolated using procedures developed by Bruce Chassy. *L. leichmannii* was grown in LCM to an OD_{600} of ~7. Care was taken to not allow the bacteria to remain in stationary phase for extended periods, since stationary phase cells are more resistant to lysis. In addition, D,L-threonine was added to the growth media as it has been

purported to interfere with cell wall cross-linking, resulting in cells that are more easily lysed (Chassy, 1976; Chassy & Giuffrida, 1980). Spheroplasts were generated with the combined action of lysozyme and mutanolysin in 10 mM Tris-HCl / 12% PEG, and were lysed with the addition of N-laurylsarcosine to the mixture with subsequent heating at 65°C for 15 min. DNA was then purified by CsCl / equilibrium centrifugation. Alternatively, the spheroplasts were lysed with the addition of SDS (5% wt/vol final concentration). DNA treated in this manner was purified by multiple extractions with phenol and phenol / $CHCl_3$ followed by incubations with ribonuclease (RNase). Mutanolysin in combination with lysozyme was critical for the reproducible recovery of high molecular weight DNA which migrated as a fairly tight band above the 23-kb molecular size marker when subjected to electrophoresis in a 1% agarose matrix. Figure 2.7 is a UV-vis spectrum of the isolated genomic DNA. The A_{260}/A_{280} ratio of ~1.9, as well as the ability of the genomic DNA to be digested with several different restriction enzymes (Figure 2.8) suggests that the DNA is free of contaminating proteins and other small molecules. Typical yields of genomic DNA from 1 L of *L. leichmannii* grown in LCM varied from 1-2 mg.

Screening of L. leichmannii Genomic DNA

The failure to isolate positive clones from the zoos in the *L. leichmannii* gene library provided by Dr. Short necessitated a revision in the strategy to be used to clone RTPR. The successful isolation of intact high molecular weight genomic DNA made it possible to consider constructing a new gene library. To decrease the number of clones in the library necessary to have greater than a 95% chance of containing the entire RTPR gene, it was decided that a subgenomic DNA library would be made. The strategy consisted of digesting the genomic

Figure 2.7: UV-visible spectrum of genomic DNA isolated from *L. leichmannii*. The spectrum was taken on a Hewlett-Packard 8452A diode-array spectrophotometer. The high A_{260}/A_{280} ratio (~1.9) suggests that the DNA is free of contaminating proteins.

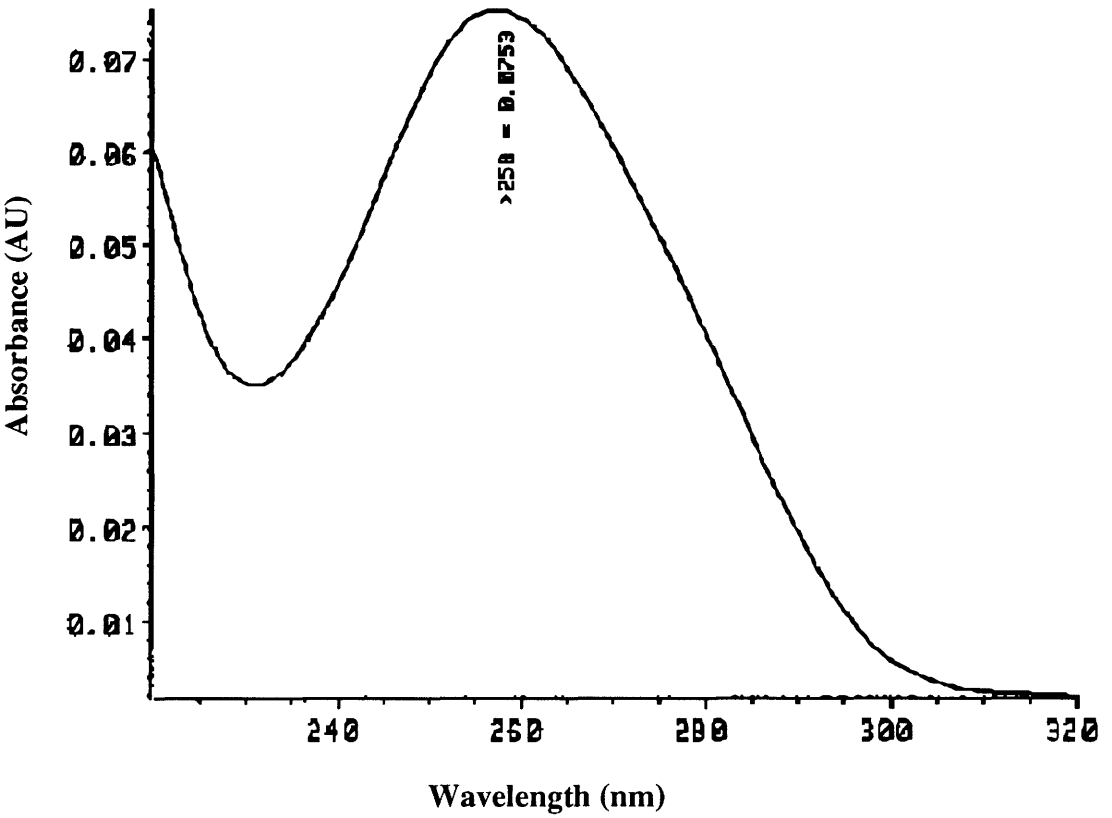
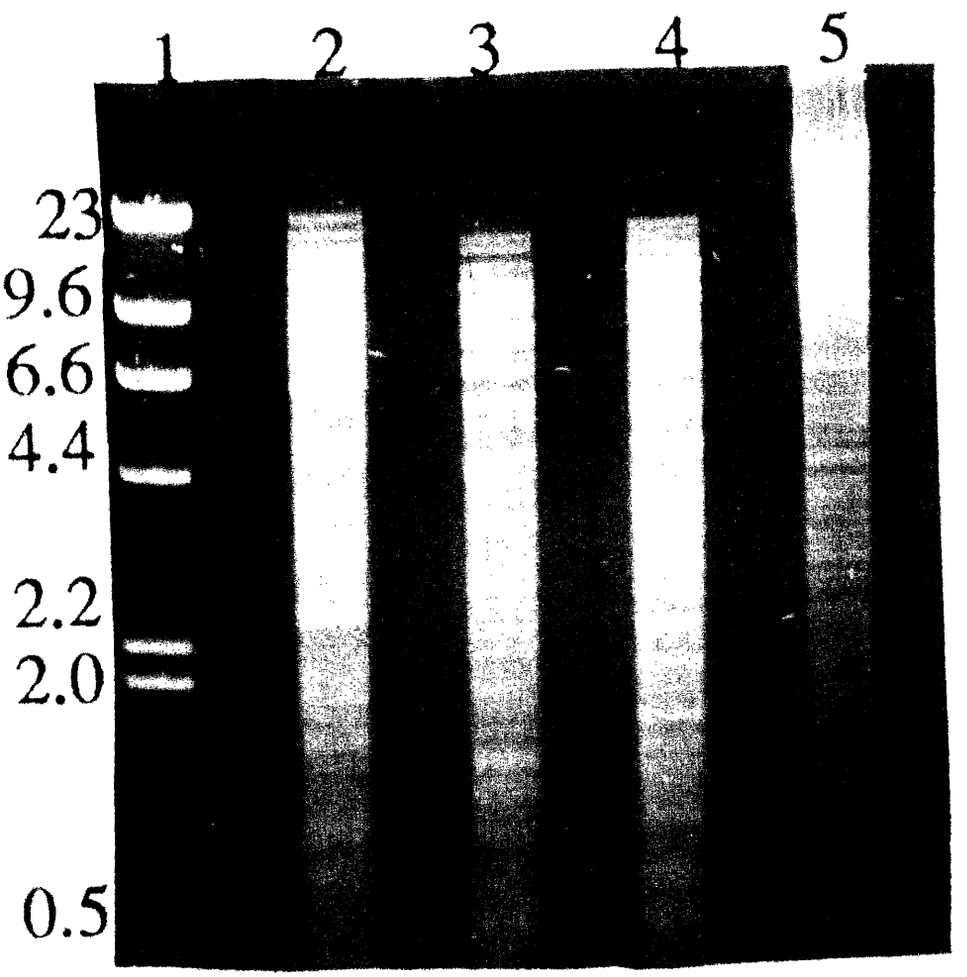

0.07
0.06
0.05
0.04
0.03
0.02
0.01
Absorbance (AU)
240
260
280
300
320
Wavelength (nm)

Figure 2.8: Restriction digest of *L. leichmannii* genomic DNA. Lane 1 contains molecular size markers (*Hin*dIII digest of λ DNA). Lane 2 contains genomic DNA digested with *Pst*I. Lane 3 contains genomic DNA digested with *Hin*dIII. Lane 4 contains genomic DNA digested with *Eco*RI. Lane 5 contains genomic DNA digested with *Bam*HI.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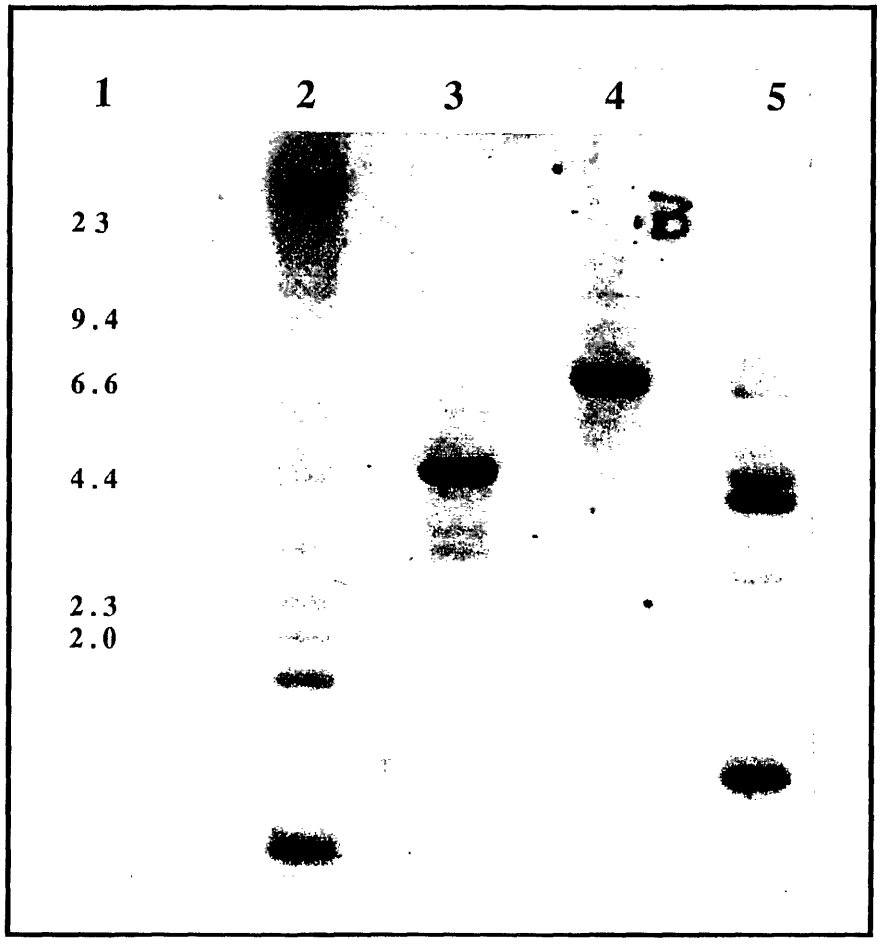
DNA with several enzymes in independent digests, and separating the fragments by agarose gel electrophoresis. Next, the DNA fragments would be transferred to a nylon membrane by the procedure of E.M. Southern (1975), and then subjected to hybridization with the inosine-containing probes. DNA from regions of the agarose gel giving rise to hybridization signals would be purified, inserted into a pUC-based plasmid, and transformed into an appropriate *E. coli* host strain. Using equation 2.1, and estimating the size of the *L. leichmannii* chromosome to be about the size of the *E. coli* chromosome ($4x10^6$ bp), it can be shown that by limiting the library to ~10% of the total chromosomal DNA, only one-tenth of the original number of clones would have to be generated in order to have a 95% chance of the library containing the entire RTPR gene. This of course assumes that the size of the inserted DNA remains the same. If the size of the insert is increased, then the number of clones needed would decrease. Likewise, if the size of the insert is decreased, the number of clones needed would increase.

L. leichmannii genomic DNA was digested with *Bam*HI, *Eco*RI, *Hin*dIII, and / or *Pst*I and screened by Southern hybridization with the two deoxyinosine-containing probes radiolabeled with ^{32}P at their 5′ ends. Genomic DNA digested with *Hin*dIII and *Eco*RI gave very clean signals when subjected to Southern hybridization with primer **I-2** (Figure 2.9). Hybridization signals with *Bam*HI and *Pst*I-digested DNA were not as clean, and probably reflect poor cutting by the respective restriction endonuclease rather than nonspecific hybridization. This is especially true of the DNA digested with *Bam*HI, as one of the two strongest signals migrates with undigested genomic DNA. Another Southern hybridization was done in the presence of labeled molecular size markers (*Hin*dIII-digested λ DNA), and a 4-fold lower amount of genomic DNA (5 μg) for each restriction digest. From the phosphorimage of the Southern

Figure 2.9: Phosphorimage of a Southern blot probed with primer **I-2**. Lane 1 contains the approximate migrations of molecular size standards (*Hin*dIII digest of λ DNA). Lane 2 contains genomic DNA digested with *Bam*HI. Lane 3 contains genomic DNA digested with *Eco*RI. Lane 4 contains genomic DNA digested with *Hin*dIII. Lane 5 contains genomic DNA digested with *Pst*I. The B on the filter is present as an orientation mark.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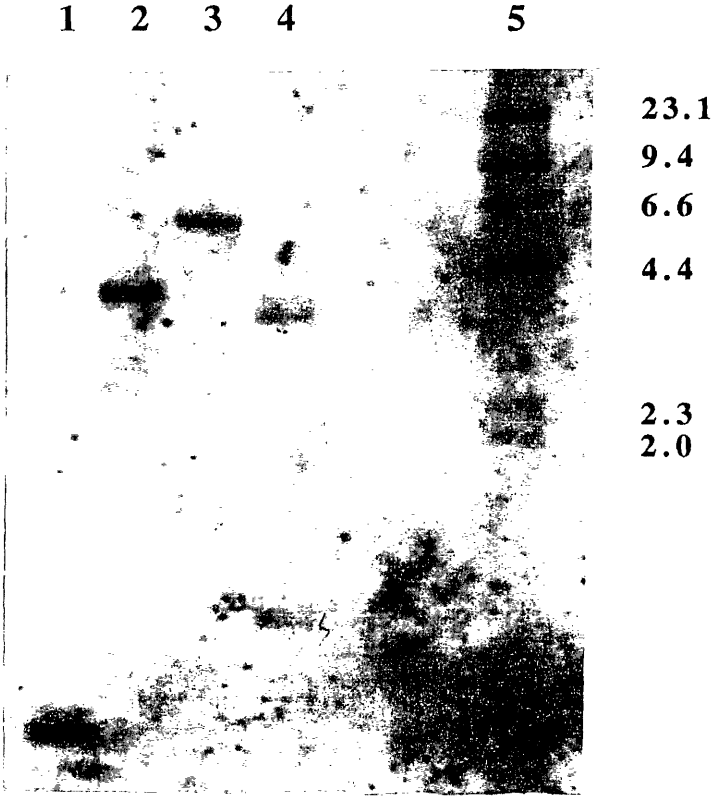
hybridization (Figure 2.10), it can be seen that the signal resulting from the *Hin*dIII digested DNA migrates just slightly faster than the 6.6-kb molecular size standard, and that the signal from the *Eco*RI-digested DNA migrates slightly faster than the 4.4-kb molecular size standard. The fragment giving rise to the hybridization signal in the *Bam*HI digested DNA lane is very small, and is estimated from its rate of migration to be less than 0.5 kb in size. Genomic DNA digested with *Pst*I appears to give rise to at least 2 bands. One band is estimated to be ~0.8 kb in size based on its rate of migration in relation to the *Bam*HI signal and the 2.0-kb molecular size marker. The other band migrates slightly faster than the *Eco*RI hybridization signal, and is estimated to be ~4 kb in size. Under more stringent washing conditions (58°C for 30 min in 2xSSC), the hybridization pattern of the *Eco*RI-digested DNA changes somewhat (Figure 2.11). Although the 4.4-kb band is present, the major hybridization signal is a doublet which migrates approximately at the rate of a 3-kb piece of linear DNA. The signals appear to be too intense under these restrictive conditions to be ascribed to background non-specific hybridization.

In light of the very small size of the hybridizing fragment resulting from the DNA digested with *Bam*HI, as well as the multiple signals obtained from the DNA digested with *Pst*I, focus was shifted to the *Hin*dIII and *Eco*RI digests of genomic DNA. Special attention was given to the *Hin*dIII digest of genomic DNA since a fragment of 6.6 kb would have a better chance than a fragment of 4.4 kb of containing the entire RTPR gene. The RTPR gene was calculated to be about 2 kb from its reported molecular weight of 76 kDa (Panagou et al., 1972). Southern hybridizations using primer **I-2** were repeated again with *Hin*dIII and *Eco*RI digests of genomic DNA, and again the same signals were observed as before (Figure 2.12). To confirm that the hybridization signals were due to RTPR, hybridizations were also done with primer **I-1**, which was made from the

Figure 2.10: Phosphorimage of a Southern blot probed with primer **I-2**, and in the presence of labeled molecular size markers. Lane 1 contains genomic DNA digested with *Bam*HI. Lane 2 contains genomic DNA digested with *Eco*RI. Lane 3 contains genomic DNA digested with *Hin*dIII. Lane 4 contains genomic DNA digested with *Pst*I. Lane 5 contains molecular size markers (*Hin*dIII digest of λ DNA) 5′ end-labeled with ^{32}P.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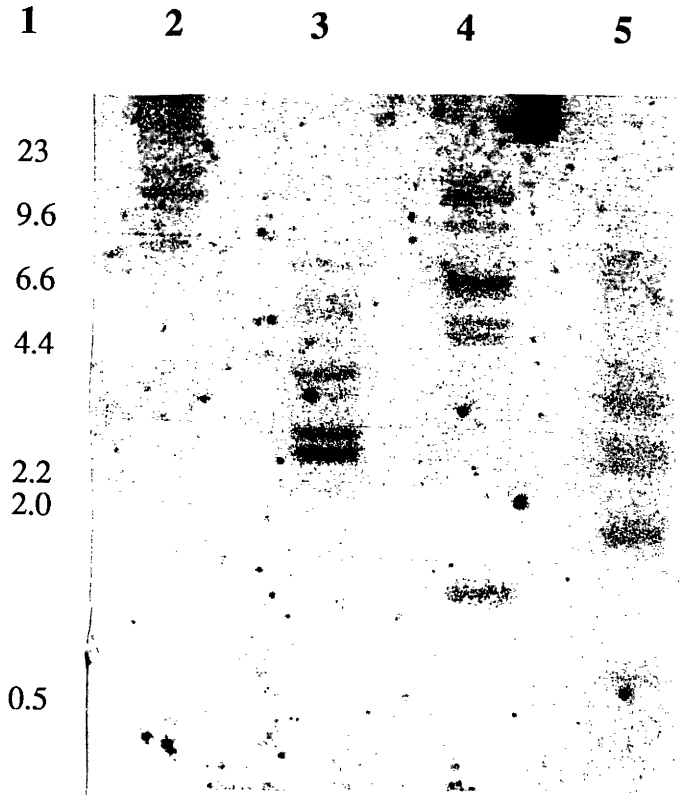
23.1
9.4
6.6
4.4
2.3
2.0

Figure 2.11: Phosphorimage of a Southern blot probed with primer **I-2**. Hybridization and washing steps are as described in **Materials and Methods**. This blot was subjected to an additional washing step at 58°C in 2xSSC for 30 min. Lane 1 contains the approximate migrations of molecular size standards (*Hin*dIII digest of λ DNA). Lane 2 contains genomic DNA digested with *Bam*HI. Lane 3 contains genomic DNA digested with *Eco*RI. Lane 4 contains genomic DNA digested with *Hin*dIII. Lane 5 contains genomic DNA digested with *Pst*I.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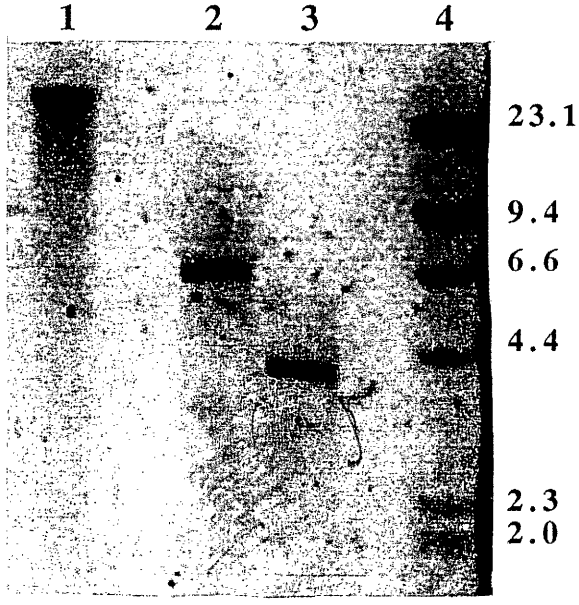
6.6
4.4
2.2
2.0
0.5

Figure 2.12: Southern blot probed with primer **I-2**. Lane 1 contains uncut *L. leichmannii* genomic DNA. Lane 2 contains genomic DNA digested with *Hin*dIII. Lane 3 contains genomic DNA digested with *Eco*RI. Lane 4 contains molecular size markers 5′ end-labeled with ^{32}P.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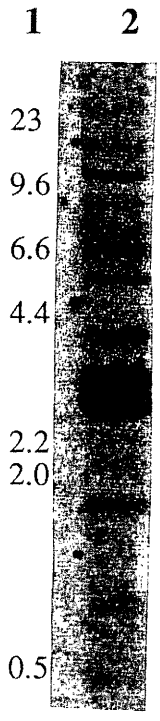
2.0

peptide sequence gained from active site labeling studies. When the protein was digested with *Eco*R1, a different type of hybridization signal was observed. A huge band which migrates in the center (~2.9 kb) of the phosphorimage (Figure 2.13) is resolved into a doublet under more stringent washing conditions (Figure 2.14). This doublet migrates at the approximate location of the doublet in Figure 2.11, and appears to be from the same target DNA sequence. This occurence of a doublet signal may be due to relatively close (~200 bp apart) *Eco*RI restriction sites which have differing degrees of accessibility. If one is cleaved readily, and the other only 50% of the time, then a doublet would result. The fact that hybridizations with primer **I-1** and primer **I-2** result in different signals suggests that the sequences complementary to these primers are separated by at least one *Eco*RI restriction site. Two Southern hybridizations to DNA digested with *Hin*dIII were done using primer **I-1**, and in both cases a fragment of ~6.6 kb hybridized to the primer (data not shown). This result suggested that this 6.6-kb fragment was relevant since it hybridized to the primers made from both the N terminus of RTPR as well as the active site peptide of RTPR. Of course, it could always be argued that since this is a restriction digest of genomic DNA, that the two primers may be annealing to two different DNA fragments that happen to be of similar size. In spite of this argument, a decision was made to create a subgenomic library containing the fragments that migrate in the 6.6-kb region of the agarose gel when the DNA is digested with *Hin*dIII.

Attempts to Create a Subgenomic Library

Given that a fragment of about 6.6 kb hybridizes with both inosine-containing primers when genomic DNA is digested with *Hin*dIII, an attempt was made to use these fragments to construct a subgenomic DNA library. The strategy consisted of digesting genomic DNA with *Hin*dIII, isolating the

Figure 2.13: Southern blot of *L. leichmannii* genomic DNA digested with *Eco*RI and probed with primer **I-1**. Lane 1 is the approximate migrations of molecular size markers (*Hin*dIII-digested λ DNA). Lane 2 contains *Eco*RI-digested genomic DNA.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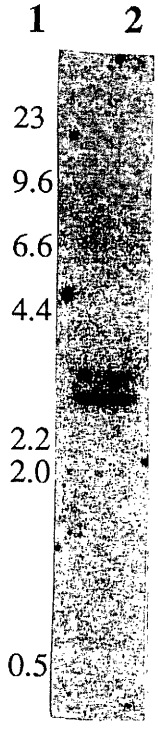
9.6
6.6
4.4
2.2
2.0
0.5

Figure 2.14: Southern blot of *L. leichmannii* genomic DNA digested with *Eco*RI and probed with primer **I-1**. Hybridization and washing steps are as described in **Materials and Methods**. However an additional washing step at 58°C in 2xSSC for 30 min was included. Lane 1 is the approximate migrations of molecular size markers (*Hin*dIII-digested λ DNA). Lane 2 contains *Eco*RI digested genomic DNA.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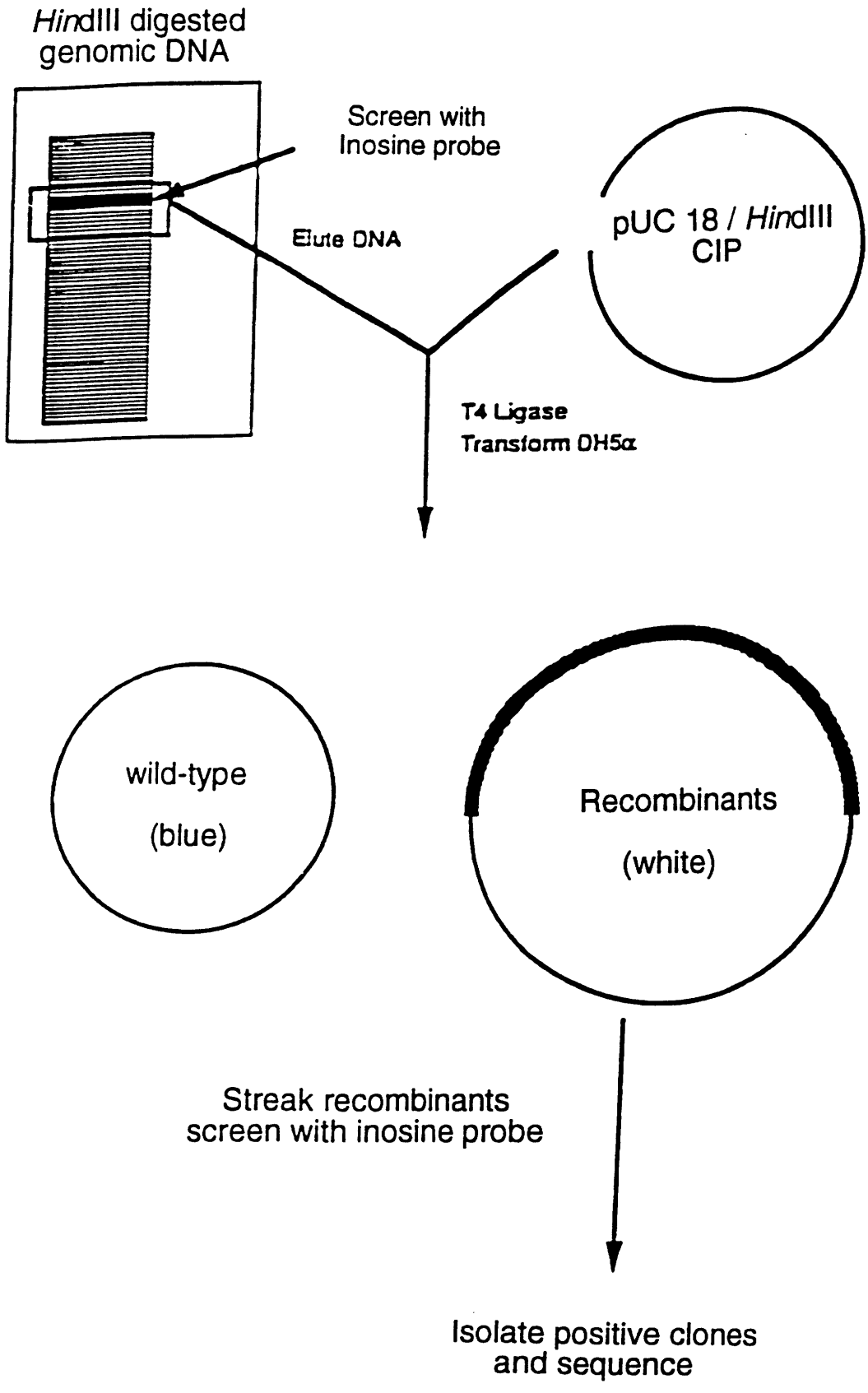
fragments which migrated at approximately as a 6.6-kb piece of linear DNA, and cloning the fragments into a suitable vector (Figure 2.15). Approximately 200 μg of genomic DNA was digested with *Hin*dIII and subjected to electrophoresis in a 1% agarose gel matrix. The gel was stained with ethidium bromide, and the region of the gel containing the 6.6-kb fragment was excised. The gel was then subjected to Southern analysis as a control to show that the correct region was indeed removed. The DNA from the agarose gel slice was purified by electroelution, and desalted by several dilutions and centrifugations in a Centricon 100 apparatus. These fragments were ligated into the multicloning region of both pUC19 and pBluescript (both of which had also been digested with *Hin*dIII), and then transformed into a suitable host bacterium. When ligations were performed with vectors that had been pretreated with CIP to remove the 5′ phosphate groups, the ligation ratio was 3 moles of vector per mole of insert. When the ligation reactions were done with vectors not previously treated with CIP, the ratio of vector to insert was 1:3. High insert to vector ratios were used to minimize the recircularization of the vector.

Both pBluescript and pUC19 contain multicloning sites within the region of the plasmid that encodes the enzyme β-galactosidase. When foreign DNA is inserted into this region, the β-galactosidase gene is interrupted, and a functional enzyme is not produced. This is used as a very good assay to determine which clones contain inserts. When transformed into a proper host strain and plated on media containing the chromogenic substrate X-Gal, recombinant clones are white, while non-recombinant clones, which produce an active β-galactosidase protein, are blue. Based on this assay, recombinant colonies were isolated and streaked in grid fashion on nylon membranes. Using equation 2.1, and assuming an average insert size of 6.6 kb as well as a total DNA population of ~20% of the normal genome (from comparing the region of the gel removed to the remainder

Figure 2.15: Strategy for creating a subgenomic DNA library. *L. leichmannii* genomic DNA would be digested with *Hin*dIII. Fragments with sizes of approximately 6.6 kb would be isolated and ligated into pUC18 that had been previously linearized with *Hin*dIII. Recombinants would be isolated as judged by their color when plated on selective media.


*Hin*dIII digested genomic DNA
Screen with Inosine probe
Elute DNA
pUC 18 / *Hin*dIII CIP
T4 Ligase
Transform DH5α
wild-type
(blue)
Recombinants
(white)
Streak recombinants screen with inosine probe
Isolate positive clones and sequence

of the gel), it was calculated that ~1000 transformants would have to be generated to have a 95% chance of finding the gene for RTPR. Colonies on the nylon membranes were then subjected to several rounds of hybridization with primer **I-2**. After approximately 200-300 colonies were screened, and no positive signals observed, a closer look at these recombinants was taken. When the white colonies were grown in liquid culture and their plasmid DNA isolated and digested with *Hin*dIII, the restriction patterns looked quite different from what was expected. In the case where the vector was dephosphorylated with CIP before the ligation reaction, two bands were distinguishable. One band migrated as if it were pUC19 without an insert (~2.7 bp), while the other band appeared to be ~2.4 kb in size. Confusingly, this restriction pattern did not appear to be a function of the β-galactosidase assay. White colonies as well as blue colonies both produced the same two bands when digested with *Hin*dIII. When the ligation reaction was performed with pUC19 previously untreated with CIP, a different restriction pattern was observed. Isolated plasmid DNA from several of the colonies produced the same restriction pattern when digested with *Hin*dIII as they did when not digested at all. This suggested that during the ligation or subsequent transformation, the *Hin*dIII site was destroyed. This might be a result of *L. leichmannii* native DNA being unstable in *E. coli* in this particular vectoral construction. During the transformation and subsequent growth, the insert comes out of the plasmid via a mechanism in which some of the base pairs around the point of ligation into the plasmid are also removed, resulting in the destruction of the *Hin*dIII site.

To test whether these anomalies were a result of errors in technique, an attempt was made to do a control ligation reaction. One set of commercially available molecular size markers is phage λ DNA digested with the restriction enzyme *Hin*dIII. This digestion produces several fragments which can be used as

standards to judge the size of any particular DNA fragment ranging from 0.5 to 23 kb. One fragment is 6.6 kb, the approximate size of the *L. leichmannii* fragments which were used in an attempt to make a subgenomic DNA library. The *Hin*dIII digest λ DNA molecular size markers were subjected to electrophoresis in a 1% agarose gel, and the 6.6-kb fragment was isolated and purified by electroelution. This fragment was then used in a ligation reaction with pUC19 which had not been treated with CIP, at a ratio of 3 moles of insert per mole of vector. DNA was isolated from several of the recombinant colonies, as judged by the β-galactosidase assay, and then digested with *Hin*dIII. In all cases, two fragments were produced, the 6.6 kb fragment of the molecular weight markers, and the 2.7 kb fragment of the plasmid. The success of this control suggested again that native *L. leichmannii* DNA was perhaps unstable in *E. coli* DH5α using the aforementioned plasmid construction.

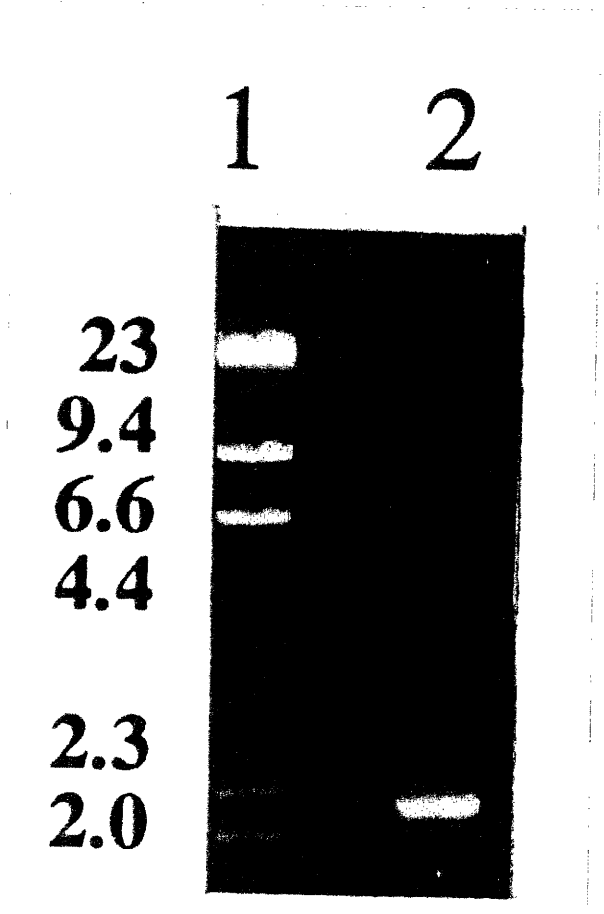
Cloning of the L. leichmannii Gene for RTPR by PCR

Yet another approach was devised to clone the *L. leichmannii* gene for ribonucleotide reductase. As discussed in Chapter 1, several experiments suggested that despite the difference in the cofactors employed, the *L. leichmannii* and *E. coli* enzymes use astonishingly similar mechanisms to carry out deoxynucleotide production (Stubbe, 1990). Furthermore, studies on each system which were designed to locate cysteine residues intimately involved in substrate turnover, resulted in the isolation of a peptide from the *L. leichmannii* enzyme which displayed a high degree of homology with a peptide isolated from the *E. coli* enzyme (Lin et al., 1987). Because the gene for the *E. coli* enzyme had been cloned, and its amino acid sequence subsequently deduced (Nilsson et al., 1988), this peptide was known to be located at the extreme C terminus of the R1 subunit. It was thus reasoned that if these two enzymes are indeed homologous,

this peptide might also occur at the extreme C terminus of the *L. leichmannii* enzyme. Using polymerase chain reaction technology, in combination with primers made from the N terminus of the protein as well as the homologous active site labeling peptide, this would effectively result in the amplification of the entire gene for the *L. leichmannii* RTPR.

Initial PCR reactions were done with equal amounts (~50 pmol) of each of the primers and a cycling protocol that included a 30 s annealing temperature at 50°C, and a 2 min extension at 72°C. No visible product was observed when up to 50% of the entire reaction was subjected to electrophoresis in a 1.5% agarose matrix and visualized by ethidium bromide staining. Another attempt in which the annealing temperature was lowered to 45°C and the extension time extended to 3 min produced identical results. A third attempt was made in which the primer concentrations were changed to better reflect their differing degrees of degeneracy. The cycling protocol was also altered to make the annealing conditions less stringent. Primer **2** which was constructed from the active site peptide is 64-fold degenerate, and 29 pmol was used in the PCR reaction. Primer **1** which was constructed from the N-terminal sequence is 192-fold degenerate, and 58 pmol was used in the PCR reaction. The cycling protocol contained a 37°C annealing temperature for 30 s, followed by a 15 s incubation at 50°C. This double annealing step was introduced to allow the correct primer to anneal, and hopefully prevent incorrect annealing by quickly going to a higher temperature. The correct primer would be elongated somewhat as *Taq* polymerase initiates the extension reaction (albeit slowly), allowing the primer to be successfully annealed to the template DNA at 50°C. However, mismatches might significantly reduce the ability of *Taq* to extend incorrect primers of this size, resulting in duplexes which are unstable at higher temperatures. As can be seen in Figure 2.16, this protocol resulted in the successful amplification of a very

Figure 2.16: Amplification of a 2.2-kb fragment from *L. leichmannii* genomic DNA by PCR. Lane 1 contains molecular size markers (*Hin*dIII digest of λ DNA). Lane 2 contains an aliquot (1/5 of the total volume) of the PCR reaction.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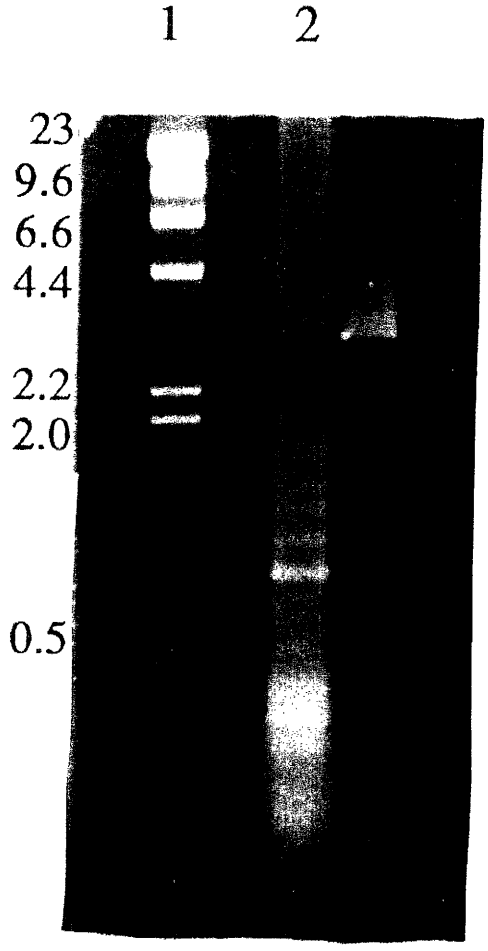
specific 2.2-kb fragment. Even more excitingly, based on the reported size of the *L. leichmannii* reductase (76 kDa), a gene of approximately 2.1 kb would be necessary to encode the protein. This experiment supported the hypothesis that the peptide gained from active site labeling studies was at the C terminus of RTPR, just as it was known from the amino acid sequence to be at the C terminus of the R1 subunit of the enzyme from *E. coli.*

Partial Cloning of the Fragment Obtained from the PCR Reaction

One of the many advantages of the PCR reaction is the ability to include at the 5′ ends of the primer, sequences which are recognized by certain restriction enzymes, and have these sequences incorporated into the amplified fragment. Upon amplification, these sites can conveniently be used to digest the fragment with the appropriate enzyme(s), and ligate it into the appropriately digested vector. As described earlier, primer **1** was engineered with a *Bam*HI restriction site at its 5′ end, while primer **2** was engineered with an *Eco*RI restriction site at its 5′ end. Several PCR reactions were run, and the amplified fragment was pooled, isolated, and subjected to restriction digestion and subsequent analysis by agarose gel electrophoresis. As shown in Figure 2.17, the PCR fragment is cleaved into a series of bands. Three or four of the bands migrate faster than the 500 bp molecular size marker, while 2 bands migrate above this molecular size marker and appear to be ~800-900 bp in size. The engineered restriction sites were thus unhelpful in cloning the 2.1-kb fragment from the PCR reaction, as one or both of the sites were represented multiple times within the amplified fragment. This figure highlights the drawback in engineering restriction sites for the cloning of a particular fragment of DNA when the sequence of the DNA is unknown.

Figure 2.17: Agarose gel analysis of PCR fragment after digestion with *Eco*R1 and *Bam*HI. Lane 1 contains the molecular size markers (*Hin*dIII digest of λ DNA). Lane 2 contains the restriction enzyme digest.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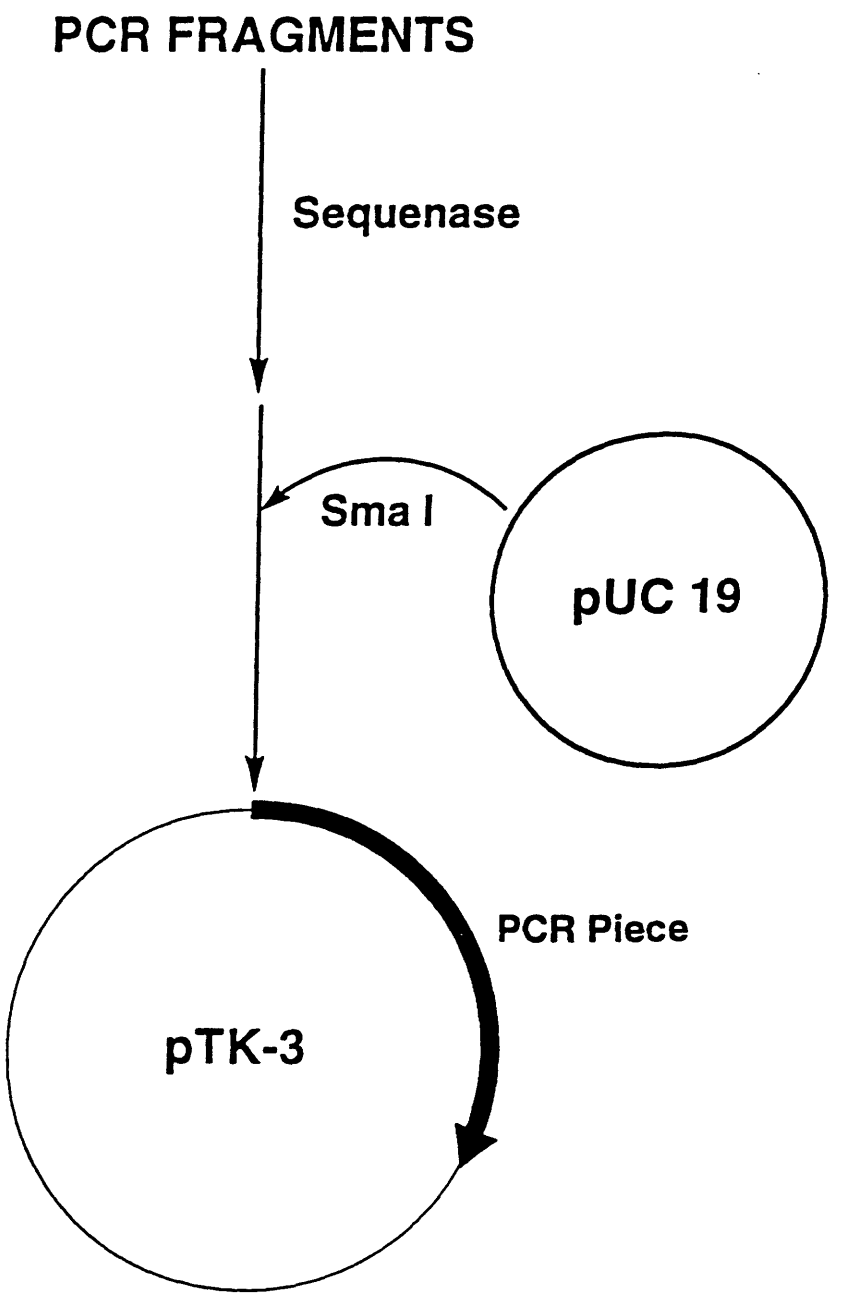
The inability to use the engineered restriction sites necessitated the formulation of an alternative ligation strategy. It was decided that the PCR fragment would be made blunt ended by treatment with T7 DNA polymerase (Sequenase), and then ligated into a pUC19 vector previously linearized with *Sma*I, a restriction enzyme that produces flush ends (Figure 2.18). Five PCR reactions were performed and pooled, and the amplified 2.1-kb fragment was isolated from a 1% agarose gel. The product was treated with Sequenase, and then ligated into pUC19 which had been previously digested with *Sma*I. The ligation was performed at a ratio of 3 moles of insert per mole of vector, since the linearized pUC19 was not pretreated with CIP. Upon completion, 2.5% (vol / vol) of the ligation mixture was used to transform *E. coli* DH5α, and the transformants were plated on selective media. Approximately 32 white colonies were obtained, of which 20 were grown in liquid culture. Plasmid DNA was isolated from the 20 colonies, and subjected to restriction digestion with *Hin*dIII, and subsequent analysis by agarose gel electrophoresis. One plasmid (pTK-3) contained a sizeable insert (~1.1 kb), but not the entire PCR fragment. However, since the insert contained the information needed to initiate the sequencing process using the universal sequencing primer (which is complementary to all pUC -based plasmids), no further attempts were made to clone the entire PCR fragment, nor to transform the remainder of the ligation mixture.

Determination of the Gene Sequence of RTPR

A large scale isolation of plasmid pTK-3 was carried out by cesium chloride / ethidium bromide centrifugation. Double-stranded sequencing was carried out by the Sanger dideoxynucleotide sequencing method using a *Taq* polymerase-based cycle sequencing method. The ability to use this particular sequencing system obviated the need to subclone the insert into a vector from

Figure 2.18: Cloning of the fragment amplified by PCR. The amplified fragments were treated with sequenase and then ligated into *Sma*I-digested pUC19 to generate the plasmid pTK-3.


PCR FRAGMENTS
Sequenase
Sma I
pUC 19
PCR Piece
pTK-3

which single-stranded DNA could be isolated. Although double-stranded sequencing can be achieved using the standard Sequenase system (US Biochemicals), the template DNA must first be denatured by treatment with NaOH, and the amount of template needed is a factor of 10 higher than for cycle sequencing with *Taq* polymerase. Also, the elevated temperature at which cycle sequencing is performed reduces the false stops generated from secondary structures in the template DNA.

The sequencing process was initiated with the universal sequencing primer, and the first 60 bases read from the cloned insert encoded 20 amino acids (8-27) of the N-terminal end of RTPR. The first 18 nucleotides, as expected, were those that encoded the N-terminal PCR primer. More importantly, the subsequent nucleotides that were not part of the PCR primers encoded all of the amino acids observed in the N-terminal peptide sequence (Table 2.5). Figure 2.19 shows the initial information obtained from sequencing with the universal primer.

This sequence was however not devoid of peculiarities. The PCR fragment was cloned into the *Sma*I site of pUC19, but the *Sma*I site was totally missing in pTK-3. Also, several other nucleotides from the multi-cloning site of pUC19 were missing, which destroyed the plasmid's *Kpn*I restriction site. The initial sequence should have read: GAA TTC GAG CTC CGG TTA CCC CGC GGA TCC GCC, with the underlined bases denoting the missing region. One explanation for this aberrant ligation reaction may be due to an activity recently found for DNA polymerases. At the very 3′ end of a template, several DNA polymerases have been shown to leave a one nucleotide overhang (Clark et al., 1987; Clark, 1988). The nucleotide base is almost always adenine, and the level at which it is present usually depends on the 3′-5′ exonuclease activity of the polymerase used. Sequenase, which has an insignificant amount of this

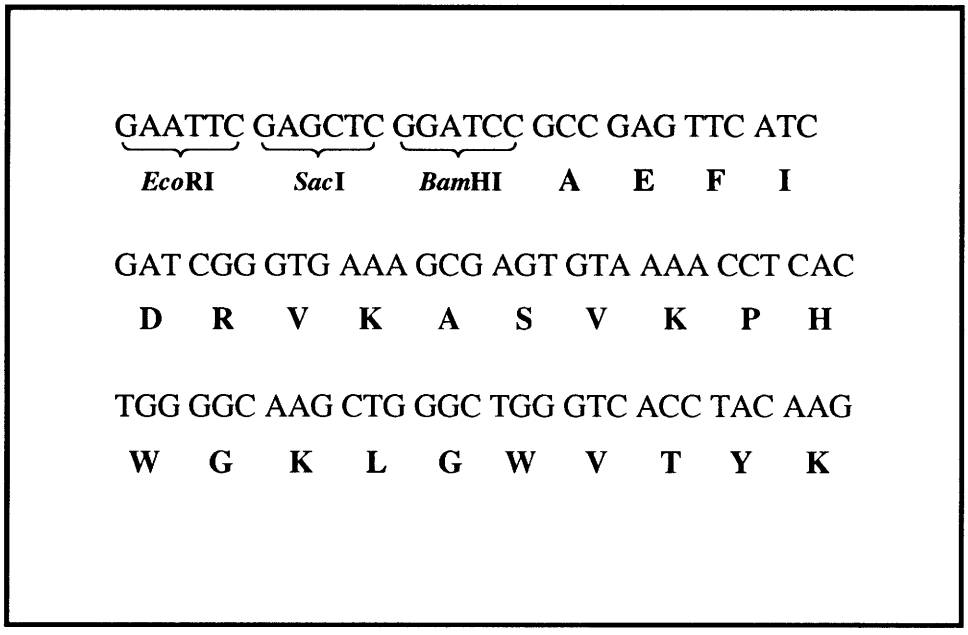


Figure 2.19: Initial sequence read from the insert of pTK-3. The bold letters are the one letter codes for the amino acids. Amino acids VK...YK are residues not encoded by the N-terminal PCR primer, but which are part of the N-terminal peptide sequence.

exonuclease activity, leaves the majority of the template with the "A" overhang, while the Klenow fragment, which has significant 3'-5' exonuclease activity, leaves little of the template with the "A" overhang. Since the PCR fragment was treated with Sequenase with the intention of producing blunt-ended DNA, most of the product generated probably contained the overhang, making it ineffective in a blunt-ended ligation reaction. Ligations which did occur were probably from aberrant chemistry, or from small amounts of shortened sequences containing blunt ends. The absence of the nucleotides "CCC" between the *Eco*R1 site and the beginning of the primer, as well as the destruction of the *Kpn*I site on the vector, suggests that the plasmid pTK-3 may have arisen from an unstable construction generated during the ligation reaction.

The sequence of the entire insert of pTK-3 was determined by successively making new primers to the ends of the previously obtained sequence. Most importantly, the entire sequence of the uncloned 2.1-kb PCR fragment could also be determined with the double-stranded sequencing method. The sequence of both strands of the 2.1-kb PCR fragment was determined. As well, the sequences of the extreme terminii of the gene, which were not flanked by the PCR primers, were determined using the purified 6.6-kb fragments from the *Hin*dIII digested genomic DNA. Although *Taq* polymerase is reported to have an error frequency of 1 in every 2000-10,000 bp (Bloch, 1991), by sequencing the uncloned PCR fragment, any mistakes that *Taq* polymerase may have made are averaged out. The gene sequence as well as the the deduced amino acid sequence of RTPR is shown in Figure 2.20. Amino acids in bold face type are parts of the peptide sequences shown in Table 2.5. The degree of peptide sequence information, as well as the distribution of the peptide sequence information lends confidence to the integrity of the sequence, especially with respect to major reading frame errors.

Figure 2.20: Nucleotide and deduced amino acid sequence of RTPR from *Lactobacillus leichmannii.* Numbers in italics on the left refer to the amino acid residues, beginning with the initial methionine. Nucleotide residues are counted by the numbers on the right in plain script. Amino acids in bold type are residues which were part of the protein sequence information outlined in Table 2.5. The underlined nucleotides at the beginning of the gene comprise the putative ribosome binding site.

```
    gaa gag tgc taa tat atg gag tgc ttt aaa aaa taa tga gac aag

    cag aaa gga cct gag aag atg agt gaa gaa ata tct ctc tcg gcg 27
                              M   S   E   E   I   S   L   S   A
    gaa ttt atc gac cgg gtg aaa gcg agt gta aaa cct cac tgg ggc 72
 10 E   F   I   D   R   V   K   A   S   V   K   P   H   W   G
    aag ctg ggc tgg gtc acc tac aag cgt acc tac gcc cgc tgg ctg 117
 25 K   L   G   W   V   T   Y   K   R   T   Y   A   R   W   L
    ccg gaa aag gga cgg agt gaa aac tgg gat gaa acc gtc aag cgg 162
 40 P   E   K   G   R   S   E   N   W   D   E   T   V   K   R
    gtg gtt gaa ggc aac atc aac ctg gat ccc cgc ctt caa gac tcc 207
 55 V   V   E   G   N   I   N   L   D   P   R   L   Q   D   S
    cca agt ctg gaa ctg aag cag agc ttg act gaa gaa gca gag cgg 252
 70 P   S   L   E   L   K   Q   S   L   T   E   E   A   E   R
    ctc tac aag ctg atc tac ggc ctg ggc gcg acg cca tct ggc cgg 297
 85 L   Y   K   L   I   Y   G   L   G   A   T   P   S   G   R
    aac ttg tgg att tcc ggg act gac tac cag aga cgg acc ggg gat 342
100 N   L   W   I   S   G   T   D   Y   Q   R   R   T   G   D
    tcc ttg aac aac tgc tgg ttt gtg gcc atc cgt ccg caa aag tat 387
115 S   L   N   N   C   W   F   V   A   I   R   P   Q   K   Y
    ggg gac agc aag atc gtg ccc agc tac ctt ggc aag cag gaa aag 432
130 G   D   S   K   I   V   P   S   Y   L   G   K   Q   E   K
    gcc gtt tcc atg cct ttc tcc ttc ctc ttt gac gaa ctg atg aag 477
145 A   V   S   M   P   F   S   F   L   F   D   E   L   M   K
    ggg ggc ggg gtt ggc ttc tca gtt gcc aga tcc aac atc agc cag 522
160 G   G   G   V   G   F   S   V   A   R   S   N   I   S   Q
    att ccc cgg gtc gac ttt gcc atc gac ctg cag ctg gtg gtc gat 567
175 I   P   R   V   D   F   A   I   D   L   Q   L   V   V   D
    gag acc agt gaa tcc tac gac gct tcc gtc aag gtc ggg gcc gtc 612
190 E   T   S   E   S   Y   D   A   S   V   K   V   G   A   V
    ggt aag aat gaa tta gtt caa gat gct gac agc atc tac tac cgc 657
205 G   K   N   E   L   V   Q   D   A   D   S   I   Y   Y   R
    ctg ccg gat acc cgg gag ggc tgg gtt ttg gcc aat gcc ctc ttg 702
220 L   P   D   T   R   E   G   W   V   L   A   N   A   L   L
    att gac ctc cac ttt gcc cag acc aac cca gac cgt aag caa aag 747
235 I   D   L   H   F   A   Q   T   N   P   D   R   K   Q   K
    ctg atc ctg gac ttg tcc gac atc cgt cct tac ggg gca gaa atc 792
250 L   I   L   D   L   S   D   I   R   P   Y   G   A   E   I
    cat ggc ttc ggc ggc acg gct tcc ggc cca atg ccc ttg atc tcc 837
265 H   G   F   G   G   T   A   S   G   P   M   P   L   I   S
    atg ctg ctg gac gtt aac gaa gtc ttg aat aat aag gct ggc ggc 882
280 M   L   L   D   V   N   E   V   L   N   N   K   A   G   G
    cgc ctt acg gct gtt gat gcc gcc gac atc tgc aac ttg atc ggc 927
295 R   L   T   A   V   D   A   A   D   I   C   N   L   I   G
    aag gca gtc gtg gcc ggc aat gtc cgc cgg tca gcg gaa ctg gcc 972
310 K   A   V   V   A   G   N   V   R   R   S   A   E   L   A
    ctg ggc tca aat gat gac cag gac ttc att tct atg aag cag gac 1017
325 L   G   S   N   D   D   Q   D   F   I   S   M   K   Q   D
    caa gag aag ctg atg cac cac cgc tgg gca tcc aac aac agc gtg 1062
340 Q   E   K   L   M   H   H   R   W   A   S   N   N   S   V
```

```
    gca gtt gat tca gcc ttc agc ggc tac cag ccg atc gct gcc ggg 1107
355  A   V   D   S   A   F   S   G   Y   Q   P   I   A   A   G
    atc cgg gaa aac ggg gag cca ggg atc gtg aac ctg gac ctg tcc 1152
370  I   R   E   N   G   E   P   G   I   V   N   L   D   L   S
    aag aac tat ggc cgg att gtt gat ggc tac cag gct gga att gac 1197
385  K   N   Y   G   R   I   V   D   G   Y   Q   A   G   I   D
    ggg gac gtg gaa ggg acc aac cct tgc ggg gag atc tcc ctg gcc 1242
400  G   D   V   E   G   T   N   P   C   G   E   I   S   L   A
    aac ggg gaa cct tgc aac ctc ttt gaa gtc ttc ccg ctg atc gcg 1287
415  N   G   E   P   C   N   L   F   E   V   F   P   L   I   A
    gaa gaa cag ggc tgg gat ttg cag gaa gtt ttt gcc tta gcg gcc 1332
430  E   E   Q   G   W   D   L   Q   E   V   F   A   L   A   A
    cgc tac gcc aag cgg gta acc ttc agt cct tat gac tgg gaa att 1377
445  R   Y   A   K   R   V   T   F   S   P   Y   D   W   E   I
    tcc cgg gaa ata att caa aag aac cgc cgg atc ggg atc tcc atg 1422
460  S   R   E   I   I   Q   K   N   R   R   I   G   I   S   M
    tcc ggg atc cag gac tgg ctt ttg acc aga ctg ggc aac cgg gtg 1467
475  S   G   I   Q   D   W   L   L   T   R   L   G   N   R   V
    gtt acc ggc ttc aag gat gac ttt gac ccg gaa acc cat gaa gct 1512
490  V   T   G   F   K   D   D   F   D   P   E   T   H   E   A
    atc aag gtg cca gtc tac gac aag cgg gcc att aag atg gtt gac 1557
505  I   K   V   P   V   Y   D   K   R   A   I   K   M   V   D
    cag ctt tac aag gcc gtg gtc aag gct gac cag gac tac agt aag 1602
520  Q   L   Y   K   A   V   V   K   A   D   Q   D   Y   S   K
    act ttg ggc tgc aat gaa tca atc aag cac acc acg gtc aag cca 1647
535  T   L   G   C   N   E   S   I   K   H   T   T   V   K   P
    tca ggc acg gtg gcc aag ttg gcc ggt gcc agt gaa ggc atg cac 1692
550  S   G   T   V   A   K   L   A   G   A   S   E   G   M   H
    ttc cac tac ggg gct tac ttg atc cag cgg atc cgt ttc cag gat 1737
565  F   H   Y   G   A   Y   L   I   Q   R   I   R   F   Q   D
    tct gac ccg ctt ttg cca gcc ttg aag gcc tgc ggc tac cgg acg 1782
580  S   D   P   L   L   P   A   L   K   A   C   G   Y   R   T
    gaa gcc gac atc tac acg gaa aac acg acc tgc gtg gaa ttc ccg 1827
595  E   A   D   I   Y   T   E   N   T   T   C   V   E   F   P
    atc aag gca gta ggg gcg gac aac cct aac ttt gcc tca gct ggg 1872
610  I   K   A   V   G   A   D   N   P   N   F   A   S   A   G
    acg gtt tca atc gcg gaa cag ttt gcc acc cag gcc ttc ttg cag 1917
625  T   V   S   I   A   E   Q   F   A   T   Q   A   F   L   Q
    act tat tgg tcg gac aac gcg gtt tcc tgc acg att act ttc cag 1962
640  T   Y   W   S   D   N   A   V   S   C   T   I   T   F   Q
    gat tcc gaa ggt gac cag gtt gaa tcc ttg ctc cgc cag tac cgc 2007
655  D   S   E   G   D   Q   V   E   S   L   L   R   Q   Y   R
    ttt atc acc aag tca acc tcg ctt ctg cca tac ttt gga ggt tcc 2052
670  F   I   T   K   S   T   S   L   L   P   Y   F   G   G   S
    ctg cag caa gcg cca aag gaa ccg atc gat aag gag act tac gaa 2097
685  L   Q   Q   A   P   K   E   P   I   D   K   E   T   Y   E
    aag cgc agc cag gaa att acc ggc aac gtg gag gaa gtc ttc agc 2142
700  K   R   S   Q   E   I   T   G   N   V   E   E   V   F   S
    cag ctg aac agc gac gtc aag gac ctg gaa ctg gtc gac cag acc 2187
715  Q   L   N   S   D   V   K   D   L   E   L   V   D   Q   T
    gac tgt gaa ggc ggc gcc tgc cca att aag taa                 2220
730  D   C   E   G   G   A   C   P   I   K
```

Discussion

The sequence of the *L. leichmanni* RTPR is the first sequence to be reported of a ribonucleotide reductase which does not require iron for catalysis. The amplification and sequencing of the gene for RTPR resulted from the fact that sequence information from both the N-terminal and C-terminal portions of the protein was available. The C-terminal sequence is intriguing, given its putative homology to the C-terminal end of the R1 subunit of the *E. coli* RDPR. In addition, the C-termini were previously implicated as being involved in catalysis by biochemical studies designed to identify the cysteines within the active site that are oxidized concomitant with substrate reduction (Lin et al., 1987). Mixed oligonucleotide probes corresponding to the C- and N-terminal ends resulted in the amplification by PCR of a 2.1-kb piece of DNA that encoded almost the entire reductase gene. The few amino acids (13 at the C terminus and 7 at the N terminus) not encoded by this 2.1-kb piece were known from peptide sequence information. Given the additional peptide sequence information (Table 2.5) dispersed throughout the entire protein, and the fact that both strands of the DNA were sequenced, the integrity of this sequence is believed to be high. This protein sequence defines a molecular mass for RTPR of 82 kDa, which contrasts with what was previously reported (76 kDa) based on SDS / PAGE and other biophysical studies (Panagou et al., 1972). This value can be compared with the molecular mass of 86.5 kDa for each protomer of the R1 subunit of the *E. coli* reductase. A list of codon usage is presented in Table 2.6. The *L. leichmannii* reductase contains 10 cysteine residues, while the reductase from *E. coli* contains 11 (Nilsson et al., 1988).

Table 2.6: Codon usage for *L. leichmannii* RTPR

TTT	F	12	TCT	S	4	TAT	Y	4	TGT	C	1
TTC	F	15	TCC	S	21	TAC	Y	23	TGC	C	9
TTA	L	2	TCA	S	9	TAA	Z	1	TGA	Z	0
TTG	L	18	TCG	S	3	TAG	Z	0	TGG	W	12
CTT	L	7	CCT	P	7	CAT	H	2	CGT	R	5
CTC	L	7	CCC	P	4	CAC	H	7	CGC	R	11
CTA	L	0	CCA	P	11	CAA	Q	7	CGA	R	0
CTG	L	28	CCG	P	9	CAG	Q	27	CGG	R	18
ATT	I	12	ACT	T	6	AAT	N	7	AGT	S	8
ATC	I	31	ACC	T	19	AAC	N	26	AGC	S	10
ATA	I	2	ACA	T	0	AAA	K	2	AGA	R	3
ATG	M	10	ACG	T	10	AAG	K	40	AGG	R	0
GTT	V	16	GCT	A	11	GAT	D	15	GGT	G	4
GTC	V	17	GCC	A	31	GAC	D	38	GGC	G	32
GTA	V	3	GCA	A	6	GAA	E	42	GGA	G	3
GTG	V	15	GCG	A	10	GAG	E	8	GGG	G	19

On the basis of extensive mechanistic studies using isotopically labeled nucleotide substrates and mechanism-based inhibitors, it has been proposed that the mechanisms of the *E. coli* and *L. leichmannii* ribonucleotide reductases are very similar (Stubbe, 1990). This mechanistic information in conjunction with a limited amount of protein sequence information suggested that the R1 subunit of RDPR might be homologous to the monomeric RTPR. The amino acid sequence of RDPR is shown in Figure 2.21. A sequence homology search between these two reductases using a variety of programs, including BLAST, FASTA, and CLUSTAL, failed to reveal any statistically significant sequence alignments. A search of the entire protein data base (GenBank, PIR, and EMBL) failed to reveal any significant sequence homology between RTPR and any

Figure 2.21: Amino acid sequence of RDPR from *E. coli*. The five underlined and bold-typed cysteines are those believed to be important during the catalytic event.

1 M N Q N L L V T K R D G S T E R I N L D K I H R V L D W A A

31 E G L H N V S I S Q V E L R S H I Q F Y D G I K T S D I H E

61 T I I K A A A D L I S R D A P D Y Q Y L A A R L A I F H L R

91 K K A Y G Q F E P P A L Y D H V V K M V E M G K Y D N H L L

121 E D Y T E E E F K Q M D T F I D H D R D M T F S Y A A V K Q

151 L E G K Y L V Q N R V T G E I Y E S A Q F L Y I L V A A C L

181 F S N Y P R E T R L Q Y V K R F Y D A V S T F K I S L P T P

211 I M S G V R T P T R Q F S S C V L I E C G D S L D S I N A T

241 S S A I V K Y V S Q R A G I G I N A G R I R A L G S P I R G

271 G E A F H T G C I P F Y K H F Q T A V K S C S Q G G V R G G

301 A A T L F Y P M W H L E V E S L L V L K N N R G V E G N R V

331 R H M D Y G V Q I N K L M Y T R L L K G E D I T L F S P S D

361 V P G L Y D A F F A D Q E E F E R L Y T K Y E K D D S I R K

391 Q R V K A V E L F S L M M Q E R A S T G R I Y I Q N V D H C

421 N T H S P F D P A I A P V R Q S N L C L E I A L P T K P L N

451 D V N D E N G E I A L C T L S A F N L G A I N N L D E L E E

481 L A I L A V R A L D A L L D Y Q D Y P I P A A K R G A M G R

511 R T L G I G V I N F A Y Y L A N D G K R Y S D G S A N N L T

541 H K T F E A I Q Y Y L L K A S N E L A K E Q G A C P W F N E

571 T T Y A K G I L P I D T Y K K D L D T I A N E P L H Y D W E

601 A L R E S I K T H G L R N S T L S A L M P S E T S S Q I S N

631 A T N G I E P P R G Y V S I K A S K D G I L R Q V V P D Y E

661 H L H D A Y E L L W E M P G N D G Y L Q L V G I M Q K F I D

691 Q S I S A N T N Y D P S R F P S G K V P M Q Q L L K D L L T

721 A Y K F G V K T L Y Y Q N T R D G A E D A Q D D L V P S I Q

751 D D G C E S G A C K I

known protein. Furthermore, only non-*E. coli* R1 RDPRs were identified as being homologous to *E. coli* R1. As mentioned in Chapter 1, a ribonucleotide reductase from *E. coli* grown under anaerobic conditions has been cloned and sequenced (Sun et al., 1993). Its amino acid sequence (Figure 2.22) shows no homology with RTPR, and only a very small amount of homology with RDPR in the N-terminal region of the proteins (Eliasson et al., 1992).

A comparison of the protein sequence of RTPR with those of the methylmalonyl-CoA mutases (Ledley et al., 1988; Marsh et al., 1989) and ethanolamine ammonia lyase (Faust et al., 1990), enzymes that catalyze AdoCbl-dependent rearrangement reactions, failed to reveal any common vitamin B_{12} binding domain. For ethanolamine ammonia lyase, some sequence similarity might have been anticipated given the similarity of its proposed mechanism (at least in the first few steps) to the postulated mechanism of RTPR (O'Brien et al., 1985; Stubbe, 1989). A sequence alignment of RTPR and methionine synthase (done by Robert Suto and Dr. Richard Finke at the University of Oregon), an enzyme that binds methylcobalamin (Banerjee et al., 1989), suggested several amino acids that might be involved in B_{12} binding (Figure 2.23). In 1992 E. Neil Marsh published the cloning and sequencing of glutamate mutase from *Clostridium tetanomorphum*. His careful sequence alignment of this mutase with methionine synthase, and methylmalonyl-CoA mutases from human, *P. shermanii*, and mouse, revealed a motif that is shared with all of the genes above as well as RTPR (Marsh & Holloway, 1992). A conserved histidine residue is flanked by a conserved aspartate (glutamate in RTPR) two residues towards the N terminus, and a conserved glycine three residues towards the C terminus (Figure 2.24). A secondary structure prediction of glutamate mutase done by Marsh, predicted an overall pattern of strands of β-sheet alternating with α-helices, and suggested that the conserved sequence DXHXXG would lie at

Figure 2.22: Deduced amino acid sequence of the ribonucleotide reductase from *E. coli* grown under anaerobic conditions. The amino acid sequence in bold type is homologous to a region in pyruvate formate-lyase, which has been shown to contain a radical on the amino acid glycine.

1 M T P H V M K R D G C K V P F K S E R I K E A I L R A A K A

31 A E V D D A D Y C A T V A A V V S E Q M Q G R N Q V D I N E

61 I Q T A V E N Q L M S G P Y K Q L A R A Y I E Y R H D R D I

91 E R E K R G R L N Q E I R G L V E Q T N A S L L N E N A N K

121 D S K V I P T Q R D L L A G I V A K H Y A R Q H L L P R D V

151 V Q A H E R G D I H Y H D L D Y S P F F P M F N C M L I D L

181 K G M L T Q G F K M G N A E I E P P K S I S T A T A V T A Q

211 I I A Q V A S H I Y G G T T I N R I D E V L A P F V T A S Y

241 N K H R K T A E E W N I P D A E R Y A N S R T I K E C Y D A

271 F Q S L E Y E V N T L H T A N G Q T P F V T F G F G L G T S

301 W E S R L I Q E S I L R N R I A G L G K N R K T A V F P K L

331 V F A I R D G L N H K K G D P N Y D I K Q L A L E C A S K R

361 M Y P D I L N Y D Q V V K V T G S F K T P M G C R S F L G V

391 W E N E N G E Q I H D G R N N L G V I S L N L P R I A L E P

421 K G D E A T F W K L L D E R L V L A R K A L M T R I A R L E

451 G V K A R V A P I L Y M E G A C G V R L N A D D D V S E I F

481 K N G R A S I S L G Y I G I H E T I N A L F G G E H V Y D N

511 E Q L R A K G I A I V E R L R Q A V D Q W K E E T G Y G F S

541 L Y S T P S E N L C D R F C R L D T A E F G V V P G V T D K

571 G Y Y T N S F H L D V E K K V N P Y D K I D F E A P Y P P L

601 A N G G F I C Y G E Y P N I Q H N L K A L E D V W D Y S Y Q

631 H V P Y Y G T N T P I D E C Y E C G F T G E F E C T S K G F

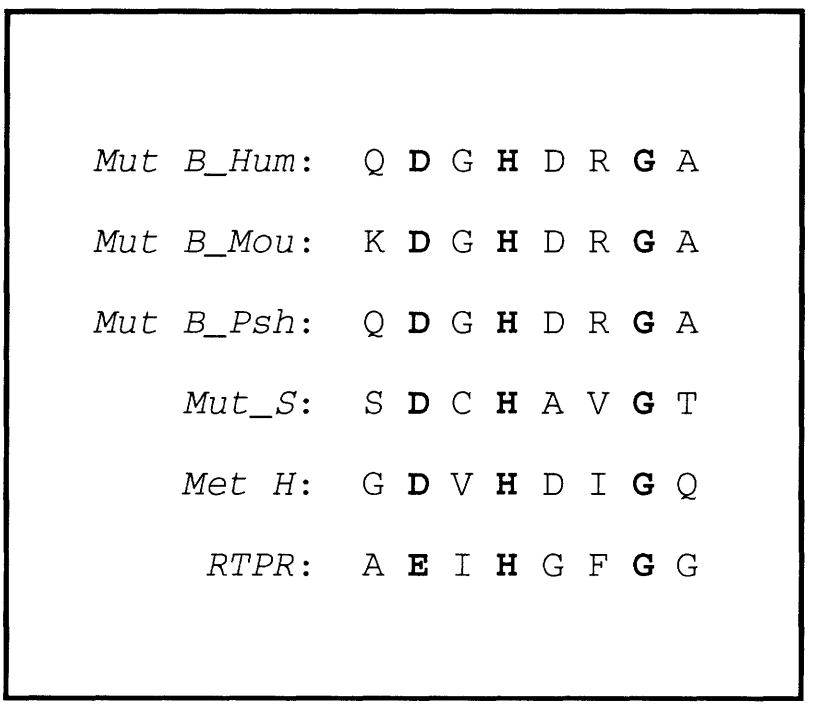
661 T C P K C G N H D A S R V S V T R **R V C G Y** L G S P D A R P

691 F N A G K Q E E V K R R V K H L G N G Q I G

Figure 2.23: Sequence alignment of the cobalamin binding domain of methionine synthase and RTPR. Amino acids in bold type are those which are identical in each protein. (Courtesy of Robert Suto and Dr. Richard Finke)


RTPR: 146VSMPFSFLFDELMKGGGVGFSVARSNISQIPRVDFAIDLQL
MetH: 639RGSKTDDTANAQQAEWRSWEVNKRLEYSLVKGITEFIEQDT
RTPR: 187VVDETSESYDASVKVGAVGKNELVQDADSIYYRLPDTREGW
MetH: 680EEARQQATRPIEVIEGPLMDGMNVVGDLFGEGKMFLPQVVK
RTPR: 228VLANALLIDLHFAQTNPDRKQKLILDLSDIRPYGAEIHGFG
MetH: 721SARVMKQAVAYLEPFIEASKEQGKTNGKMVIATVKGDVHEI
RTPR: 269GTASGPMPLISMLLDVNEVLNNKAGGRLTAVDAADICNLIG
MetH: 762GKNIVGVVLQCNNYEIVDLGVMVPAEKILRTAKEVNADLIG
RTPR: 310KAVVAGNVRRSAELALGSNDDQDFISMKQDQEKLMHHRWAS
MetH: 803LSGLITPSLDEMVNVAKEMERQGFTIPLLIGGATTSKAHTA
RTPR: 351NNSVAVDSAFSGYQPIAAGIRENGEPGIVNLDLSKNYGRIV
MetH: 844VKIEQNYSGPTVYVQNASRTVGVVAALLSDEQRDDFVARTR
RTPR: 392DGYQAGIDGDVEGTNPCGEISLANGEPCNL
MetH: 885KEYETVRIQMGRKKPRTPPVTLEAARDNDF

Figure 2.24: Sequence alignment of a conserved motif from amino acid sequences of methylmalonyl-CoA mutases, methionine synthase, and RTPR. Amino acids in bold type are identical in all proteins.


Mut B_Hum: Q **D** G **H** D R **G** A
Mut B_Mou: K **D** G **H** D R **G** A
Mut B_Psh: Q **D** G **H** D R **G** A
Mut_S: S **D** C **H** A V **G** T
Met H: G **D** V **H** D I **G** Q
RTPR: A **E** I **H** G F **G** G

a β-turn between two strands of β-sheet (Marsh & Holloway, 1992). A secondary structure prediction of RTPR using a program written by Burkhard Rost and Chris Sander, also predicted basically a pattern of strands of β-sheet alternating with α-helices (Rost & Sander, 1993). The sequence EIHGFG lies in a loop region separated by strands of β-sheet. Due to the lack of proteins displaying significant homology with RTPR, the secondary structure prediction is only approximately 60-65% accurate. The exact location of the conserved sequence awaits the solving of the three-dimensional structure of methionine synthase, the only B_{12} dependent protein for which diffraction quality crystals have been obtained (Luschinsky et al., 1992).

While RTPR and *E. coli* RDPR do not exhibit any statistically significant sequence homology, this does not preclude the possibility that sequence fragments may be conserved by function. Our previous studies on the *E. coli* RDPR have allowed us to propose a model in which five cysteines on the R1 subunit are involved in nucleotide reduction. Two cysteines (positions 754 and 759) at the C-terminal end of the R1 protein are proposed to shuttle electrons from thioredoxin (the *in vivo* reductant) to the active-site cysteines (positions 225 and 462), which are proposed to be directly involved in nucleotide reduction (Mao et al., 1992a; Mao et al., 1992b). A fifth cysteine (Cys 439) is proposed to be converted into the protein radical that is required to initiate 3′ hydrogen atom abstraction from the nucleotide substrate (Mao et al., 1992). A search of the protein sequence of RTPR was therefore made for cysteine-containing pepetides with sequence similarities to the peptides surrounding the catalytically important cysteines of the *E. coli* R1 subunit. This search revealed two fragment similarities shown below.

***RTPR*:** **DLELVDQTD_C^{731}EGGAC^{736}PIK**

***RDPR*:** **DLVPSIQDDGC^{754}ESGAC^{759}KI**

***RTPR*:** **TNPC^{408}GEISLA**

***RDPR*:** **SNLC^{439}LEIALP**

The alignment of the cysteines in the C-terminal region of both reductases suggests that Cys-731 and Cys-736 of RTPR might function to shuttle electrons from the *in vivo* reductant (thioredoxin) to the active site cysteines. Consistent with this proposal is the observation that the *L. leichmannii* reductase can be reduced by the *E. coli* thioredoxin (Lin et al., 1987).

The alignment of Cys-408 in RTPR with a fragment containing Cys-439 of RDPR is particularly intriguing. Cys-439 in RDPR has been proposed to be the protein radical that initiates catalysis by hydrogen atom abstraction from the nucleotide substrate. Given that the putative 5'-deoxyadenosyl radical generated from homolysis of the carbon-cobalt bond of AdoCbl has been shown not to directly initiate catalysis by 3' hydrogen atom abstraction (Stubbe, 1989), it is interesting to speculate that its function is to generate the thiyl radical of RTPR Cys-408, which then initiates the reduction process.

Efforts to locate by sequence gazing the two cysteines thought to be oxidized directly concomitant with substrate reduction have been unsuccessful. However, previous biochemical studies with RTPR have been interpreted to indicate that Cys-119 is close in three-dimensional space to Cys-731 and Cys-736 (Lin et al., 1987). Analogy with similar studies on the *E. coli* RDPR indicating that Cys-225 is directly involved in substrate reduction (Mao et al., 1989) suggests that Cys-119 of RTPR might be an active-site reductant. A candidate for the second

cysteine of RTPR directly involved in substrate reduction, Cys-419, is highly speculative. This cysteine is 11 amino acids removed from Cys-408, while in the *E. coli* RDPR Cys-462, the second cysteine involved in substrate reduction, is 23 residues removed from Cys-439. In a sequence alignment (done by Dr. Britt-Marie Sjöberg of the University of Stockholm Sweden) (Figure 2.25) of the R1 subunits of all of the non-heme iron-requiring reductases, the region demarcated by cysteines 439 and 462 in the *E. coli* enzyme is relatively conserved, but the distance between the cysteines ranges from 13-22 amino acids.

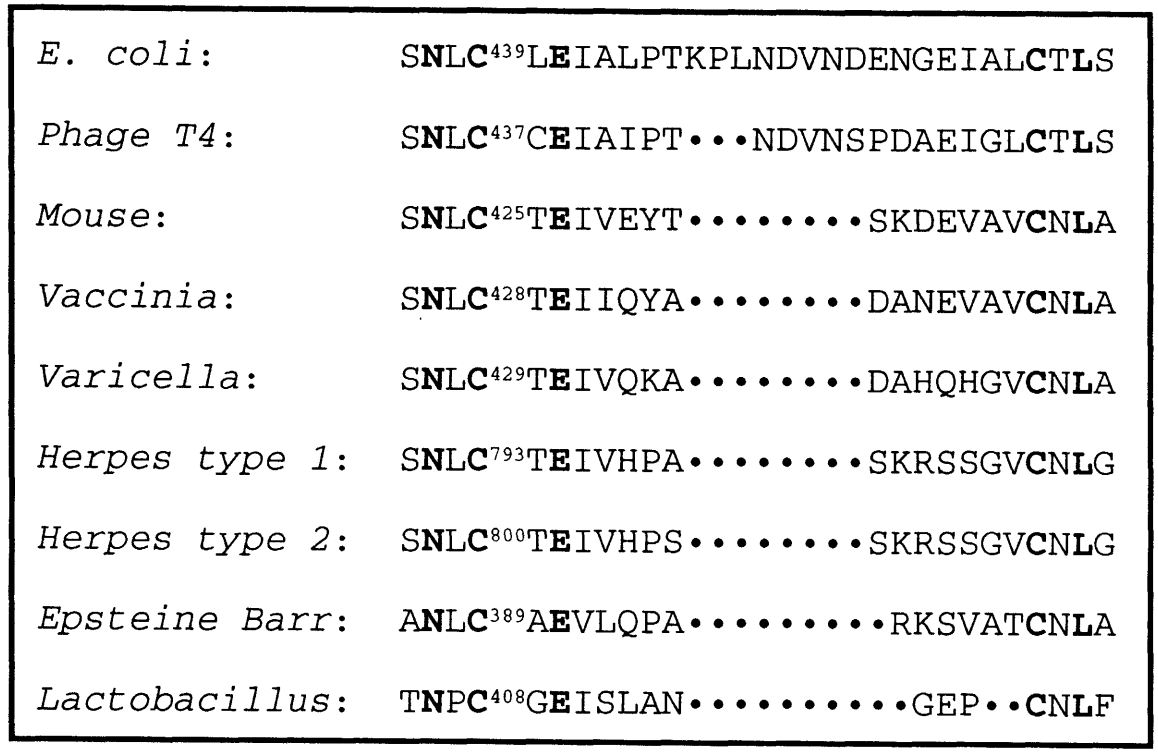
Finally, the sequence search of RTPR revealed a third region with sequence homology to RDPR:

RTPR: Y^{457}DWEISREIIQ

RDPR: Y^{599}DWEALRESIK

The function of this region of RDPR is unknown, although the tryptophan is conserved in all of the non-heme iron-requiring reductases sequenced to date (Eriksson & Sjöberg, 1989).

Figure 2.25: Alignment of a conserved region of several of the non-heme iron-dependent reductases with RTPR of *L. leichmannii*. This region contains cysteines 419 and 462 of *E. coli*, which have been previously shown to be important in catalysis. Amino acids in bold type are identical in all of the aligned proteins.


E. coli: SNLC439LEIALPTKPLNDVNDENGEIALCTLS
Phage T4: SNLC437CEIAIPT•••NDVNSPDAEIGLCTLS
Mouse: SNLC425TEIVEYT••••••••SKDEVAVCNLA
Vaccinia: SNLC428TEIIQYA••••••••DANEVAVCNLA
Varicella: SNLC429TEIVQKA••••••••DAHQHGVCNLA
Herpes type 1: SNLC793TEIVHPA••••••••SKRSSGVCNLG
Herpes type 2: SNLC800TEIVHPS••••••••SKRSSGVCNLG
Epsteine Barr: ANLC389AEVLQPA•••••••••RKSVATCNLA
Lactobacillus: TNPC408GEISLAN••••••••••GEP••CNLF

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Chapter 3:

Expression, Purification, and Characterization of Ribonucleoside Triphosphate Reductase from *Lactobacillus leichmannii*

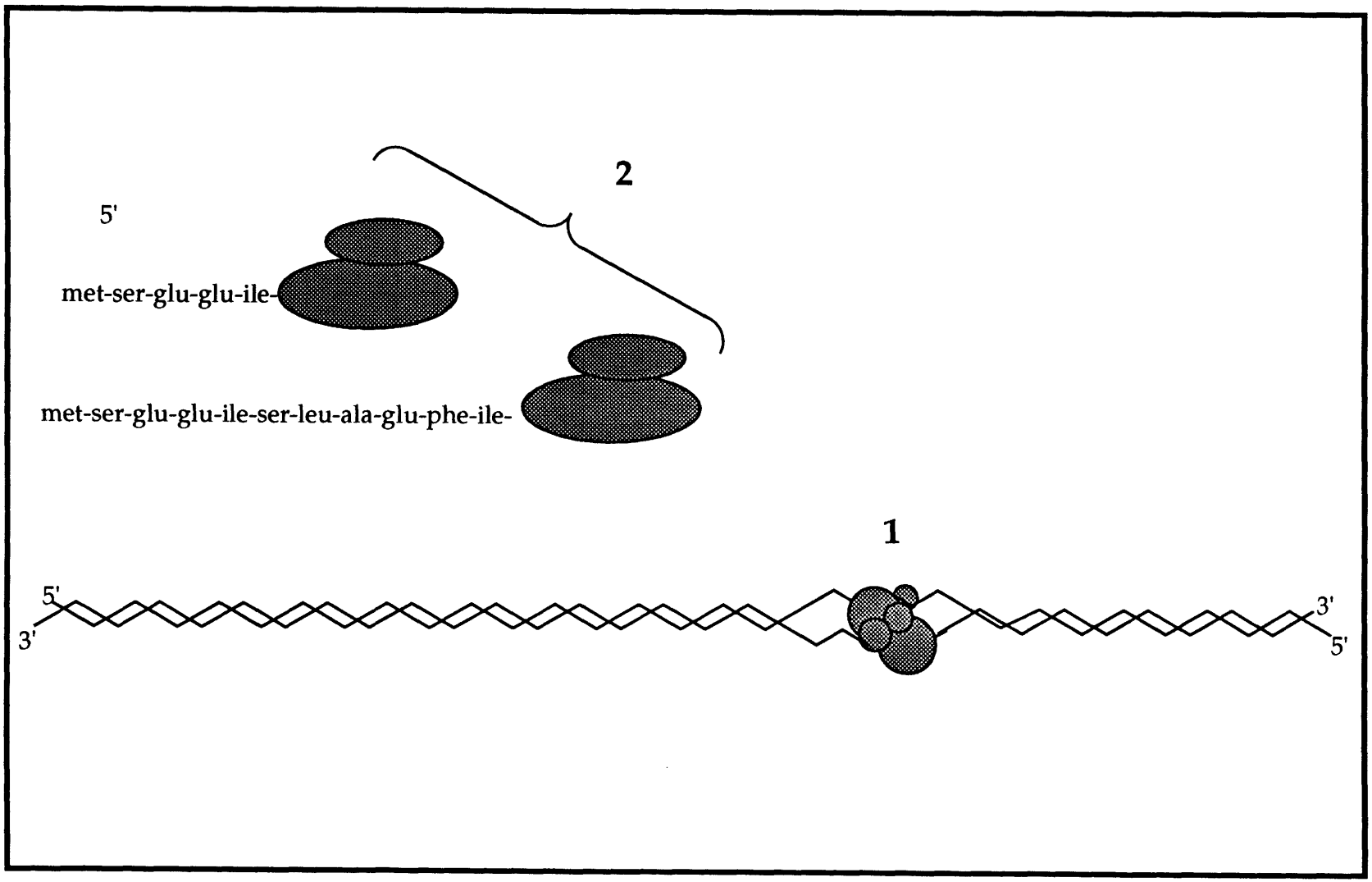
Recombinant DNA technology has greatly facilitated the understanding of the structure, mechansim, and cellular regulation of enzymes. As described in Chapter 2, genes for enzymes can be isolated and cloned, and from their sequences the primary structures (i.e. amino acid sequences) of the corresponding enzymes can be deduced. In Chapter 4 we shall see how a technique that is termed site-directed mutagenesis can be used to alter any of the amino acids which comprise an enzyme whose gene has been cloned. These mutant proteins can then be analyzed to see what effect the mutation has on catalysis, regulation, secretion, or a host of other cellular functions, perhaps allowing distinct physiological roles to be assigned to specific amino acids. However, these experiments, and particularly those which involve a variety of biophysical techniques [e.g. electron paramagnetic resonance (EPR), Mössbauer spectroscopy, and X-ray crystallography] can require large quantities of pure enzyme. Fortuitously, the wealth of knowledge that has accumulated within the last three decades concerning the cellular and molecular biology of the *Escherichia coli* bacterium has enabled biological scientists not only to clone genes for proteins, but also to manipulate *E. coli* to produce large amounts of cloned proteins.

The process of overproducing or overexpressing proteins in bacteria, yeast, or even baculoviruses, is only an exploitation of the many mechanisms of regulation which are already present within the organism. It is therefore beneficial to understand how simple prokaryotes initiate and regulate protein synthesis in order to appreciate the mechanisms and techniques by which they can be made to synthesize foreign proteins at levels that can approach 40% of the total protein in the cell. The biochemistry by which prokaryotes such as *E. coli* transform a segment of DNA into one or more proteins is generally thought of as a two-step process. It begins with the synthesis of an RNA transcript of the DNA

in a process that is termed transcription. In the second step, translation, ribosomes use the RNA message to determine the sequence of amino acids that must be assembled to give the corresponding protein (Figure 3.1). All of the proteins which are encoded by an organism's DNA are not syntheisized simultaneously however. *E. coli*, for example, have over 5000 genes, the products of many of which enable the bacterium to survive and/or thrive under a wide range of environmental conditions. It would be energetically costly for the bacterium to constitutively synthesize proteins for which it has no immediate need. Protein synthesis must therefore be regulated in diverse ways to allow *E. coli* and other organisms to respond rapidly to environmental changes.

In prokaryotes most of the regulation of protein synthesis takes place at the transcriptional level. Unlike eukaryotic genes, prokaryotic genes whose protein products are involved in particular pathways are often found contiguously grouped together in an operon. The basic layout of an operon includes a group of structural genes which are all under the same system of regulation, an upstream promoter region which is where RNA polymerase binds the DNA and initiates transcription of the structural genes, and downstream termination signals which alert RNA polymerase to cease transcription. Although the RNA molecule is polycistronic (i.e. more than one gene is located on one RNA molecule) each gene product has its own AUG start codon and stop codon (UAA, UAG, or UGA). The promoter region of an operon usually plays the central role in the regulation of the synthesis of the structural genes. Promoters in *E. coli* are characterized by conserved sequences at positions -35 and -10 (the first nucleotide transcribed into RNA is defined as +1). The consensus sequences at these positions are:

Figure 3.1: A simple schematic showing the process by which proteins are synthesized from a DNA message. In the first step (**1**), the enzyme RNA polymerase makes an RNA copy of the DNA template in a process called transcription. In the second step (**2**), which is called translation, ribosomes use the RNA message to assemble the sequence of amino acids that comprise a given protein.

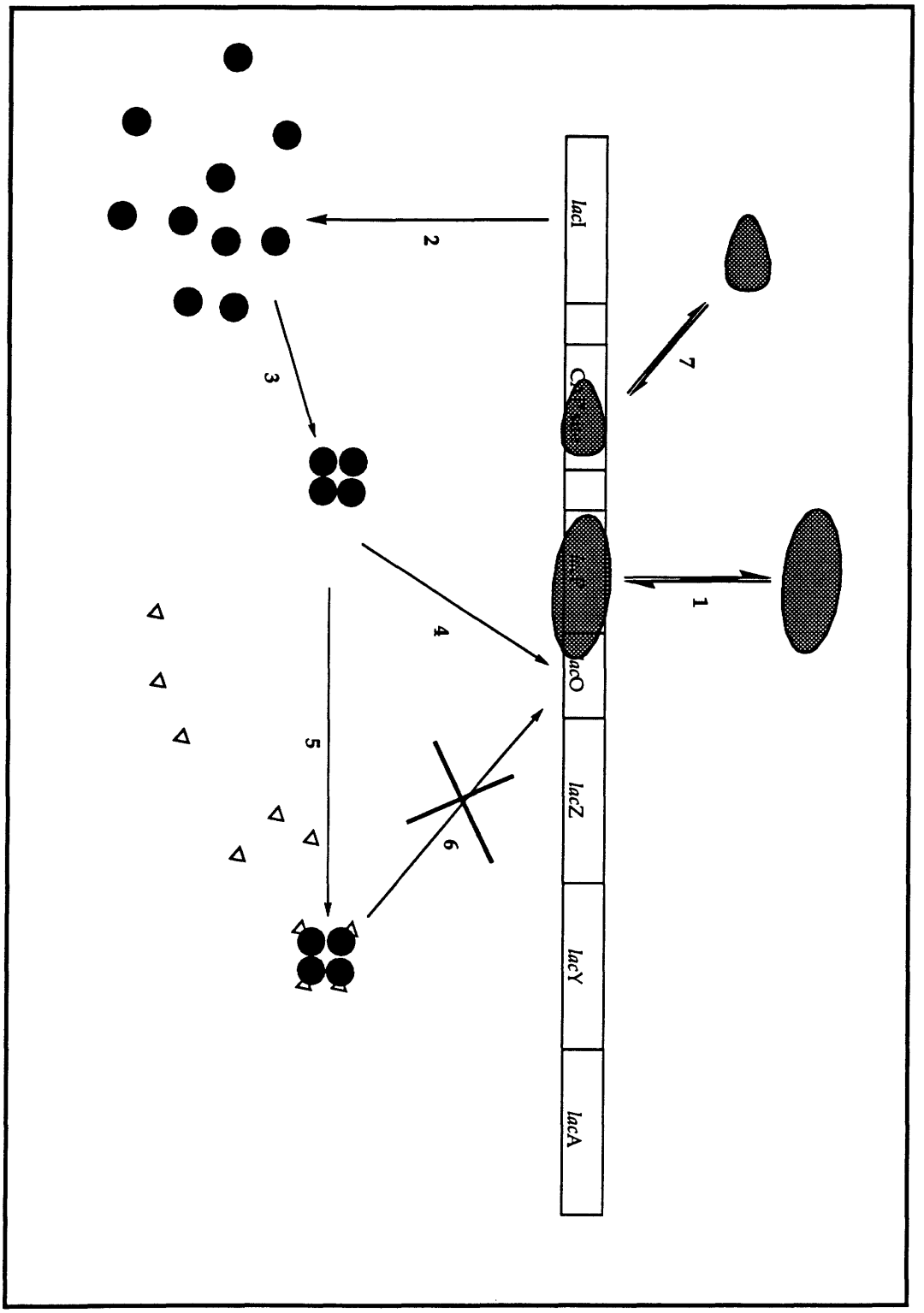

2
5'
met-ser-glu-glu-ile-
met-ser-glu-glu-ile-ser-leu-ala-glu-phe-ile-
1
5'
3'
3'
5'

position -35	T_{85} T_{83} G_{81} A_{61} C_{69} A_{52}
position -10	T_{89} A_{81} T_{50} A_{65} A_{65} T_{100}

with the numbers in italics indicating how often (in percent) the specified nucleotide occurs at the denoted position in promoter sequences of over 100 genes (Beebee & Burke, 1992). In general, the closer a promoter is to the above consensus sequences, the stronger it is.

Although promoters can be strong, weak, or somewhere between these two extremes, this form of regulation is not modulated by environmental stimulii; protein synthesis is constitutive, and the concentration inside of the cell of a given protein is dependent upon the strength of the promoter and the half-lives of the mRNA transcript and the protein. Rigid regulation of protein synthesis relys on the ability of various factors to make RNA polymerase more or less specific for a given promoter. This is exemplified by the best characterized operon - the lactose operon. The lactose or *lac* operon (shown in Figure 3.2) is composed of 3 structural genes: *lac*Z, *lac*Y, and *lac*A, which encode the proteins β-galactosidase, lactose permease, and thiogalactoside transacetylase. In addition, a repressor protein encoded by the *lac*I gene, and two non-protein-encoding sequences, *lac*O and *lac*P (representing the operator and promoter regions), are components of the *lac* operon. The *lac* repressor is generally present at ~40 copies per cell, and has an affinity for the operator that is ~4×10^6 times that of random DNA sequences (Beebee & Burke, 1992). Binding of the repressor (as a tetramer) to the operator blocks the binding of RNA polymerase to the promoter site, resulting in the inhibition of transcription. Mutations in the operator sequence are dominant, and result in constitutive transcription of the structural genes. Mutations in the *lac* repressor also result in constitutive transcription of the structural genes; however, these mutations are recessive and

Figure 3.2: The *lac* operon. The transcription of the structural genes on the lac operon (*lac*Z, *lac*Y, and *lac*A) is initiated with the binding of RNA polymerase to the operon's promoter region - *lac*P (**1**). The *lac*I gene is reponsible for making a repressor protein (**2**) which binds to the operator sequence (*lac*O) as a tetramer (**3-4**) and prevents the binding of RNA polymerase to the promoter. In the event that sufficient allo-lactose is present in the cell, it binds to the repressor protein (**5**) and prevents it from binding to the operator region (**6**). This subsequently allows RNA polymerase to bind to the promoter and begin the process of transcription. The *lac* operon is also positively stimulated. Under conditions of low glucose, cyclic AMP associates with catabolite activator protein (CAP), and this complex binds to a site near the promoter region (**7**). This association with the CAP site subsequently stimulates the binding of RNA polymerase to the promoter region.


lacI
lacO
lacZ
lacY
lacA
1
2
3
4
5
6
7

can be overcome by supplying wild-type repressor. The *lac* operon regulates a bacterium's ability to use lactose as a carbon source rather than glucose which is used normally. When glucose levels are sufficient, the *lac* operon is repressed. When glucose levels are low, cyclic adenosine monphosphate (cAMP) associates with catabolite activator protein (CAP), and this complex binds to another region of the operon and induces the binding of RNA polymerase. However the *lac* operon is still not activated unless the concentration of lactose inside of the cell is sufficient enough for it to be used as a carbon source. In the event that enough allo-lactose is present, it binds to the repressor and induces a conformational change which causes the repressor to dissociate from the operator. Experimentally, compounds that mimic allo-lactose, [e.g. isopropyl thio β-D-galactoside (IPTG)] are usually used since β-galactosidase hydrolyzes allo-lactose.

Because of the high degree to which the *lac* operon has been characterized, vectors employing this system of regulation were among the first to be constructed for the heterologous expression of cloned genes. The *lacUV5* promoter has historically been the most important in terms of the overexpression of foreign proteins in *E. coli.* It contains two mutations in the -10 consensus sequence which strenghten protein synthesis and also allows it to be resistant to repression by glucose (Reznikoff & Gold, 1986). Since multicopy plasmids tend to titrate the *lac* repressor, the overexpression of cloned genes is often carried out in *E. coli* strains (*lacI*^{Q}) which have been engineered to overproduce the repressor. This insures that overproduction is not initiated until the allotted time. This is most important when expressing proteins such as insulin which are lethal to *E. coli.*

Some of the most successful vectors for the heterologous expression of cloned genes in *E. coli* have been those containing chimeras of the *lacUV5*

promoter. The most celebrated of these promoters is the *tac* promoter, in which the -35 region of the *lacUV5* promoter is replaced with the -35 region of the promoter from the tryptophan operon. As its name implies, this operon contains the genes for the synthesis of this aromatic amino acid from the precursor chorismate, and is another well-studied and beautiful example of transcriptional regulation. The -35 region of the *trp* promoter, unlike that of the *lac* promoter, bears the consensus sequence described above. The resulting chimera, in which consensus sequences are present in both of the critical regions of the promoter, affords a new promoter which is 10-fold stronger than the *lacUV5*. As with the *lacUV5* promoter, the *tac* promoter is repressed when expression is carried out in *E. coli* strains that are *lacI*^{Q}. In analogous fashion, the promoter is induced to full strength with IPTG. The success of the *tac* promoter is highlighted by the many genes that have been expressed with this system, and the many new expression vectors that have been constructed which employ this promoter.

Recently a new expression system that is worth mention has been developed, and is based on the RNA polymerase isolated from bacteriophage T7 (Tabor & Richardson, 1985). This bacteriophage RNA polymerase is very specific for its own promoter sequence, and does not initiate the expression of any of the *E. coli* chromosomal genes. In addition, it is highly processive, extremely active, and can synthesize RNA transcripts at rates that are several fold higher than the *E. coli* RNA polymerase (Ausubel et al., 1987). The gene to be expressed is cloned into a vector that contains a T7 promoter (e.g. pT7 series or pET series) and the resulting construct is transformed into *E. coli* lysogen BL21(DE3) which contains an inducible T7 RNA polymerase gene (Tabor & Richardson, 1985; Studier & Rosenberg, 1990). Alternatively, the T7 polymerase can be provided by an additional plasmid that contains the T7 RNA polymerase gene under the control of a separate promoter. One of the many advantages of this system is that it can

be used to express genes that may be toxic to *E. coli*, since the cellular polymerases do not recognize the T7 promoter. Another advantage is that the T7 RNA polymerase is not inhibited by rifampicin, a compound that binds to one of the subunits of bacterial RNA polymerases, and prevents the initiation step of transcription. This compound can be added to the growth media at a given time to halt the synthesis of host proteins while having little effect on the synthesis of the plasmid-encoded protein. Upon the addition of [^{35}S]methionine, expression can be monitored in a time-dependent fashion by autoradiography.

As mentioned above, transcription is only half of the two-step process of protein synthesis. As soon as the transcript is synthesized, ribosomes attach to the mRNA and begin the process of translation. The initiation of translation is often the rate-limiting step in protein expression. All of the factors that govern this process are not completely understood; however, based on empirical data, a set of rules has been developed as a guide for optimizing heterologous gene expression in *E. coli*. Two of the most important interactions involved in the initiation of translation are the interaction between the anticodon of the initiator tRNA and the AUG start codon, and the interaction between the ribosome and a sequence just upstream of the AUG start codon. This latter sequence is called the Shine-Dalgarno sequence of the ribosome binding site. The first rule in maximizing translation in *E. coli*, is to introduce a Shine-Dalgarno sequence of at least four nucleotides that is as close as possible to the consensus sequence AGGAGG. The optimal spacing between this sequence and the ATG start codon is anywhere between 5 and 13 nucleotides, with 9 usually being a good first approximation. Secondly, the region between the Shine-Dalgarno sequence and the start codon should be AT rich. In addition, the two nucleotides preceeding the ATG start codon (positions -1 and -2) should be T's, and the nucleotide following the ATG start codon (position +4) should be A if possible, or G.

Moreover, the nucleotide in position +5 should be G (MacFerrin et al., 1990). These rules are very good guidelines for the overproduction of cloned proteins in *E. coli*; however, they by no means guarantee the success of the process. In the event that satisfactory expression is not achieved, other factors such as the stability of the mRNA transcript as well as the codon usage of the foreign gene need to be considered.

This chapter describes the procedures used to clone RTPR into plasmid pKK223-3, and to overexpress it under the control of the *tac* promoter. In addition, reported herein is the purification of RTPR to near-homogeneity, as well as the characterization of the recombinant enzyme to show that it is indistinguishable from that isolated from cultures of *L. leichmannii.*

Materials and Methods

Materials

Immobilon poly[vinylidene difluoride] (PVDF) membranes (0.45 μm pore were purchased from Millipore. Ampicillin, ethidium bromide, protamine sulfate, ATP, and AdoCbl, were purchased from Sigma. Nick columns were from Pharmacia. Dithiothreitol and D_2O were purchased from Mallinckrodt. DE-52 anion-exchange resin was from Whatman. Kanamycin was purchased from United States Biochemical. The dsDNA Cycle Sequencing system, T4 DNA ligase, isopropyl thio β-D-galactoside (IPTG), and competent *E. coli* HB101 cells were from GIBCO/BRL. AmpliTaq DNA polymerase was from Perkin-Elmer/Cetus. The TA cloning system was purchased from Invitrogen. Centricons, ultrafiltration devices, and membranes were purchased from Amicon. Restriction endonucleases were purchased from New England Biolabs.

E. coli thioredoxin (TR) (specific activity, 50 units/mg) and thioredoxin reductase (TRR) (specific activity, 800 units/mg) were isolated from

overproducing strains SK3981 and K91/pMR14 (Lunn et al., 1984; Russel & Model, 1985). *E. coli* strain JM105 was obtained from Pharmacia. Plasmid pKK223-3 was a generous gift from Dr. Oliver Peoples of MIT. Oligonucleotide primers used for expression-cassette PCR were obtained from the MIT Biopolymers Laboratory, or Oligos Etc. of Wisonville, OR, and are listed in Table 3.1.

General UV-vis absorption spectra were recorded on a Hewlett Packard 8452A diode-array spectrophotometer.

Table 3.1: Primers used for expression-cassette PCR.

Primer	Primer Sequence
1	5' d(GCC-GGC-GAA-TTC-AGG-AGA-AAA-TAT-TAT-GAG -TGA-AGA-AAT-ATC-TCT-CTC-C)3'
2	5' d(GAA-GAC-TTC-AAA-GAG-GTT-GCA-AGG)3'
3	5' d(GGC-GCG-AAG-CTT-ACT-TAA-TTG-GGC-AGG-CGC-C)3'
4	5' d(CTA-CCA-GGC-TGG-AAT-TGA-CGG)3'

Expression-Cassette Amplification of the N-terminal Half of the RTPR Gene

The nucleotide sequence of RTPR was used to generate PCR primers for the amplification of the N-terminal half of the gene. The N-terminal primer **1**, was 49 bases in length, and contained in addition to the first 24 bases of the RTPR gene, an 8-base transitional spacing element flanked by a consensus *E. coli* ribosome binding site and the ATG start codon. The alternate strand primer **2** was 24 bases in length and complementary to a region of the RTPR gene (bp 1252-1275) 23 bases downstream of a unique *Bgl* II restriction site. The

amplification reaction contained in a final volume of 100 μL: each primer at 1 μM, 10 μL of 10x PCR buffer (500 mM KCL / 100 mM Tris-HCl pH 8.3 / 15 mM $MgCl_2$ / 0.15% gelatin), all four dNTPs (each at 0.2 mM), and 1.2 μg of genomic DNA. The reaction mixture was overlaid with 100 μL of paraffin oil and heated to 94°C for 5 min. *Taq* polymerase (2.5 U) was added under the oil layer and 35 cycles of the following program were run: 94°C for 1 min, 45°C for 1 min, and 72°C for 3 min. Upon completion, an aliquot consisting of 1/4 of the total reaction volume was removed for analysis of the amplification reaction by electrophoresis in a 1% agarose gel.

The concentration of the 1.2-kb amplified fragment was estimated from ethidium bromide staining of the agarose gel, and the fragment was ligated into the Invitrogen TA vector (pCR1000) at a molar ratio of 1:4 (vector:insert). The ligation reaction included in a total volume of 10 μL: 50 ng of pCR1000, 100 ng of the amplified 1.2-kb fragment, 1 μL of 10x T4 DNA ligase buffer (500 mM Tris-HCl pH 7.8 / 100 mM $MgCl_2$ / 200 mM DTT / 10 mM ATP / 500 μg/mL BSA), and 7 Weiss units of T4 DNA ligase. The reaction was allowed to proceed at 12°C for 16 h, and a 1 μL aliquot of the ligation mixture was used to transform competent *E. coli* DH5α (Sambrook et al., 1989). Approximately 1/5 of the transformation mixture was spread on LB plates containing kanamycin (50 μg/mL) and X-Gal (0.008% wt/vol). Clones that contained inserts were identified by their white phenotype, and several were chosen for further characterization by appropriate restriction digestion and subsequent electrophoresis in a 1% agarose matrix. Several clones that contained plasmids possessing the correct restriction pattern were subjected to sequencing around the points of ligation into the vector. Of the clones that contained the correct sequences, clone 6α4 was chosen to be used to construct the full-length RTPR gene.

Expression-Cassette Amplification of the C-terminal Half of the RTPR Gene

The procedure for the amplification and isolation of the C-terminal half of the gene is very similar to that described for the N-terminal half. The C-terminal primer, **3**, was 31 bases long. It contained a *Hin*dIII restriction site at its extreme 5′ end, and the complementary sequence of the last 21 bases of the RTPR gene, including the stop codon. The alternate strand primer, **4**, was 21 bases long and complementary to a sequence of the RTPR gene (bp 1179-1199) 30 bases upstream of a unique *Bgl* II restriction site. A 1-kb fragment was amplified and ligated into pCR1000 as described above for the N-terminal half of the RPTR gene. Recombinant clones (as judged by their white phenotype when plated on X-Gal) were screened for inserts by appropriate restriction digestion and subsequent electrophoresis in a 1% agarose matrix. Several plasmids that possessed the correct restriction pattern were screened further by DNA sequencing around the point of ligation of the insert into the vector. Of the clones that contained the correct sequences, clone 3α1 was chosen to use to construct the full-length RTPR gene.

Cloning of the Full-length RTPR Gene into Plasmid pKK223-3

Plasmid DNA was isolated from 10-mL overnight cultures of clones 6α4 and 3α1 grown in LB media supplemented with kanamycin (50 μg/mL) using Qiagen Midi columns according to the manufacturer's recommendations. Plasmid 6α4 (5.5 μg) was digested (in a total volume of 50 μL) with 16 U of *Bgl* II, and 40 U of *Eco*R1 in the buffer specified for the use of *Bgl* II (50 mM Tris-HCl, pH 7.9 / 100 mM NaCl / 10 mM $MgCl_2$ / 1 mM DTT). Plasmid 3α1 (17 μg) was digested (in a total volume of 50 μL) with 40 U of *Bgl* II and 20 U of *Hin*dIII in the recommended buffer for *Hin*dIII (10 mM Tris-HCl, pH 7.9 / 10 mM $MgCl_2$ / 50 mM NaCl / 1 mM DTT). The restriction digestions were allowed to proceed

for 4 h at 37°C, and then were diluted to 200 μL and divided into 10 equal portions which were loaded onto a 1.5% agarose gel. The DNA was subjected to electrophoresis in Tris-acetate / EDTA (TAE) buffer for a period sufficiently long such that the insert of plasmid 6α4 (1246 bp) could be resolved from the *Bgl* II/*Eco*R1 fragment of the vector (1170 bp). The 6α4 insert as well as the 3α1 insert was excised from the agarose gel with a sterile razor blade, and the DNA was electroeluted from the agarose plug as described in Chapter 2 (Isolation of the 6.6-kb *Hin*dIII Fragments). The DNA was concentrated using a Centricon 100, and then desalted on a Pharmacia Nick column. Plasmid pKK223-3 (10 μg) was digested for 3 h at 37°C (in a total volume of 200 μL) with 100 U of *Eco*RI and 200 U of *Hin*dIII in the recommended buffer for *Eco*RI (50 mM NaCl / 100 mM Tris-HCl, pH 7.5 / 5 mM $MgCl_2$ / 0.05% Triton X-100). The digestion reaction was divided into 8 equal portions which were subsequently subjected to electrophoresis in a 1% agarose matrix. The vector was excised from the agarose gel with a sterile razor blade, and subsequently electroeluted from the plug and purified as described above. It was then used in a three-fragment ligation reaction along with the isolated inserts from plasmids 3α1 and 6α4. The ligation reaction contained in a volume of 10 μL: 100 ng of fragment 3α1, 100 ng of fragment 6α4, 200 ng of pKK223-3, 2 μl of 5x ligase buffer [250 mM Tris-HCl pH 7.6 / 50 mM $MgCl_2$ / 5 mM ATP / 5 mM DTT / 25% (wt/vol) polyethylene glycol-8000], and 1 unit of T4 DNA ligase (BRL). The ligation reaction, in which the molar concentration of each insert was twice that of the vector, was incubated for 20 h at 16°C.

Expression of RTPR in E. coli HB101

Competent *E. coli* HB101 was thawed on ice, and a 100 μL aliquot of the bacteria was placed into a sterile polypropylene Falcon tube (17x100 mm, Becton

Dickinson). A 2 μL aliquot of the ligation mixture was added to the competent cells, and the resulting mixture was incubated on ice for 30 min. The mixture was subsequently heated at 42°C for 45 s, and then allowed to stand at room temperature for 2 min. A 500 μL aliquot of SOC media (Sambrook et al., 1989) previously equilibrated to 37°C was added to the mixture, and the mixture was incubated at 37°C for 1 h with aeration (300 rpms). A 400 μL aliquot of the resulting transformation mixture was spread on large (150x15 mm) LB plates supplemented with ampicillin (50 μg/mL), and then the plates were incubated at 37°C for 20 h. Of the nine colonies which were isolated, 3 were approximately 1/2 the size of the other 6. Each of the individual colonies was used to inoculate 5 mL of the above LB liquid media, and the resulting cultures were incubated at 37°C until a saturated culture was obtained (10-14 h). Plasmid DNA was isolated from each of the colonies using Qiagen Mini I columns according to the manufacturer's directions, and then digested with *Eco*R1. Upon subsequent electrophoresis in a 1% agarose matrix, two of the fast-growing colonies and all three of the slow-growing colonies were judged to have the correct construction. Aliquots (100 μL) from cultures of these 5 colonies were used to inoculate 5 mL of LB media supplemented with ampicillin (50 μg/mL) and 1 mM IPTG. The cultures were grown to saturation, and a 1-mL aliquot of each was used for analysis by SDS/PAGE according to the procedure of Laemmli (1970). Each aliquot was pelleted by centrifugation, and the pellet was subsequently resuspended in 100 μL of H_2O and 100 μL of 5x Laemmli buffer [0.3 M Tris-HCl, pH 6.8 / 10% (wt/vol) SDS / 50% (vol/vol) glycerol / 25% (vol/vol) 2-mercaptoethanol / 0.005% (wt/vol) bromophenol blue]. Each sample was boiled for 3-5 min and then subjected to SDS/PAGE in a 10% polyacrylamide matrix. The three slow-growing colonies expressed a protein that migrated with

RTPR isolated from *L. leichmannii*. One of the three (heretofore referred to as pSQUIRE) expressed RTPR at a much greater level than the other two.

Purification of Recombinant RTPR

A 1-mL saturated culture of *E. coli* HB101/pSQUIRE was used to inoculate 500 mL of LB media. The culture was grown to 37°C with aeration to late logarithmic phase (doubling time, 45 min). The bacteria were pelleted by centrifugation at 9000xg for 25 min, and the pellet was resuspended in 20 mL of buffer A (200 mM potassium phosphate, pH 7.2 / 1 mM 2-mercaptoethanol / 1 mM EDTA / 0.1 mM phenylmethylsulfonyl fluoride) equilibrated at 4°C. All subsequent steps were performed at 4°C, and all buffers contained 1 mM 2-mercaptoethanol. The cells were disrupted by passage through a French pressure cell at 16,000 psi, and the suspension was recentrifuged at 9000xg for 25 min. Protamine sulfate (1% in buffer A) was added to the supernatant (22 mL) to give a final concentration of 0.25%. Addition took place over 10 min, and the resulting solution was stirred for an additional 20 min. The precipitate was subsequently removed by centrifugation at 9000xg for 30 min. EDTA was added to the supernatant (27 mL) to a final concentration of 5 mM, and the supernatant was brought to 40% saturation with $(NH_4)_2SO_4$ (243 g/L) over 20 min. The solution was stirred for an additional 50 min, and the precipitate was isolated by centrifugation at 9000xg for 30 min. The pellet was dissolved in 1 mL of buffer B (10 mM potassium phosphate, pH 7.2 / 1 mM 2-mercaptoethanol) and desalted on a Sephadex G-25 column (1.6x45 cm), using buffer B as the eluate. The protein-containing fractions were pooled (20 mL) and loaded onto a DE-52 anion-exchange column (2.5x5.5 cm) equilibrated in buffer B. The column was washed with 50 mL of buffer B, followed by 50 mL of 100 mM potassium phosphate (pH 7.2). RTPR was eluted with 150 mM potassium phosphate (pH 7.2). Fractions

containing RTPR activity were pooled (41 mL) and exchanged into buffer C (10 mM potassium phosphate, pH 6.8 / 1 mM 2-mercaptoethanol) by diluting and concentrating the fractions in an Amicon ultrafiltration apparatus fitted with a PM-30 membrane. The protein was loaded onto a hydroxylapatite column (2.5x7 cm) equilibrated in buffer C, and the column was washed with 50 mL of the same buffer. The protein was eluted with a 400-mL linear gradient from 10 mM to 200 mM potassium phosphate. RTPR eluted at 75 mM buffer. The protein fractions were pooled, concentrated to 20 mg/mL, dialyzed against 100 mM sodium citrate, pH 5.5 / 1 mM dithiothreitol / 1 mM EDTA / 0.25% sodium azide / 20% (vol/vol) glycerol, and stored in aliquots at -80°C.

RTPR Activity Determination

RTPR was assayed by a modification of the procedure of Blakley (Blakley, 1978). The assay mixture contained in a final volume of 500 µL: 50 mM potassium phosphate (pH 7.5), 4 mM EDTA, 1 M NaOAc, 100 mM ATP (pH 7), 54 µM *E. coli* TR, 64 nM *E. coli* TRR, 0.2 mM NADPH, 8 µM AdoCbl, and 0.1-0.2 nM RTPR. All components except AdoCbl were added to a 0.7-mL cuvette and equilibrated in a 37°C water bath for 3-5 min. Subsequent to recording the background rate of NADPH oxidation (ε=6220 M^{-1} cm^{-1}), AdoCbl was added to initiate the reaction. The specific activity of RTPR (µmol of dATP formed per min per mg of enzyme) was then calculated as the difference of these two rates.

Kinetic Characterization of Recombinant RTPR

Initial rates of dATP production were determined by the spectrophotometric assay using TR/TRR/NADPH as described above. Each assay was carried out at different concentrations of ATP (0.054 mM, 0.162 mM, 0.27 mM, 0.54 mM, 2.7 mM, and 4.0 mM), using 0.4 nM wild-type or recombinant

RTPR. In order to obtain a K_m value for AdoCbl, the ATP concentration in the assay mixture was held constant at 10 mM, and each assay was performed at different concentrations of AdoCbl (0.11 μM, 0.32 M, 0.54 μM, 1.1 μM, 2.2 μM, 5.4 μM, 8.1 μM, and 10.8 μM). The reciprocal initial velocities were plotted as a function of the reciprocal substrate concentrations to insure that the double-reciprocal plots were linear. The kinetic constants V_{max} and K_m were determined from a fit of the initial velocities to a FORTRAN version of the program HYPER (Cleland, 1979) to Eq 1, where v is the initial rate, V_{max} is the maximum velocity, and K_m is the Michealis -Menton constant.

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad \text{Eq 1}$$

Analysis of the Ability of dGTP to Stimulate the Reduction of ATP using Recombinant RTPR

The reaction was carried out as described above for the analysis of dATP production by the NADPH-dependent spectrophotometric assay, except that NaOAc was replaced with varying concentrations of dGTP (0 μM, 2 μM, 6 μM, 10 μM, 20 μM, 40 μM, 100 μM, 175 μM, 200 μM, 350 μM, and 700 μM).

Transformation of pSQUIRE into E. coli JM105 and Expression of RTPR under the Control of an Inducible System

E. coli JM105 was made competent by the following procedure. The bacteria were streaked onto LB plates supplemented with streptomycin (25 μg/mL), and grown overnight at 37°C. A single colony was used to inoculate

5 mL of the same liquid media, and the culture was grown to saturation at 37°C. A 100-µL aliquot of this culture was used to inoculate 50 mL of LB media (pH 7.2), which was subsequently grown to an OD_{600} of 0.3 (spectrophotometer blanked with media) and then immediately placed on ice. A 20-mL aliquot was removed and pelleted by centrifugation at 10,000xg for 20 min. The pellet was resuspended in 7.5 mL of 50 mM $CaCl_2$ that had been equilibrated to 4°C, and the mixture was incubated on ice for 8 min. The mixture was centrifuged again as described, and the pellet was resuspended in 1 mL of 50 mM $CaCl_2$. A 200-µL aliquot of the bacterial suspension was added to a Falcon tube (17x100 mm, Becton Dickinson) containing 100 µL of 100 mM Tris-HCl (pH 7.4). Plasmid pSQUIRE (20 ng) was added, and the resulting mixture was incubated on ice for 10 min. The tube was removed from the ice, and immediately immersed in a water bath equilibrated at 42°C, and incubated for 2 min at this temperature. The mixture was allowed to stand at room temperature for 10 min, following which a 1-mL aliquot of LB media (pH 7) containing 0.2% dextrose was added. The transformation mixture was incubated for 1 h without agitation in a water bath equilibrated to 37°C. Subsequent to this incubation, a 200-µL aliquot was spread on LB-agar plates supplemented with ampicillin (50 µg/mL).

A single colony was used to inoculate 5 mL of LB media supplemented with ampicillin (50 µg/mL), which was subsequently grown to saturation at 37°C with aeration. A 100-µL aliquot of this culture was subsequently used to inoculate 60 mL of the above media, and the resulting culture was grown at 37°C with aeration. Every 30-60 min an aliquot was removed to check the OD_{600}. At an OD_{600} of 0.9, IPTG was added to the culture through a 0.2 µm sterile filter (Millipore) to a final concentration of 1 mM . Before the addition of IPTG, and every hour subsequent to it, a 1-mL aliquot was removed and prepared for SDS/PAGE as described above.

Expression of RTPR in D_2O/Minimal Media and Purification of the Deuterated Protein

E. coli JM105/pSQUIRE was streaked onto M9 minimal media-agar plates (Sambrook et al., 1989) supplemented with thiamine (0.00005%), $MgSO_4$ (1 mM), and ampicillin (50 μg/mL), and allowed to grow at 37°C overnight. A single colony was used to inoculate 50 mL of the above liquid media, and the culture was grown at 37°C with aeration. IPTG (1 mM final concentration) was added to the culture once it had attained an OD_{660} of 0.5, and expression was allowed to take place for 8 h. The expression of RTPR was monitored by SDS/PAGE before induction with IPTG, and every 1-3 h after induction using the procedure of Laemmli as described above.

A 100-μL aliquot of the above culture was then added to 5 mL of the above minimal media containing 50% D_2O, and the resulting culture was allowed to grow to saturation at 37°C. A 100-μL aliquot of this culture was removed and added to 5 mL of minimal media containing 80 % D_2O, and this culture was subsequently grown to saturation. Lastly, a 100-μL aliquot of the 80% D_2O culture was used to inoculate 100 mL of the above minimal media containing ≥95% D_2O. This culture was grown at 37°C (doubling time ~3 h), with the OD_{660} being monitored every 1-3 h. At an OD_{660} of 0.5, IPTG was added to a final concentration of 1 mM, and the expression of RTPR was allowed to proceed for 15 h.

Deuterated RTPR was purified according to the procedures outlined above with the following modifications. Upon the initial pelleting of the bacteria, they were resuspended in 2 mL of buffer A. After precipitating the protein with $(NH_4)_2SO_4$, the pellet was dissolved in 500 μL of buffer B , and the protein was desalted on a Sephadex G-25 column (0.75x25 cm). The protein-containing fractions (A_{280}) were pooled and loaded onto a DE-52 column (1.25x5 cm)

equilibrated in buffer B. The column was washed with 10 mL of buffer B, followed by 10 mL of 100 mM potassium phosphate (pH 7.2). RTPR was then eluted with 150 mM potassium phosphate (pH 7.2). It was concentrated and dialyzed against 100 mM sodium citrate, pH 5.6 / 1 mM dithiothreitol / 1 mM EDTA / 0.25% sodium azide / 20% (vol/vol) glycerol, and stored at -80°C.

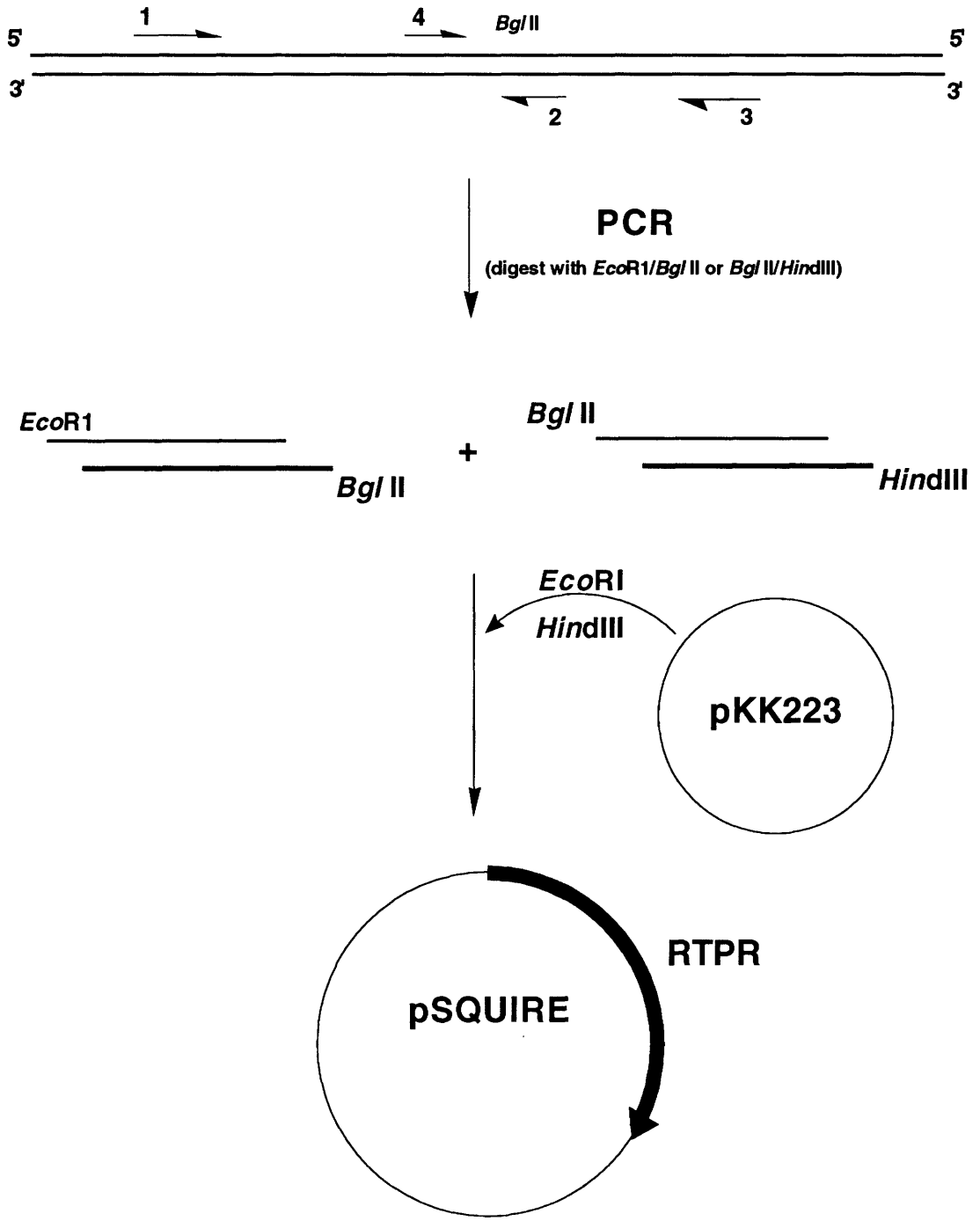
Results

Expression-Cassette Cloning of the Entire RTPR Gene into Plasmid pKK223-3

The recent method by MacFerrin *et al.* (MacFerrin et al., 1990) for maximizing the efficiency of translation of cloned genes in *E. coli* allowed the formulation of the strategy outlined in Figure 3.3 for the cloning of the RTPR gene. The polymerase chain reaction was used to incorporate at the front of the gene necessary elements for efficient translation. These elements, which were engineered into the N-terminal PCR primer, included an 8-base transitional spacing element flanked by a consensus Shine-Dalgarno sequence and the ATG start codon of the RTPR gene. In addition, the 5' terminus of the N-terminal primer included an *Eco*R1 restriction site to facilitate the cloning of the amplified fragment, and a GC clamp to aid the restriction enzyme in cutting at the terminus of the DNA (Figure 3.4). The C-terminal primer included the RTPR gene's natural TAA stop codon as well as a *Hin*dIII restriction site at its 5' end.

The cloning process diverged somewhat from the traditional approach of amplifying a segment of DNA and ligating it directly into the appropriately-digested expression vector. The gene for RTPR was amplified in two halves,

Figure 3.3: Strategy for the cloning of the RTPR gene. PCR primers containing restriction sites at their 5' ends were constructed from the gene sequence of RTPR. The N-terminal primer included the elements necessary for efficient translation in *E. coli*, while the C-terminal primer included the gene's natural TAA stop codon. The gene was amplified in two halves, each of which was ligated into the Invitrogen vector pCR1000. Each vector was transformed into *E. coli* DH5α and subsequently isolated in large quantities. The inserts were removed by appropriate restriction digestion, and ligated together into plasmid pKK223-3 to give pSQUIRE.


1
4
Bgl II
5'
3'
5'
3'
2
3
PCR
(digest with EcoR1/Bgl II or Bgl II/HindIII)
EcoR1
Bgl II
+
Bgl II
HindIII
EcoRI
HindIII
pKK223
pSQUIRE
RTPR

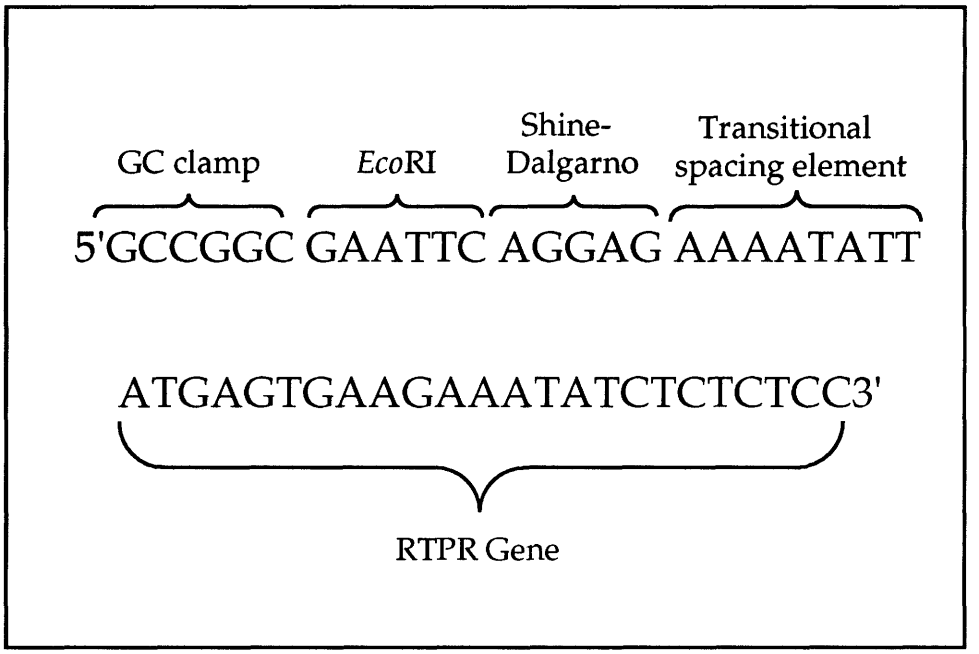


Figure 3.4: Construction of the N-terminal PCR primer for expression-cassette PCR.

each of which was then ligated separately into the Invitrogen vector pCR1000. The choice of this vector was governed by the high efficiency with which DNA amplified by PCR can be ligated directly into it. In addition, the ligation reaction requires no initial restriction digestion to create cohesive ends on either the vector or the amplified fragment. The success of this vector is due to the fact that it is commercially available in a linearized form such that both its 5' terminii have T overhangs. As described in Chapter 2, many DNA polymerases (including *Taq* polymerase) leave an A overhang at the 3' end of the template. The extent to which this A overhang is present among a population of amplified fragments depends largely upon the level of the 3'→5' exonuclease activity of the polymerase (Clark et al., 1987; Clark, 1988). *Taq* polymerase possesses no exonuclease activity; and ligations with DNA amplified with this polymerase are 50-fold more efficient using the Invitrogen vector pCR1000 than the corresponding ligations with blunt-ended DNA (Mead et al., 1991). The plasmid (6α4) containing the N-terminal half of the gene, as well as the plasmid (3α1) containing the C-terminal half of the gene, was transformed into *E. coli* and isolated in large quantities. The inserts from these plasmids were then used to construct the full-length RTPR gene, by exploiting a unique *Bgl* II restriction site which each insert shared. Two lines of reasoning suggested this approach. The first is that plasmid pKK223-3 often yields better expression when the N terminus of the gene of interest is ligated into the *Eco*RI restriction site of the plasmid (personal communication Dr. Oliver Peoples). The presence of an *Eco*RI restriction site within the RTPR gene precludes a facile ligation into the expression vector since the C-terminal *Eco*RI-*Hin*dIII fragment would be expected to ligate much more efficiently than that which would give the correct construction - an *Eco*RI/*Eco*RI - *Eco*RI/*Hin*dIII three-fragment ligation into pKK223-3. The second line of reasoning is based on the anticipation of making

mutants of cysteines that are believed to be important in catalysis. As mentioned in Chapter 2, the two C-terminal cysteines (731 and 736) as well as C408 are believed to be important in catalysis as determined from a sequence comparison of RTPR with the *E. coli* RDPR. The C-terminal cysteines can easily be altered by PCR with a mutant primer since they lie at the extreme C terminus of the gene. However, the unique *Bgl* II restriction site within the RTPR gene that is present on both plasmid 6α4 and 3α1 can be used to easily alter C408 and C419 by PCR (For a detailed description of the mutagenesis of these cysteines, see Chapter 4). In short, a C408X mutant primer containing the *Bgl* II restriction site can be used in combination with primer **1** to amplify the N-terminal half of the gene containing the mutated amino acid. The amplified fragment can then be ligated into the expression vector along with the C-terminal half of the gene from plasmid 3α1. Likewise, a C419X mutant can be made using a primer containing a *Bgl* II restriction site in combination with primer **3** to amplify the C-terminal half of RTPR containing the mutated amino acid. This fragment can then be ligated into the expression vector in combination with the N-terminal half of the gene from plasmid 6α4. By maintaining the two halves of RTPR on separate plasmids, a well-characterized and ample source of the components of RTPR is always available in a construct that can be easily manipulated.

Expression of RTPR in E. coli HB101

An aliquot of the three-fragment ligation into pKK223-3 was transformed into *E. coli* strain HB101 - a strain which does not overproduce the *lac* repressor. Two distinct phenotypes were observed upon plating the transformation mixture. The resulting colonies either grew slowly (20-24 h vs 10-12 h to see a colony which grows at a normal rate) and were small in size, or grew at normal rates and were of the expected size. Of the 9 colonies which were obtained after

transformation and plating, 6 were fast-growers, while 3 were slow-growers. The slow growers maintained the same phenotype in liquid culture as well. Another sample of the ligation mixture was again transformed into *E. coli* HB101 to determine if the occurence of two phenotypes was reproducible. In this case twenty-six colonies were obtained, with most having the slow-growing phenotype. Two of the fast-growers and all three of the slow growers from the initial transformation were shown to have the correct construction upon restriction digestion and subsequent electrophoresis of DNA preparations from cultures of the 9 colonies. Cultures of these 5 colonies, in which IPTG (1 mM) was added at the time of the initial inoculation, were subjected to SDS/PAGE by the procedure of Laemmli (1970) to monitor the expression of RTPR. Neither of the two fast-growers overproduced RTPR as determined by comparison of the Coomassie Brilliant Blue-stained gel with that containing an authentic sample of RTPR from *L. leichmannii*. All three of the slow-growers, however, exhibited relatively large bands which migrated with the authentic RTPR sample (data not shown). The overproduction of one of these slow-growers appeared to be twice as good as the other two, and was accordingly chosen as the recombinant RTPR.

An experiment was carried out to determine the optimal time in which to add IPTG to the culture. Three separate cultures of *E. coli* HB101/pSQUIRE were grown under varying conditions. Culture C was grown in the presence of 1 mM IPTG. Culture B was grown in media containing no added IPTG, and culture A was "induced" with the addition of IPTG to 1 mM at an OD_{660} of 0.5. Figures 3.5 and 3.6 show the results of this experiment. As can be visualized, there appears to be no dependence of IPTG on expression of RTPR from pSQUIRE. All three cultures confer the same amount of expression regardless of when IPTG is added, or whether it is added at all. The only difference between the three cultures is that C grew at a slower rate (slightly less than twofold) than A and B.

Figure 3.5: Expression of RTPR (cultures A and B). Lanes 1-5 (culture A) contain samples in which IPTG was added to the growing culture at an A_{600} of 0.9. Aliquots were removed at 1 h intervals and monitored for expression by SDS/PAGE. Lane 1 is a sample taken before the addition of IPTG. Lanes 2-5, are 1 h, 2 h, 3 h, and 4 h subsequent to the addition of IPTG. Lanes 6-10 (culture B) contain samples in which no IPTG was added to the growing culture. Lane 6 contains a sample removed at an A_{600} of 0.6. Lanes 7-10 contain samples removed at 1 h, 2 h, 3 h, and 4 h subsequent to the intial sample. Lane 11 contains a sample of RTPR isolated from cultures of *L. leichmannii*.

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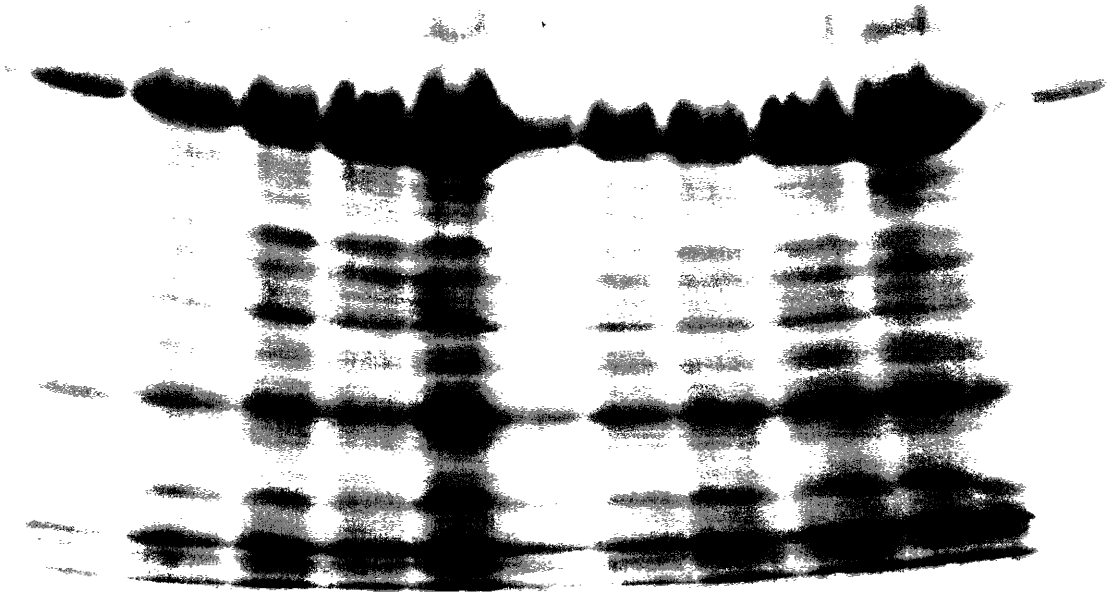
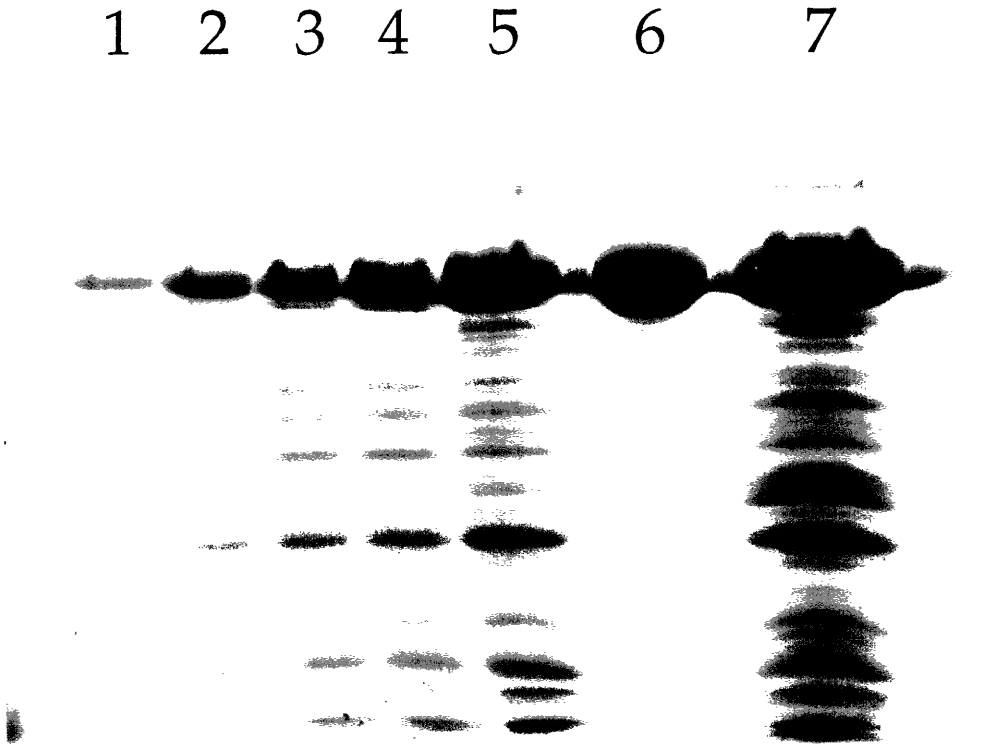


Figure 3.6: Expression of RTPR (culture C). Lanes 1-5 contain samples which were grown in the presence of IPTG from the inital inoculation. Lane 1 contains a sample removed at an A_{600} of 0.45. Lanes 2-5 contain samples removed at 1 h, 2 h, 3 h, and 4 h subsequent to the initial sample. Lane 6 contains a sample of RTPR isolated from cultures of *L. leichmannii*. Lane 7 contains a saturated culture of RTPR grown in the absence of IPTG.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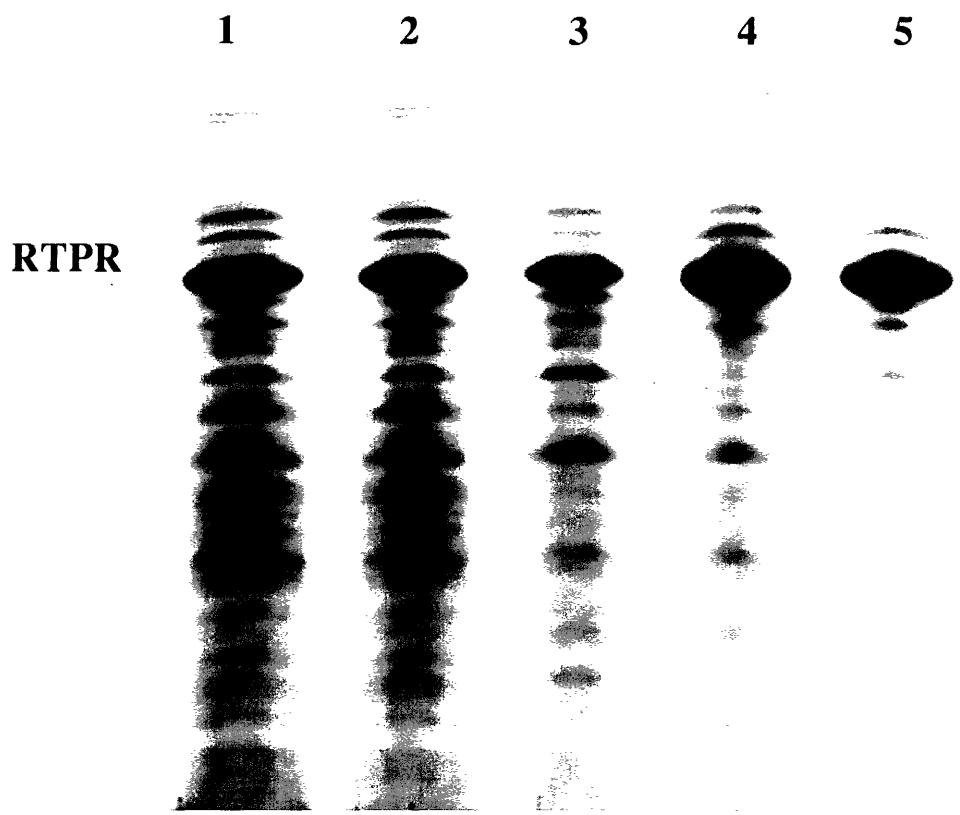
A 50 mL culture of *E. coli* HB101/pSQUIRE was grown to saturation, and a crude extract was prepared as described in the methods for the purification of RTPR. The specific activity of the crude was determined to be ~0.4 U/mg, which is 10 to 20-fold greater than that from cultures of *L. leichmannii* (0.02-0.04 U/mg) (Harris, 1984). Moreover, this activity was absolutely dependent upon the addition of AdoCbl to the assay mixture.

Purification of Recombinant RTPR

Recombinant RTPR was purified using procedures very similar to those used to purify RTPR from *L. leichmannii* (Ashley et al., 1986). Protamine sulfate at 0.1% (wt/vol) was much more effective in precipitating nucleic acids than streptomycin sulfate, which is used routinely in the *L. leichmannii* isolation. Two additional protease inhibitors other than PMSF were tested for their ability to stabilize RTPR activity throughout the isolation. The addition of tosyllysine chloromethylketone and tosylphenylalanine chloromethylketone to the crude preparation (each at a concentration of 1 mM) resulted in a 70% loss of activity of RTPR as determined by the TR/TRR/NADPH spectrophotometric assay. This inhibition was not studied in detail, however, these compounds are inhibited by alkylating agents. Therefore it is believed that inhibition of RTPR most likely occurs via the alkylation of the C-terminal cysteines by the added compounds.

Table 3.2 summarizes a typical protein isolation from a 500-mL saturated culture of *E. coli* HB101/pSQUIRE [~2.5 g (wet wieght)]. Approximately 90 mg of protein that is greater than 95% pure can be obtained from a half-liter culture of the recombinant bacteria. This yield is comparable to what was obtained from 200 g of *L. leichmannii* cell paste (Harris, 1984; Ashley et al., 1986). Moreover, the specific activity of 1.5 $\mu mol \bullet min^{-1} \bullet mg^{-1}$ is comparable to the best reported specific activities of non-recombinant RTPR. Figure 3.7 illustrates again the high

Figure 3.7: SDS/PAGE (10%) analysis of the purification of RTPR. Lane 1 is the crude lysate. Lane 2 is after the protamine sulfate step. Lane 3 is after the ammonium sulfate step. Lane 4 is after the DE-52 anion-exchange column. Lane 5 is after the hydroxylapatite column.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RTPR

level of heterologous expression that is conferred by plasmid pSQUIRE, and indicates the purity of RTPR throughout each step of the purification as judged by Coomassie Brilliant Blue staining. Elution profiles of both the DE-52 and hydroxylapatite columns (data not shown) indicate that RTPR mutants can be isolated by essentially pooling the huge peaks which elute at 150 mM potassium phosphate (pH 7.2) for the DE-52 column, and 75 mM potassium phosphate (pH 6.8) for the hydroxylapatite column. This is important since a good affinity column for this protein is not available, and several of the mutants would be expected to have no easily-assayable phenotypes.

Table 3.2: Purification of RTPR from *E. coli* HB101/pSQUIRE

Step	Protein (mg)	Specific activity (units/mg)	Total units
Crude	660	0.4	230
Protamine sulfate	648	0.4	228
$(NH_4)_2SO_4$/Sephadex G-25	272	0.6	171
DE-52	131	1	130
Hydroxylapatite	90	1.5	134

Characterization of Recombinant RTPR

Recombinant RTPR was characterized in several different ways. Firstly, the RTPR gene on plasmid pSQUIRE was sequenced to verify its identity to the gene sequence reported in Chapter 2. Secondly, an amino acid analysis and N-terminal sequence analysis of the recombinant protein was carried out

essentially as described in Chapter 2 for the non-recombinant RTPR. However, the samples were submitted to the MIT Biopolymers Laboratory instead of the Harvard Microchemistry Facility. Table 3.3 summarizes the amino acid composition of recombinant RTPR, as well as the non-recombinant RTPR that was used to obtain N-terminal sequence information for cloning. In addition, previous determinations by Georgianna Harris (1984) and Panagou *et al.* (1972) are included. The amino acid analysis by Harris agrees best with the amino acid composition of RTPR deduced from its gene sequence, with the analysis by the Harvard Microchemistry Facility of the non-recombinant RTPR agreeing the least. The composition of the recombinant RTPR is in fair proximity with that deduced from the gene sequence except in the cases of the amino acids methionine and lysine. Methionine is often difficult to quantify by automated methods, and is best characterized by treatment of the protein with cyanogen bromide and isolation of the the resulting fragments by HPLC. The discrepancy with respect to lysine is not understood at present, however, the variability between the results of the two most recent determinations (Booker) suggests that it could be due to experimental variance. The N-terminal sequence of the recombinant RTPR (S-E-E-I-S-L-S-A-E-F) is identical with that of the non-recombinant protein, as well as with the amino acid sequence deduced from the gene sequence. It is interesting to note that the N-terminal methionine is also processed, as it is in the enzyme isolated directly from *L. leichmannii.*

The third method employed to characterize recombinant RTPR consisted of obtaining kinetic constants for both substrate (ATP) and cofactor (AdoCbl). A comparison of these values to those of the non-recombinant enzyme which were determined under the same set of conditions is listed in Table 3.4. Although these constants were determined from a fit to the kinetic program HYPER as

described in the methods, Lineweaver-Burk plots were also constructed (Figures 3.8 and 3.9) to show that the data were indeed linear. As can be seen in Table 3.4,

Table 3.3: Amino acid composition of recombinant RTPR.

Amino acid	Harris[a]	Panagou[b]	Booker (non-recombinant)[c]	Booker (recombinant)[d]
ASX (86)[f]	83	78	111	84
GLX (84)	80	78	96	102
SER (55)	44	54	72	59
GLY (58)	57	56	80	75
HIS (9)	10	8	7	12
ARG (37)	35	33	38	39
THR (35)	33	32	32	36
ALA (58)	58	55	66	67
PRO (31)	27	31	21	41
TYR (27)	25	24	26	31
VAL (51)	47	46	35	49
MET (10)	10	8	2	4
ILE (45)	40	39	31	40
LEU (62)	n.d.	59	58	65
PHE (27)	24	25	22	24
LYS (42)	42	43	48	30

[a](Harris, 1984). [b](Panagou et al., 1972). [c, d]This work. [f]parentheses indicate the number of residues as determined from the gene sequence.

the K_ms and V_{max}s for the recombinant and non-recombinant enzymes are in very good agreement for both the substrate and cofactor.

Table 3.4: [a]Kinetic constants for recombinant and non-recombinant RTPRs.

	ATP		AdoCbl	
RTPR	K_m (mM)	V_{max}	K_m (μM)	V_{max}
Recombinant	1.2±0.11	1.1 U/mg	1.1±0.07	1.2 U/mg
Non-recombinant	1.1±0.12	0.85 U/mg	1.59±0.21	1.1 U/mg

[a]Kinetic constants obtained with 1 M NaOAc, and TR/TRR/NADPH.

Additional studies were carried out to show the ability of the allosteric effector (dGTP) to stimulate the reduction of substrate (ATP). Initial experiments were focused on determining the concentration of dGTP that yields the maximum rate of dATP production. Figure 3.10 is a plot of the initial rate of ATP reduction as a function of dGTP concentration. RTPR exhibits turnover in the absence of allosteric effector; however, dGTP at 150-200 μM increases the rate of ATP reduction by a factor of 6. Higher concentrations of dGTP appear to be inhibitory. At a concentration of 350 μM dGTP the rate is 69% of the maximum; and at a concentration of 5.2 mM dGTP it is appromoxmately equal to the rate of ATP reduction in the absence of allosteric effector (data not shown). This data suggests that the allosteric effector is able to bind to the substrate site at high concentrations. Further studies carried out to obtain a K_m for substrate (ATP) in the presence of 150 μM dGTP also showed an interrelatedness of substrate and allosteric effector. Figure 3.11 shows that at high substrate concentrations the

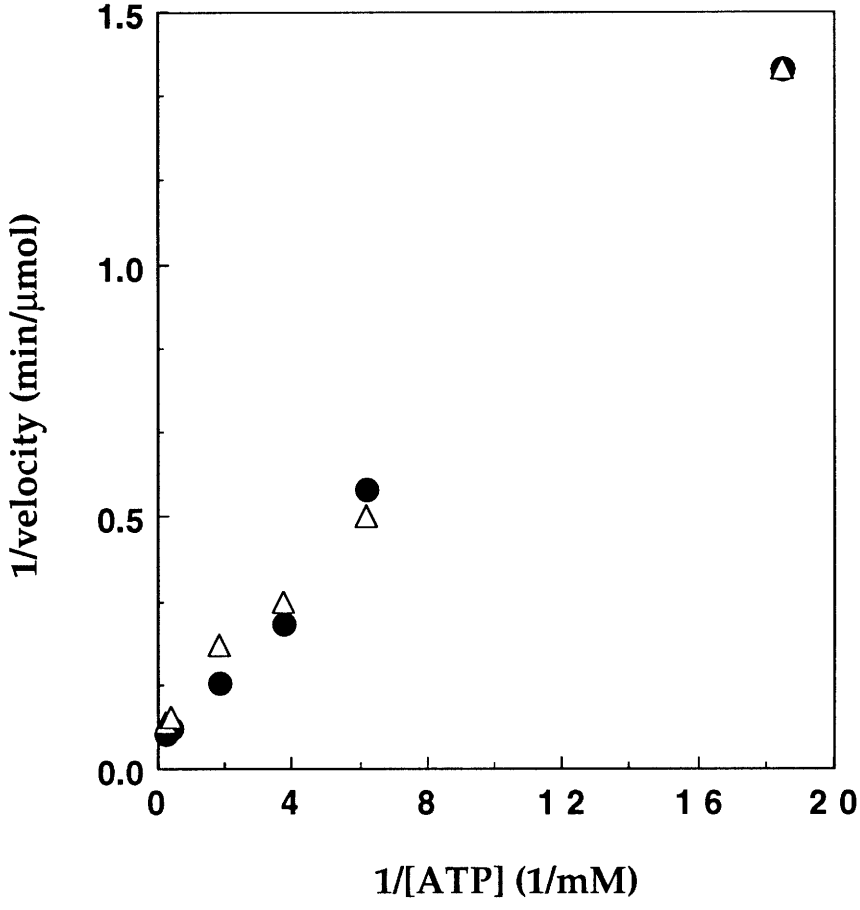


Figure 3.8: Lineweaver-Burk plot of initial rate data vs [ATP]. Concentrations varied from 0.054 mM to 4.05 mM ATP. (●) Recombinant RTPR. (Δ) Non-recombinant RTPR.

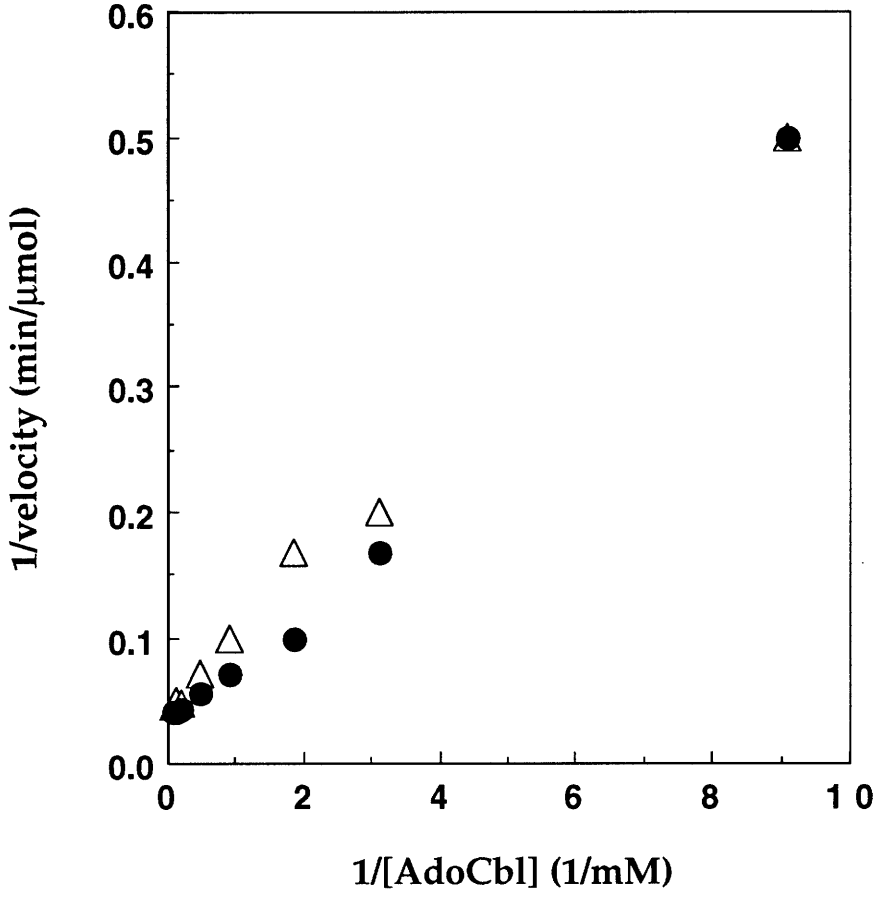


Figure 3.9: Lineweaver-Burk plot of initial rate data vs [AdoCbl]. Concentrations were varied from 0.11 to 10.8 μM AdoCbl. (●) Recombinant RTPR. (Δ) Non-recombinant RTPR.

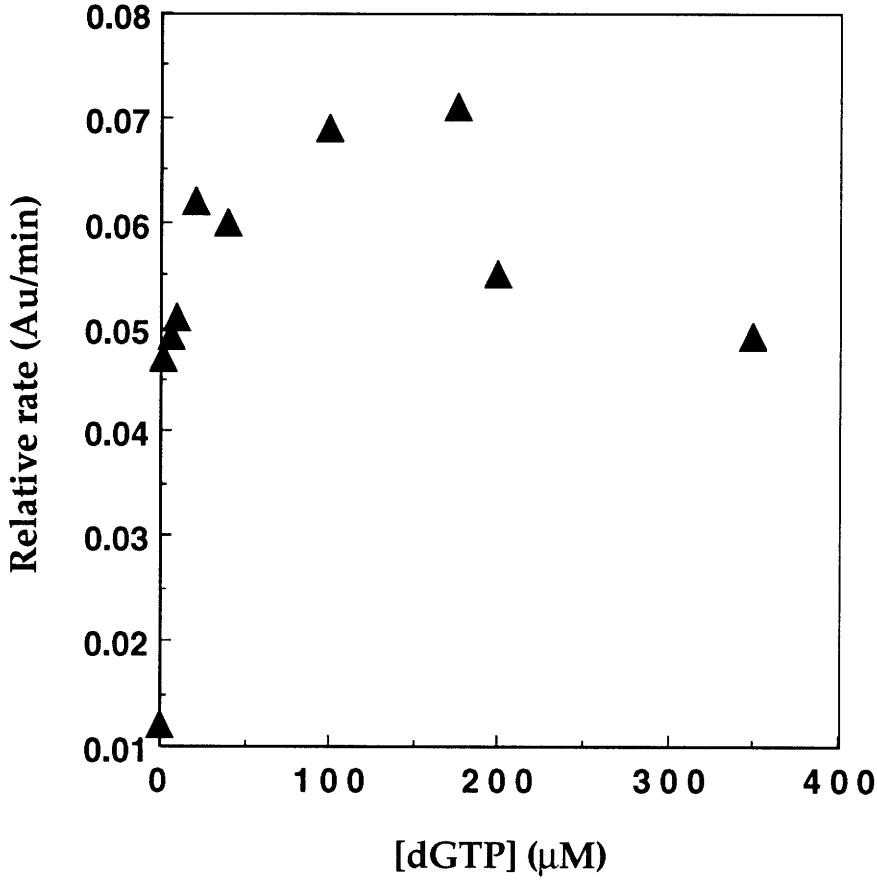


Figure 3.10: Initial rate of dATP production as a function of [dGTP]. dGTP concentrations were varied from 0 to 350 μM.

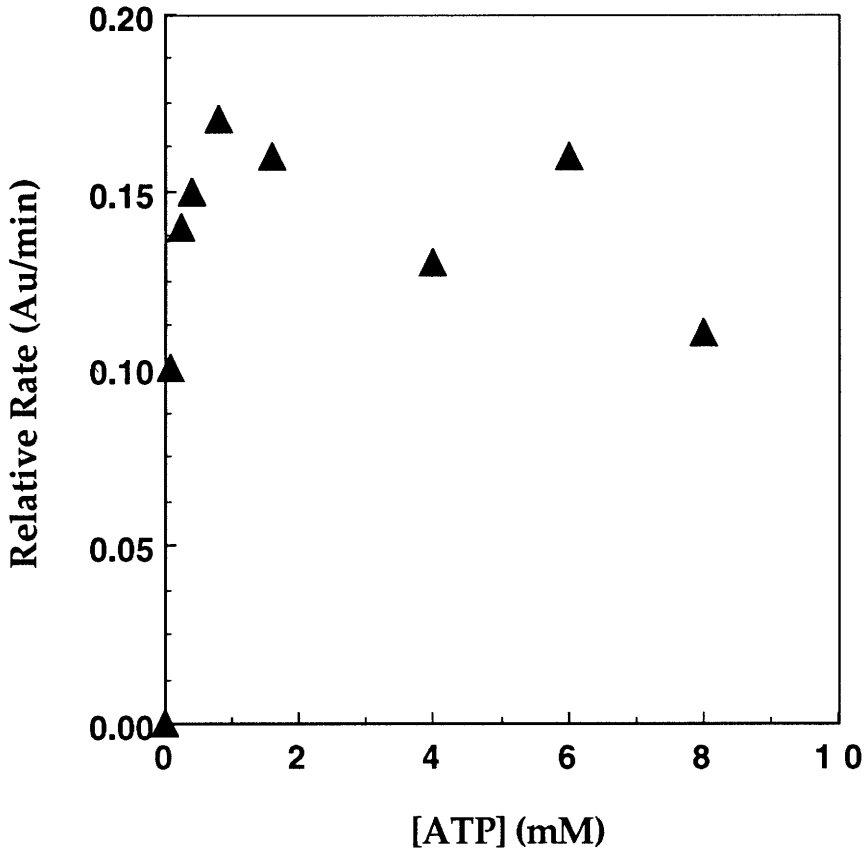


Figure 3.11: Initial rate of ATP reduction as a function of [ATP]. Initial rates were obtained in the presence of 150 μM dGTP. ATP concentrations were varied from 0.08 to 8 mM.

initial rates of ATP reduction begin to drop off. A previous study which was initiated to characterize the kinetics of RTPR isolated from *L. leichmannii* also reported non-Michaelis-Menten kinetics (Vitols et al., 1967). Interestingly, the degree of substrate inhibition of turnover was found to be dependent upon the concentration of the allosteric effector. In the case of CTP reduction, substrate inhibition was observed at concentrations greater than 10 mM in the absence of dATP - the allosteric effector for CTP reduction. In the presence of 1 mM dATP, inhibition was observed at CTP concentrations of 2 mM. Similar results were obtained with ATP and dGTP; however, the details of the data were not reported. Nonetheless, these experiments reported herein show that the recombinant RTPR is kinetically similar to the non-recombinant protein. Under identical conditions, they each have approximately the same K_ms and V_{max}s for substrate and cofactor. The recombinant protein is stimulated by the allosteric effector dGTP, and also exhibits substrate inhibition at sufficiently high concentrations of ATP - characteristics which the non-recombinant protein also portrays.

Inducible Expression of RTPR in E. coli JM105

The high level of expression of RTPR in *E. coli* HB101 places a great deal of strain on the bacterium, since a large amount of its energy is used to produce a foreign protein which has no bearing on its metabolism. Constitutive expression of a protein at this level can lead to mutations which reduce or abolish the expression of the foreign protein. Because these bacteria would be expected to have normal doubling times (20 min vs 45-60 min for HB101/pSQUIRE), bacteria which express RTPR at decent levels would be selected against. It was therefore desirable to place plasmid pSQUIRE in an *E. coli* strain that would allow the expression of RTPR to be induced at a given time. Unlike *E. coli* HB101, JM105

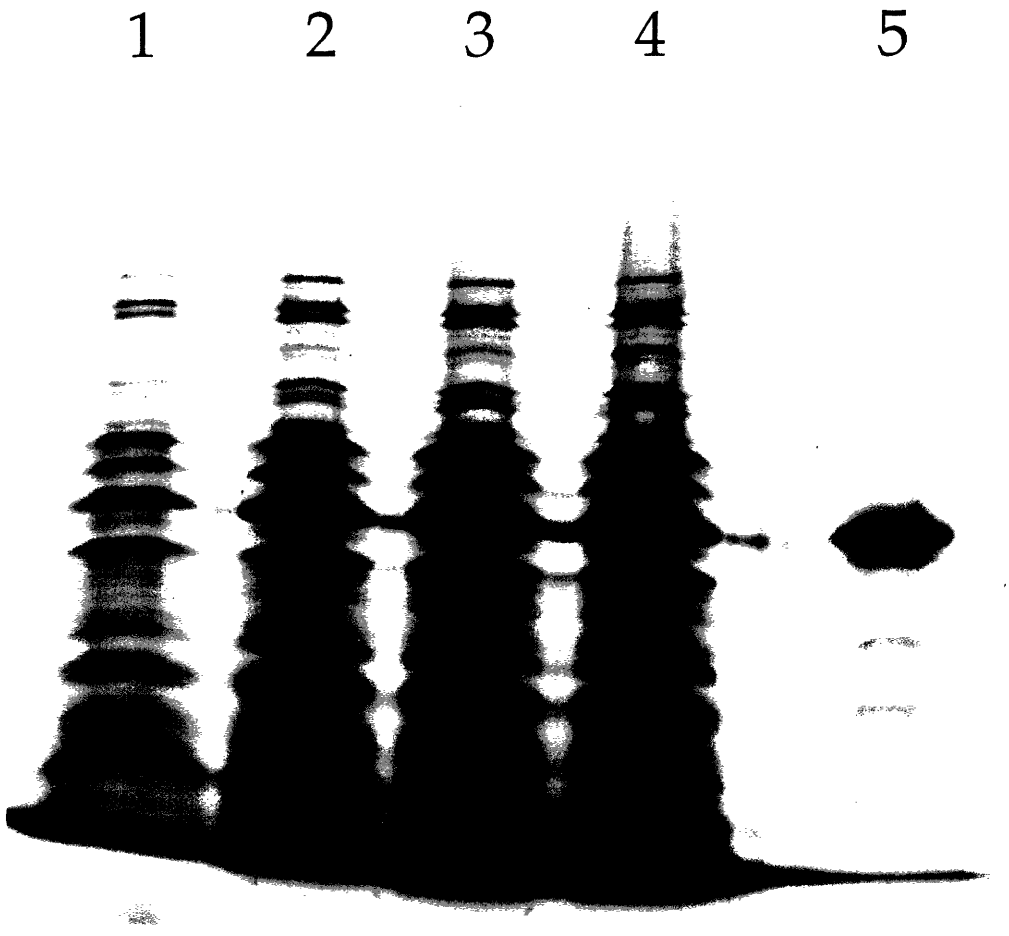
contains the *lacI*^{Q} genotype, which allows the bacterium to synthesize the *lac* repressor in sufficient quantities to repress the expression of genes which are controlled by a *lac* promoter. As described in the introduction to this chapter, IPTG can then be added at the desired time to induce the expression of the cloned gene.

Plasmid pSQUIRE was transformed into *E. coli* JM105, and grown in LB media (doubling time, 30 min) supplemented with ampicillin (50 μg/mL). Figure 3.12 is a Coomassie Brillian Blue-stained SDS/PAGE gel (10%) of the progress of the induction experiment. Before induction, very little RTPR production is exhibited. However, subsequent to the addition of 1 mM IPTG, the levels of RTPR become quite substantial within the first hour. From a progress curve of specific activities of crude cell lysates vs time after induction, it appears that the highest specific activity of RTPR (0.2 U/mg) occurs after ~3-4 h of growth subsequent to induction.

Expression of RTPR in Minimal Media Containing D_2O as the Solvent

One of the advantages in using *E. coli* JM105 to express RTPR from pSQUIRE, is that the protein can be overproduced under a variety of conditions. One set of conditions which is of interest is in minimal media. In this case, specifically-labeled amino acids which might help deconvolute particular aspects of the mechanism of catalysis could be added to the medium. In the same fashion, it was of interest to produce totally-deuterated RTPR in order to look for the participation of a protein backbone radical (perhaps glycine as in pyruvate formate lyase) in catalysis. Initial efforts were focused on growing *E. coli* HB101/pSQUIRE in minimal media; however, they were unsuccessful. Although this bacterial strain is auxotrophic for leucine, addition of this amino acid to the media did not stimulate growth. *E. coli* JM105/pSQUIRE was

Figure 3.12: Inducible expression of RTPR in *E. coli* JM105. Lane 1 is before induction. Lanes 2-4 are 1 h, 2 h, and 3 h after the addition of 1 mM IPTG to a culture having an A_{600} of 0.9.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therefore used to express RPTR in minimal media. Under the conditions described in the methods, the bacteria had a doubling time of ~1 h. These bacteria were gradually adapted to minimal media which contained D_2O (≥95%) as the solvent. As shown in Figure 3.13, these D_2O-adapted bacteria could be induced to express RTPR with the addition of 1 mM IPTG . To show that the deuterated protein was indeed active, the bacteria were pelleted, and RTPR was isolated through the DE-52 column as outlined in the methods. The specific activity of the deuterated protein was determined using the TR/TRR/NADPH assay, and was found to be 1.3 U/mg.

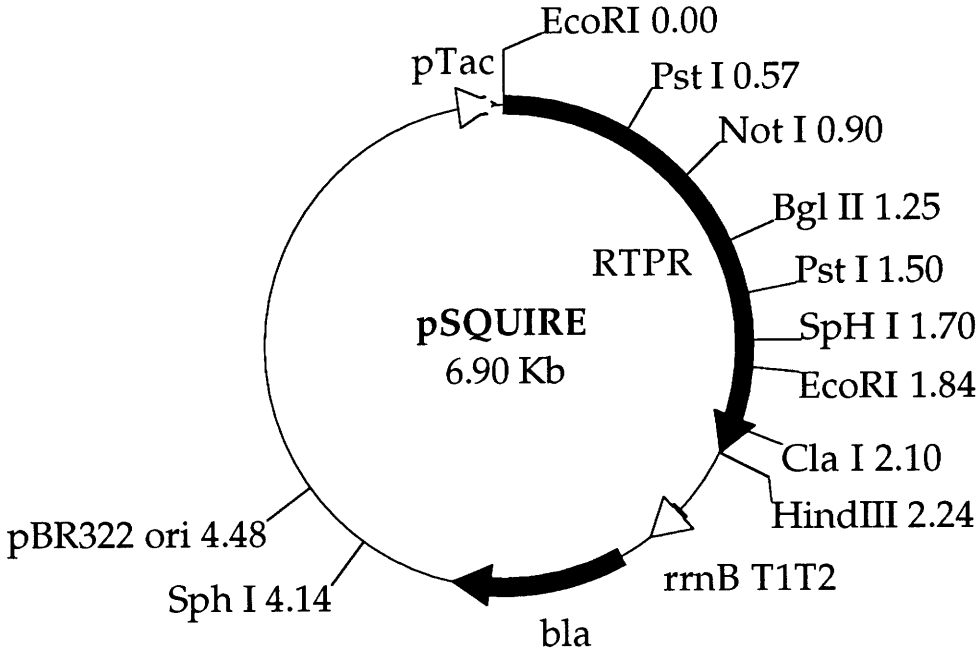
Discussion

The successful cloning and overexpression of RTPR in *E. coli* was largely due to the isolation and sequencing of the RTPR gene. Using the rules outlined in the introduction for maximizing the efficiency of translation of foreign proteins in *E. coli*, a set of primers was constructed to be used to amplify and clone the RTPR gene. The gene was amplified in two halves, and then ligated into the vector pKK223-3, where the expression of RTPR was under control of the *tac* promoter. Figure 3.14 shows a map of the resulting plasmid pSQUIRE, containing important restriction sites that can be used for further subcloning or the making of mutants. When pSQUIRE is transformed into *E. coli* strain HB101, the expression of RTPR is consitutive. As mentioned in the introduction to this chapter, this is due to this strain's inability to overproduce the *lac* repressor protein. This strain overproduces pSQUIRE at levels that approach 30-40% of the total cellular protein. In addition, the ability to express the protein at this level in the absence of the inducer IPTG is an added benefit since this compound is

Figure 3.13: Expression of RTPR in minimal media with D_2O as the solvent. Lane 1 is before induction. Lane 2 is 6 h after the addition of 1 mM IPTG to a culture having an A_{600} of 0.5. Lane 3 is 15 h after induction.

1 2 3

Figure 3.14: Circular map of plasmid pSQUIRE showing unique restriction sites as well as those which may be important for subcloning.


EcoRI 0.00
pTac
Pst I 0.57
Not I 0.90
Bgl II 1.25
RTPR
Pst I 1.50
pSQUIRE
6.90 Kb
SpH I 1.70
EcoRI 1.84
Cla I 2.10
HindIII 2.24
pBR322 ori 4.48
rrnB T1T2
Sph I 4.14
bla

relatively expensive. When pSQUIRE is transformed into *E. coli* strain JM105 - a strain which does produce the repressor in high quantities - the expression of RTPR is minimal until IPTG is added. The ability to express RTPR in minimal media with D_2O as the solvent opens up several biophysical techiques that may allow protein radicals to be observed during catalysis.

RTPR was purified using procedures that are very similar to that which has been previously described. It is gratifying that from 500 mL of *E. coli* HB101/pSQUIRE, greater than 80 mg of essentially pure protein can be obtained. This is roughly equal to the amount of protein that was routinely obtained from a 300-L culture of *Lactobacillus leichmannii*. All means employed to characterize the recombinant protein suggested that there was no difference between the recombinant and non-recombinant forms of RTPR. Both proteins exhibited very similar K_m values for both the substrate (ATP) and cofactor (AdoCbl). In addition, the N terminus of the recombinant protein is identical to the N terminus of the non-recombinant protein. Their N-terminal identity is even extended to the cellular processing of the N-formylmethionine, as both proteins begin with the second amino acid serine.

The successful cloning, sequencing, and overexpression of RTPR has opened up a wide variety of new techniques which can be used to explore the mechanism of this amazing enzyme at an even more detailed level. As will be described in the following two chapters, protein analogs or site-directed mutants will be used to probe the function of several cysteines which are believed to be involved in catalysis, as well as study the mechanism by which the tritium in [5'-^{3}H]AdoCbl equilibrates with water.

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Chapter 4:

Evidence for the Participation of Five Cysteine Residues in Ribonucleotide Reduction

The ribonucleotide reductases have been classified based on their cofactor requirement and quarternary structure. Class I reductases are represented by the ribonucleoside diphosphate reductase (RDPR) isolated from *E. coli*. Enzymes in this class are tetramers constructed of two homodimeric proteins, R1 and R2. The smaller protein, R2, houses a diferric iron center-tyrosyl radical cofactor which is absolutely necessary for substrate turnover. Other reductases in this class are from mammalian systems, as well as the Herpes Simplex viruses (Averett et al., 1983; Dutia, 1986; Stubbe, 1990b; Reichard, 1993b). Class II enzymes are structurally the simplest of all the reductases, and are represented by the ribonucleoside triphosphate reductase (RTPR) from *Lactobacillus leichmannii* (Blakley, 1978; Lammers & Follmann, 1983). RTPR functions as a single polypeptide of 82 kDa (Booker & Stubbe, 1993), and requires coenzyme B_{12} (AdoCbl) for dNTP production. The enzyme isolated from *Brevibacterium ammoniagenes* is the prototype for class III reductases. This enzyme has an absolute requirement for manganese, and is composed of two proteins, the smaller of which is a dimer, and the larger a monomer (Willing et al., 1988b; Willing et al., 1988a). Recently a ribonucleotide reductase from *E. coli* grown under anaerobic conditions has been isolated. This class IV enzyme is a dimer, and is proposed to use a [4Fe-4S] cluster in combination with S-adenosylmethionine and other small molecules to carry out substrate reduction (Eliasson et al., 1992; Reichard, 1993a; Reichard, 1993b).

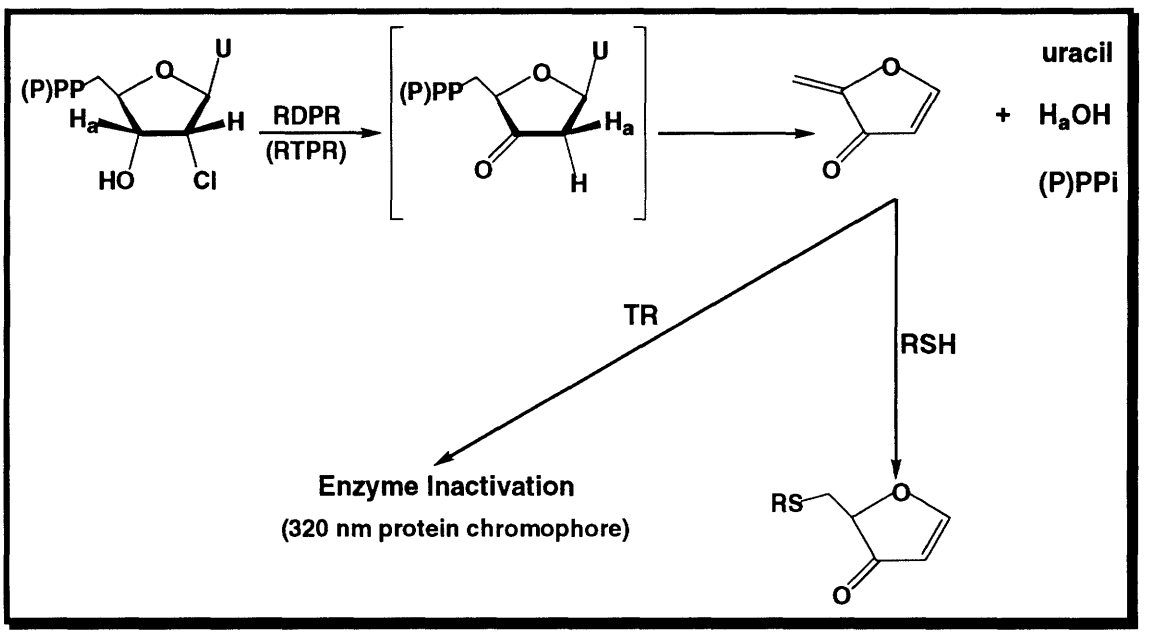
Despite the differences in quarternary structure, cofactor requirement, as well as primary sequence, evidence suggests that at least two reductases, those from *L. leichmannii* and *E. coli*, operate by very similar mechanisms of catalysis. Firstly, both enzymes contain cysteine residues which become oxidized to a cystine concomitant with each substrate turnover event (Vitols et al., 1967b;

Thelander, 1974). In both systems the redox-active disulfide can be rereduced by an *in vivo* reductant, thioredoxin, or by small organic dithiols such as dithiothreitol (DTT), a requirement for multiple turnovers. Secondly, both enzymes initiate the catalytic event by a protein radical-mediated hydrogen atom abstraction from the 3' carbon of the nucleotide substrate (Stubbe et al., 1981; Stubbe et al., 1983). This hydrogen atom is subsequently returned back to the same position in the deoxynucleotide product. In each system a protein radical is postulated to be generated by the metallocofactor (Stubbe, 1990a). Thirdly, both enzymes display remarkably similar phenotypes when presented with 2'-halogenated-2'-deoxynucleotides. Studies with the mechanism-based inhibitor 2'-chloro-2'-deoxyuridine di(tri)phosphate revealed that subsequent to 3' C-H bond cleavage, each enzyme catalyzed the formation of 3'-keto-2'-deoxyuridine 5'-di(tri)phosphate in which the hydrogen originally at the 3' carbon of the sugar is returned stereospecifically to the β face of the 2' carbon of the sugar or is transferred to the solvent (Scheme 4.1). This intermediate then collapses to liberate free base (uracil), pyrophosphate (tripolyphosphate), and a 2-methylene-3(2H)-furanone which in each case alkylates the protein yielding a chromophore at 320 nm (Harris et al., 1984; Lin et al., 1987; Ashley et al., 1988).

The mechanism of ribonucleotide reduction has also been investigated with the aid of protein analogs. Recent site-directed mutagenesis studies on the RDPR from *E. coli* have allowed a model to be proposed in which five cysteine residues act in concert to carry out the reduction process (Aberg et al., 1989; Mao et al., 1989; Mao et al., 1992a; Mao et al., 1992b; Mao et al., 1992). All of these catalytically important cysteines are located on the R1 subunit of the enzyme, which is the subunit that binds the substrates as well as the allosteric effectors. Two C-terminal cysteines (754 and 759), function to deliver reducing equivalents

from thioredoxin into the active site disulfide of RDPR (formed concomitant with substrate reduction) thus regenerating active enzyme. The active site cysteines

Scheme 4.1: Interaction of ribonucleoside di(tri)phosphate reductase with 2'-chloro-2'-deoxyuridine 5'-di(tri)phosphate



(225 and 462) function to directly reduce the nucleotide substrate. Lastly, a fifth cysteine is proposed to be converted into a thiyl radical via e^-/H^+ transfer to the diferric iron center-tyrosyl radical cofactor on the second subunit, R2. This thiyl radical is then proposed to initiate catalysis by abstracting the 3′ hydrogen atom of the substrate. While this model for the role of multiple cysteines in catalysis by the *E. coli* reductase is moderately compelling, the interpretation of the results was blemished by the presence of contaminating wildtype (wt) R1 in all of the mutants examined (Aberg et al., 1989; Mao et al., 1992a; Mao et al., 1992b; Mao et al., 1992). The cloning, sequencing, and expression, of the enzyme from

L. leichmannii (Chapters 2 & 3) (Booker & Stubbe, 1993) have now made similar studies possible on a different class of reductase. Furthermore, since the *L. leichmannii* reductase uses ribonucleoside triphosphates as opposed to ribonucleoside diphosphates, the ability to express it in *E. coli* has eliminated the problem of contaminating wt in the mutants generated. The results of the studies of the interactions of five C→S site-directed mutants of RTPR with CTP are presented herein. The phenotypes of these mutants are strikingly similar to the corresponding *E. coli* RDPR mutants providing convincing evidence in conjunction with the mechanistic studies, that the active sites of these enzymes are structurally similar.

Materials and Methods

Materials

Dithiothreitol was purchased from Mallinckrodt. [2-^{14}C]Cytidine 5'-diphosphate (CDP; 2.0 GBq/mmol), [1',2'-^{3}H]deoxyguanosine 5'-triphosphate (dGTP; 1.3 TBq/mmol) and [γ-^{32}P]ATP (222 Tbq/mmol) were purchased from New England Nuclear. Pyruvate kinase (specific activity 470 U/mg), L-lactic dehydrogenase (specific activity 925 units/mg), phosphoenolpyruvate (PEP), 5'-deoxyadenosine (5'-dA), cytidine 5'-triphosphate (CTP), CDP, deoxyadenosine 5'-triphosphate (dATP), cytosine, 2'-deoxycytidine (dC), NADPH, and coenzyme B12 (AdoCbl) were purchased from Sigma. Alkaline phosphatase (specific activity 3143 units/mg) from calf intestine (CIP) was purchased from Boehringer Mannheim. Restriction endonucleases were purchased from New England Biolabs. AmpliTaq DNA polymerase was from Perkin-Elmer/Cetus, and ultrapure dNTPs were from Pharmacia. T4 DNA ligase was purchased from GIBCO/BRL. Centricons and membranes for Amicon ultrafiltration devices were obtained from Amicon. Anion-exchange resin AG1-X2 (50-100 mesh) was

purchased from Bio-Rad. [5′-^{3}H]AdoCbl ($1x10^{7}$ cpm/μmol) was a generous gift from Professor H.P.C. Hogenkamp of the University of Minnesota (Minneapolis).

UV-visible absorption spectra were recorded on a Hewlett-Packard 8452A diode-array spectrophotometer. All scintillation counting was performed on a Packard 1500 liquid scintillation analyzer using 8 mL of S¢INT-A XF scintillation cocktail (Packard) per 1 mL of aqueous reaction. High Pressure Liquid Chromatography (HPLC) was carried out using a Beckman 110 Solvent Delivery Module, 421A Controller, and a 163 Variable Wavelength Detector, in combination with an Alltech Econosil C_{18} column. SDS/PAGE was performed as described by Laemmli (1970).

Wild-type *L. leichmannii* RTPR (specific activity 1.5 U/mg) was isolated from the *E. coli* overproducing strain pSQUIRE/HB101 (Chapter 3). *E. coli* thioredoxin (TR) (specific activity, 50 units/mg) and thioredoxin reductase (TRR) (specific activity, 800 units/mg) were isolated from overproducing strains SK3981 (Lunn et al., 1984) and K91/pMR14 (Russel & Model, 1985). *E. coli* strain JM101 was obtained from Pharmacia. Chromosomal DNA from *L. leichmannii* (ATCC 7830) was isolated as described in Chapter 2. Oligonucleotide primers used for DNA sequencing, mutagenesis, and the polymerase chain reaction (PCR) were obtained from the MIT Biopolymers Laboratory, or Oligos Etc. of Wilsonville, OR.

Preparation of [2-^{14}C]CTP

[^{14}C]CTP was not commercially available and therefore had to be synthesized. [2-^{14}C]CDP (~50 μCi) was diluted with cold CDP to a specific activity of ~$6.5x10^{6}$ cpm/μmol, and the sample was lyophilized to dryness. The reaction included in a volume of 1 mL: 10 mM CDP, 50 mM phosphoenolpyruvate, 100 mM imidazole (pH 7.6), 62 mM $MgSO_4$, 120 mM KCl,

and 100 U of pyruvate kinase. The reaction was incubated at 37°C, and the extent of reaction was measured by removing a 5-μL aliquot from the reaction mixture and adding it to an assay mixture containing in a final volume of 1 mL: 80 μM NADH, 62 mM $MgSO_4$, 120 mM KCl, 100 mM imidazole (pH 7.6), and 50 U of lactate dehydrogenase. The mixture was incubated for 5 min at 37°C, and the absorbance at 340 nm ($\varepsilon = 6220\ M^{-1}cm^{-1}$) was recorded. Under these conditions conversion of CDP to CTP was complete within 2 h. The reaction mixture was diluted with H_2O to 50 mL and loaded onto a DEAE Sephadex A25 column (2.5 x 7 cm) previously equilibrated in H_2O. The column was eluted with a 200x200-mL linear gradient from 0 to 1 M triethylammonium bicarbonate (TEAB) pH 7.4, and fractions of 8 mL were collected. The product-containing fractions, which eluted at approximately 0.5 M TEAB, were pooled and evaporated to dryness by rotary evaporation. The residue was redissolved in 1 mL of water, and then applied to a Sep-pak C18 cartridge (Millipore) which had been pretreated with 10 mL of methanol followed by 10 mL of H_2O. The cartridge was washed with H_2O until the A_{260} of the filtrate dropped to below 0.1 (~10 mL). The filtrate was lyophilized to dryness, and then redissolved in water.

Preparation of Site-Directed Mutants

All site-directed mutants were prepared by Dr. Joan Broderick. The primers used for the preparation of the mutants are shown in Table 4.1. Mutants C731S, C736S, and the double mutant C731&736S were constructed using a single PCR. Since these cysteines are located at the extreme C terminus of RTPR, the C-terminal half of the gene was amplified with the appropriate mutant primer, **2**, **3**, or **4** (each of which contains a stop codon and a *Hin*dIII restriction site at its 5′ end), and an alternate strand primer, **1**, which is complementary to a

Table 4.1: Primers used for mutagenesis.

Primer	Sequence	Range (bp)
1	5′-d(CTACCAGGCTGGAATTGACGG)-3′	1179-1199
2	5′-d(GGCGCGAAGCTTACTTAATTGGGCAGGCGCC GCCTTCA**G**AGTCGGTCTGGTC)-3′	2177-2220
3	5′-d(GGCGCGAAGCTTACTTAATTGGG**G**AGGCGCC GCCTTCACAGTC)-3′	2186-2220
4	5′-d(GGCGCGAAGCTTACTTAATTGGG**G**AGGCGCC GCCTTCA**G**AGTCGGTCTGGTC)-3′	2177-2220
5	5′-d(AACCCTTGCGGGG*AGATCT*CCCTGGCCAACG GGGAACCT**A**GCAACCTCTTTGAA)-3′	1216-1269
6	5′-d(GGCGCGAAGCTTACTTAATTGGGC AGGCGCC)-3′	2199-2220
7	5′-d(CCCGTTGGCCAGGG*AGATCT*CCCCGC**T**AGGG TTGGTCCCTTC)-3′	1205-1247
8	5′-d(GCCGGC*GAATTC*AGGAGAAAATATTATGAGT GAAGAAATATCTCTCTCC)-3′	1-24
9	5′-d(GATTCCTTGAACAACT**C**CTGGTTTG TGGCCATC)-3′	340-373
10	5′-d(GAAGACTTCAAAGAGGTTGCAAGG)-3′	1251-1274
11	5′d(GATCAAGTTGGAGATGTCGGC)-3′	902-923

Underlined bases represent stop codons in primers **2,3,4, & 6**, or the ribosome binding site and start codon in primer **8**. Bases in italics represent the *Bgl* II site in primer **5 & 7**, and the *Eco*RI site in primer **8**. Bases in bold type represent the appropriate mutation.

region 39 bp upstream of a unique *Bgl* II site. This *Bgl* II site separates the gene into two halves, each of which has been cloned separately into the Invitrogen plasmid pCR1000 (Chapter 3). The plasmid containing the N-terminal half of the gene is 6α4, and the plasmid containing the C-terminal half is 3α1. The PCR reaction included in a volume of 100 μL: 10 μL of 10x PCR buffer (500 mM KCl / 100 mM Tris-HCl, pH 8.3 / 15 mM $MgCl_2$ / 0.1% gelatin), 1.2 μg of genomic DNA, 1 μM of each primer, and all four dNTPs each at 0.2 mM. The mixture was overlaid with 100 μL of paraffin oil and heated at 94°C for 5 min. *Taq* polymerase (5 U) was added under the oil layer, and 35 cycles of the following program were run: 1 min at 94°C, 1 min at 60°C, and 3 min at 72°C. The last cycle included an additional 10 min incubation at 72°C. Subsequent to amplification, the mutant DNA was purified using the USBioclean kit (US Biochemicals) according to the manufacturer's specifications. The purified DNA was digested with *Bgl* II and *Hin*dIII and the resulting *Bgl* II/*Hin*dIII fragment, and the N-terminal *Eco*RI/*Bgl* II fragment generated from plasmid 6α4, were ligated into the expression vector pKK223-3 (which had been previously digested with *Eco*RI and *Hin*dIII).

Mutant C419S was constructed in a similar manner; however, the mutant primer, **5**, spanned the unique *Bgl* II restriction site of the RTPR gene. Primer **5** was used in combination with a primer, **6**. Subsequent to amplification, this C419S mutant fragment was digested, purified, and ligated into pKK223-3 as described for the C-terminal mutants.

Mutant C408S was also constructed using a single PCR procedure. However in this case the N-terminal half of the gene was amplified using the mutant primer, **7**. Primer **7** in analogous fashion to primer **5**, also spanned the unique *Bgl* II site of the RTPR gene. It was used in combination with a primer, **8**, which is complementary to the extreme N terminus of the RTPR gene sequence. In addition to encoding the first 24 bases of the RTPR gene, primer **8** contained

an *Eco*RI restriction site upstream of an *E. coli* consensus ribosome binding site. Subsequent to amplification as already described, the resulting fragment was digested with *Eco*RI and *Bgl* II. Upon purification from a 1% agarose gel using USBioclean, this N-terminal *Eco*RI/*Bgl* II fragment and the C-terminal *Bgl* II/*Hin*d III fragment of RTPR from plasmid 3α1 were ligated into *Eco*RI/*Hin*dIII-digested pKK223-3.

Cysteine 119 could not be easily accessed by a single PCR reaction because it does not lie near a unique restriction site in the RTPR gene. It was therefore necessary to perform two PCR reactions in order to amplify the mutant DNA such that both of its ends contained unique restriction sites. The conditions for the first PCR reaction were as previously described using a mutant primer, **9**, and an alternate strand primer **10**. The first PCR reaction introduced the mutation at C119, and amplified the region of the RTPR gene between C119 and the unique *Bgl* II restriction site (bp 340-1229). The product was purified from a 1% agarose gel and used as a primer in combination with primer **8** in a second PCR reaction. The conditions for the amplification were as described above, except that the concentration of the primers was 0.1 μM. The product of this second PCR reaction, which amplified the N-terminal region of the gene from the *Eco*RI site to the *Bgl* II site, was purified from a 1% agarose gel using USBioclean, and digested with *Eco*RI and *Bgl* II. This *Eco*RI/*Bgl* II fragment and the C-terminal *Bgl* II/*Hin*dIII fragment from plasmid 3α1, were then ligated into *Eco*RI/*Hin*dIII-digested pKK223-3.

Mutant C305S was made with the Oligonucleotide-directed *in vitro* Mutagenesis system (version 2.1) from Amersham, using the mutant primer, **11**. The mutagenesis was carried out on a 1685 bp *Eco*RI/*Sph* I fragment cloned into M13mp18. Subsequent to mutagenesis as described by the manufacturer, dsM13 containing the mutated RTPR fragment was isolated by standard methods and

purified on a Qiagen plasmid Mini I column according to the manufacturer's directions. The mutated codon was located on a 351 bp fragment defined by an upstream *Bgl* II site, and a downstream *Not* I site. This fragment was isolated subsequent to digestion with these restriction enzymes and electrophoresis in a 1% agarose matrix. Upon purification using USBioclean, this *Bgl* II/*Not* I fragment was ligated into pSQUIRE from which the wt *Bgl* II/*Not* I fragment had been removed.

The integrity of each mutant sequence was verified using the dideoxy chain-termination method of Sanger (Sanger et al., 1977) in combination with the dsDNA Cycle Sequencing system from GIBCO/BRL. Primers, which were labeled with [γ-^{32}P]ATP, were 21-24 bases in length, and spaced approximately 150 bp apart. The entire PCR-amplified portion of the mutated gene sequence of each mutant was sequenced. In the case of the C305S mutant, the 351-bp *Bgl* II/*Not* I fragment was sequenced.

Growth and Expression of Mutants

Plasmid pSQUIRE containing the appropriate mutant was transformed into *E. coli* JM101 and grown to saturation (10-12 h) in Luria broth supplemented with ampicillin (50 μg/mL). No isopropyl thio-β–D-galactoside was needed to induce the expression of RTPR. The mutant RTPRs were isolated by procedures identical to that which was previously described for wild type (wt)-RTPR (Chapter 3) except that care was taken to insure that the column resins had never previously been used to isolate the wt protein.

Enzyme Assays Using [2-^{14}C]CTP and NaOAc

RTPR or the appropriate mutant was exchanged into 2 mM HEPES (pH 7.5) using a Sephadex G-50 column, and its concentration was determined

by UV absorption ($E^{1\%}$=13.3 at 280 nm) (Blakley, 1978). A typical assay contained in a final volume of 510 μL: 50 mM HEPES (pH 7.5), 4 mM EDTA, 1 M NaOAc, 30 mM DTT, 1 mM [2-^{14}C]CTP (specific activity 7.5x10^5-1.5x10^6 cpm/μmol), 8 μM AdoCbl, and 0.10-0.12 nmol of wt or mutant RTPR. Alternatively, DTT was replaced with TR (108 μM), TRR (0.5 μM), and NADPH (2 mM). The reaction mixture was incubated for 5 min at 37°C, and an aliquot (100 μL) containing everything except AdoCbl was removed at the zero time point. All assays were carried out in the dark under dim red light, and at various times subsequent to the addition of AdoCbl, a 100 μL aliquot was removed and quenched in 200 μL of 2% perchloric acid. The contents were neutralized with 180 μL of 0.2 N NaOH and 50 μL of 0.5 M Tris-HCl, pH 8.5 / 1 mM EDTA. Subsequent to the addition of 10 U of CIP, the reaction was incubated at 37°C for 1.5 h. To each reaction vial was added 30 μL of carrier cytosine and dC (120 nmol), and the reaction was diluted to ~1.5 mL with H_2O. Each reaction was loaded onto 0.75x7 cm AG1-X2 columns (borate form, 50-100 mesh) prepared by the method of Steeper and Steuart (1970). Each column was washed with 12 mL of H_2O, and a 1-mL portion of the eluate was subjected to scintillation counting. A 10-mL aliquot of the remaining 11 mL was concentrated to ~1 mL for reverse phase HPLC analysis using an Econosil C_{18} column with H_2O as the eluate. The column was washed with H_2O at a flow rate of 1 mL/min, and 1-mL fractions were collected and analyzed by scintillation counting. Cytosine and dC eluted isocratically in H_2O at ~7 and 20 min respectively.

Enzyme Assays Using [2-^{14}C]CTP and Allosteric Effector

Enzyme reaction mixtures were identical to that described above except that NaOAc was replaced with 0.12 mM dATP and 1 mM $MgCl_2$. Subsequent to

the addition of AdoCbl to initiate the reaction, 100-μL aliquots were removed at various time intervals and quenched in 50 μL of 2% perchloric acid. The contents were neutralized with 45 μL of 0.2 N NaOH and 20 μL of 0.5 M Tris-HCl, pH 8.5 / 1 mM EDTA. CIP (5U) was added, and the reaction mixture was incubated at 37°C for 1.5 h, and then analyzed for cytosine and dC formation as described above.

Single-Turnover Experiments with Mutants C731S, C736S, and C731&736S

Wt- RTPR or the appropriate mutant (37 nmol) was pre-reduced (in a total volume of 200 μL) for 20 min at 37°C with 30 mM DTT in 50 mM HEPES (pH 7.5). This mixture was loaded onto a Sephadex G-50 column (0.75x7 cm) equilibrated in 2 mM HEPES (pH 7.5) to remove the DTT. Fractions containing protein (A_{280} nm) were pooled and concentrated. A 50-μL aliquot (9-12 nmol) was added to the reaction mixture which contained in a final volume of 510 μL: 50 mM HEPES (pH 7.5), 4 mM EDTA, 1 mM [2-^{14}C]CTP (specific activity $1.4x10^6$ cpm/μmol), 0.12 mM dATP, 1 mM $MgCl_2$ and 50 μM AdoCbl. Alternatively, 1 M NaOAc replaced the dATP and $MgCl_2$. The reaction mixture was incubated for 5 min at 37°C, and an aliquot (100 μL) containing everything except AdoCbl was removed at the zero time point. Subsequent to the addition of AdoCbl, 100-μL aliquots were removed at 1, 3, 10, and 20 min, quenched and analyzed as described above.

Characterization of Oxidized RTPR

Pre-reduced RTPR (29 nmol) was added to a solution containing in a final volume of 200 μL: 50 mM HEPES (pH 7.5), 4 mM EDTA, 1 mM CTP, 50 μM AdoCbl, and 1 M NaOAc, and pre-incubated for 2 min at 37°C. The reaction was initiated with AdoCbl and incubated for 3 min at 37°C. It was then loaded onto a

Sephadex G-50 column (0.75x7 cm) equilibrated in 2 mM HEPES (pH 7.5). The protein-containing fractions were pooled, and concentrated to ~200 μL in a Centricon 30 ultrafiltration device. A 150-μL aliquot (17 nmol) of this enzyme was added to the single-turnover assay solution described above containing 1 mM [2-^{14}C]CTP (specific activity 1.4x10^6 cpm/μmol), and 1 M NaOAc. Subsequent to the removal of a 100-μL aliquot (t=0 min), the assay was initiated by the addition of AdoCbl. Additional 100-μL aliquots were removed at 10, 20, 30, and 40 min, and quenched and worked up as described above.

Determination of Product Production with Mutants C119S and C419S

The assay contained in a final volume of 250 μL: 25 mM HEPES (pH 7.5), 1 M NaOAc, 4 mM EDTA, 1 mM [2-^{14}C]CTP (specific activity 1.2x10^6 cpm/μmol), 20 μM TR, 0.12 μM TRR, 0.2 mM NADPH, 2.5 nmol of C419S or C119S, and 80 μM AdoCbl. Alternatively, DTT (30 mM) replaced the TR/TRR/NADPH reducing system, and/or dATP (0.12 mM) and $MgCl_2$ (1 mM) was substituted for NaOAc. The reaction mixture was incubated for 5 min at 37°C, and an aliquot containing everything except AdoCbl, which was used to initiate the reaction, was removed at the zero time point. After a 30 min incubation at 37°C for C119S or a 60 min incubation for C419S, another 100-μL aliquot was removed, quenched, and worked up as already described depending on whether the assay was conducted with NaOAc or with dATP and $MgCl_2$.

Analysis of the Ability of C408S to Catalyze Nucleotide Reduction

The assay solution contained in a final volume of 310 μL: 50 mM HEPES (pH 7.5), 4 mM EDTA, 50 μM AdoCbl, 1 mM [2-^{14}C]CTP (specific activity 1.5x10^6 cpm/μmol), 24 μM C408S RTPR, 0.12 mM dATP, 1 mM $MgCl_2$, 108 μM TR, 0.5 μM TRR, and 2 mM NADPH. Alternatively, dATP and $MgCl_2$ were

replaced with 1 M NaOAc, and/or TR/TRR/NADPH was replaced with 30 mM DTT. Subsequent to a 5-min pre-incubation at 37°C, a 100-μL aliquot was withdrawn (t=0), and the reaction was initiated with the addition of AdoCbl. Additional 100-μL aliquots were removed at 10 and 30 min, and quenched for 2 min in a boiling water bath. Cytosine and dC production was analyzed by HPLC as described above.

Circular Dichroism Spectra

Circular dichroism (CD) spectra were recorded on an AVIV Model 62DS Circular Dichroism Spectrometer (Lakewood, N.J.) attached to a CompuAdd 320 computer. Samples included 9 μM wt RTPR or 12 μM mutant C408S in 10 mM potassium phosphate buffer (pH 7.2) / 1 mM DTT. A baseline containing all components except protein was run, and then the protein spectrum was obtained from 200-260 nm at 37°C using a 0.5 mm path-length cell.

Characterization of C305S RTPR

The assay solution contained in a final volume of 510 μL: 50 mM HEPES (pH 7.5), 4 mM EDTA, 2 mM NADPH, 108 μM TR, 0.5 μM TRR, 1 mM [2-^{14}C]CTP (specific activity 1.5x10^6 cpm/μmol), 1 M NaOAc, 50 μM AdoCbl, and 0.2 μM enzyme. Subsequent to a 5-min incubation at 37°C, a 100-μL aliquot was removed (t=0), and the reaction was initiated with the addition of AdoCbl. Additional 100-μL aliquots were removed at 2, 4, 6, and 8 min, and quenched in 2% perchloric acid. Cytosine and dC production was analyzed by HPLC as described above. Assays were also performed in which NaOAc was replaced with dATP (120 μM) and $MgCl_2$ (1 mM).

Results

Preparation of Site Directed Mutants

Five cysteines have been targeted for mutagenesis so that a comparison can be made between their phenotypes and those of the corresponding *E. coli* mutants. Cysteines 119, 408, 419, 731, and 736, were converted to serines using the PCR reaction as described in detail in the methods. Additionally, cysteine 305 was changed to a serine using an *in vitro* mutagenesis kit from Amersham. All mutant plasmids were transformed into *E. coli* JM101, and individual colonies were screened for the expression of RTPR by SDS/PAGE of saturated overnight cultures (Laemmli, 1970). The integrity of each mutant was confirmed by DNA sequencing. Mutant C736S was observed to contain an additional mutation in which nucleotide 1619 was converted from an A to a T. This change resulted in the conversion of amino acid 540 from a glutamine to a leucine. All other mutants had the expected sequences. The mutant proteins were purified as described previously and portrayed the same characteristics as the wt protein throughout each step of the purification. Each mutant protein, except for C305S, was purified to ≥ 95% homogeneity, with typical yields ranging from 50-80 mg per 500 mL of a saturated overnight culture.

At the outset of determining the phenotypes of each of these mutants, it was noticed that there was variability in the level of product production among different preparations of the same mutant protein when the assays were performed with [^{14}C]CTP of high specific activity. Control experiments revealed that the variability was the result of small amounts of wt RTPR contamination in the mutant preparation. This contamination resulted from isolating mutant proteins on resin that was previously used to isolate wt RTPR. Although the resin was routinely cleaned with high concentrations of various salts (1 M

potassium phosphate or NaCl) these conditions were not stringent enough to remove the trace amounts of wt protein. These results highlight the degree of prudence that must be exercised when attempting to set very low limits of detection for mutants that are expected to have no catalytic activity. The method of cleaning the resin was therefore changed to mild acid and/or base washes; and as a precaution, each distinct mutant was isolated on resin dedicated to that particular protein.

RTPR Assays

One of the many unique aspects of both the *L. leichmannii* and *E. coli* reductases is the pattern of allosteric regulation that governs which of the 4 nucleotide substrates is reduced (Reichard, 1988). The *L. leichmannii* enzyme contains a single substrate site which binds each of the four ribonucleoside triphosphates, and a separate allosteric site which binds TTP, dGTP, dATP, and dCTP. Appropriate effector binding enhances the rate of substrate turnover ~5 fold (Vitols et al., 1967a). The binding of dGTP stimulates the reduction of ATP. Likewise, dATP stimulates dCTP production, TTP stimulates dGTP production, and dCTP stimulates dUTP production (Beck, 1967; Chen et al., 1974). This elaborate array of allosteric control of substrate turnover is abrogated with the addition of high concentrations of certain ions such as acetate (Jacobsen & Huennekens, 1969). One molar NaOAc allows each of the four NTPs to be reduced to dNTPs in the absence of any effectors with turnover numbers that are almost identical to those observed in the presence of effectors.

In addition to the requirement for an allosteric effector, RTPR also requires a reductant that rereduces the active site disulfide generated concomitant with substrate reduction. It is generally believed that *in vivo*, TR provides RTPR with reducing equivalents which come ultimately from NADPH in a process that is

mediated by the flavoprotein TRR (Moore et al., 1964). *In vitro* however, the *E. coli* TR (which has been cloned and overexpressed) can serve this purpose. Alternatively, reducing equivalents can be provided by small dithiol molecules such as dihydrolipoic acid and dithiothreitol (Beck et al., 1966).

Because of the complexity of the reaction with regard to the mechanism of substrate reduction and allosteric activation, the investigation of ribonucleotide reduction presented herein is under a defined set of conditions. The reduction of CTP is investigated in the presence of dATP or sodium acetate, using the *E. coli* TR or DTT as the reductant. Each mutant (or wt protein) is thus assayed under four different sets of conditions. The choice of [^{14}C]CTP as substrate is dictated by the ease of product analysis, and this substrate was synthesized from the corresponding [^{14}C]CDP using pyruvate kinase in conjunction with PEP as the phosphate donor (Worthington, 1988).

Characterization of C731S, C736S, and the Double Mutant C731&736S

Previous studies of Mao *et al.* (1989; 1992a) using C754S and C759S R1 mutants of the *E. coli* RDPR suggested that the function of these C-terminal cysteines was to shuttle reducing equivalents from TR into the active site via disulfide interchange. The similarity in sequence between cysteines 754 and 759 of the *E. coli* RDPR and the C-terminal cysteines, 731 and 736 of RTPR, in conjunction with biochemical studies of Lin *et al.* (1987) suggested that these cysteines in the *Lactobacillus* enzyme might serve a similar function. If indeed these cysteines provide a pathway for TR to rereduce the active site disulfide of RTPR, then two phenotypic characteristics would be predicted. In the presence of the TR/TRR/NADPH reducing system, a single turnover of CTP to dCTP would be expected. Subsequent turnovers would be prohibited since the vehicle for shuttling reducing equivalents into the active site has been destroyed. On the

other hand, small organic dithiols such as DTT or dihydolipoic acid might be expected to bypass these C-terminal cysteines providing reducing equivalents directly to the active site disulfide.

Our model predicts that for wt RTPR under single-turnover conditions a maximum of 2 equivalents of dCTP should be produced if the C-terminal cysteines can completely rereduce the active site disulfide subsequent to production of the first dCTP. In the same fashion, each of the C-terminal mutants should only be capable of producing 1 dCTP. This prediction was tested by pre-reducing the enzyme with DTT, and then removing the reductant by size-exclusion chromatography. The enzyme was then treated with the substrate in the absence of either reductant, and in the presence of dATP or NaOAc. These experiments indicate that C731S, C736S, and C731&736S RTPRs all result in the production of 0.6-0.8 equivalents of dCTP (Table 4.2). The wt RTPR on the other hand results in the production of 1.4 equivalents of dCTP. The substoichiometric amounts of dCTP for each of the mutants and the wt RTPR relative to the predicted value is not understood. From control studies it is believed that this is not a consequence of partial oxidation of the pre-reduced enzyme before the initiation of the one-turnover experiment. It may be significant that in analogous experiments using the *E. coli* RDPR 2.6 dCDPs are observed per R1 dimer in which four sets of cysteines are available to produce a maximum of 4 dCDPs. For each of the *E. coli* C-terminal mutants, as well as the C-terminal double mutant, equivalents of dCDP ranged from 0.9-1.2 in single-turnover experiments (Mao et al., 1989; Mao et al., 1992b). Despite the fact that the number of equivalents of dNTP per equivalent of enzyme is lower than expected, it is significant that in each C-terminal mutant, the number is approximately 1/2 of that observed with the wt-RTPR.

Table 4.2: Phenotypes of the C-terminal RTPR mutants.

Protein (condition)	Specific Activity DTT (μmol/min/mg)	Specific Activity TR/TRR/NADPH (μmol/min/mg)	Single Turnover (equiv of dCTP)
Wildtype (acetate)	1.5[a]	1.2	1.4
Wildtype (dATP)	0.1	1.0	1.5
C731S (acetate)	0.9	$< 5x10^{-5}$	0.8
C731S (dATP)	0.095	$< 5x10^{-5}$	0.8
C736S (acetate)	1.6	$< 5x10^{-5}$	0.6
C736S (dATP)	0.13	$< 5x10^{-5}$	0.6
C731 & 736 (acetate)	1.8	$< 5x10^{-5}$	0.7
C731 & 736S (dATP)	0.09	$< 5x10^{-5}$	0.7

[a]freezing and thawing of RTPR results in loss of activity from 1.5 to 1.0 U/mg

Experiments carried out in the presence of reductant corroborate the above hypothesis. Wt RTPR has a specific activity of 1-1.2 $\mu mol \bullet min^{-1} \bullet mg^{-1}$ when assayed in the presence of the thioredoxin reducing system using either the allosteric effector (dATP) for CTP reduction, or in the presence of NaOAc. Subsequent to the initial turnover, each of the C-terminal C→S mutants produce dCTP at a rate that is less than $5x10^{-5}$ that of the wt RTPR. This rate represents the lower limit of detection of this particular assay. When the assay is conducted using DTT as the reductant, the rate of turnover for each of the mutants under a defined set of conditions is similar to that of the wt protein. These results provide strong support for the integrity of the enzyme (since it can generate dCTP), and the role of the C-terminal thiols as redox shuttles.

Studies of the kinetics of the single-turnover reaction using HPLC analysis to examine product production provided some unexpected results (Figure 4.1). After an initial burst of radioactivity, a second, much slower process occurs, giving rise to biphasic kinetics. The amount of dCTP in the first time point (1 min) however, is equivalent to the amount observed in the 20-min time point, suggesting that nucleotide reduction is very fast. Based on HPLC analysis the slow reaction is attributed to the production of cytosine and some yet uncharacterized species which elutes at 14-15 min under the conditions described in the methods section. This uncharacterized species is much more prevalent in assays conducted in the presence of NaOAc, and is approximately equal to the amount of cytosine observed after a 20-min incubation (Table 4.3). The amount of cytosine and this uncharacterized peak is usually negligible in the first time point of the assay, suggesting that it might be due to chemistry subsequent to active site disulfide formation. In order to test this hypothesis, wt RTPR was treated with substrate for 2 min at 37°C in a single-turnover experiment (absence of reductant), and then the protein was separated from the small molecules by size-exclusion chromatography. The pre-oxidized enzyme was then incubated with the radioactive substrate and analyzed for the time-dependent production of dCTP, cytosine, and the unknown species (Figure 4.2). After 40 min, ~0.29 eq of cytosine and 0.28 eq of the unknown species were produced, with no detectable dC. The kinetics of cytosine and the unknown species are essentially linear for the first 20 min, and then tapers off during the subsequent 20 min. The structure of this unknown species is presently under investigation. A slow formation of cytosine using oxidized *E. coli* RDPR has also been observed (G. Yu, unpublished results). These results are also very similar to previous studies of the interaction with ClUDP with oxidized *E. coli* RDPR. The oxidized enzyme

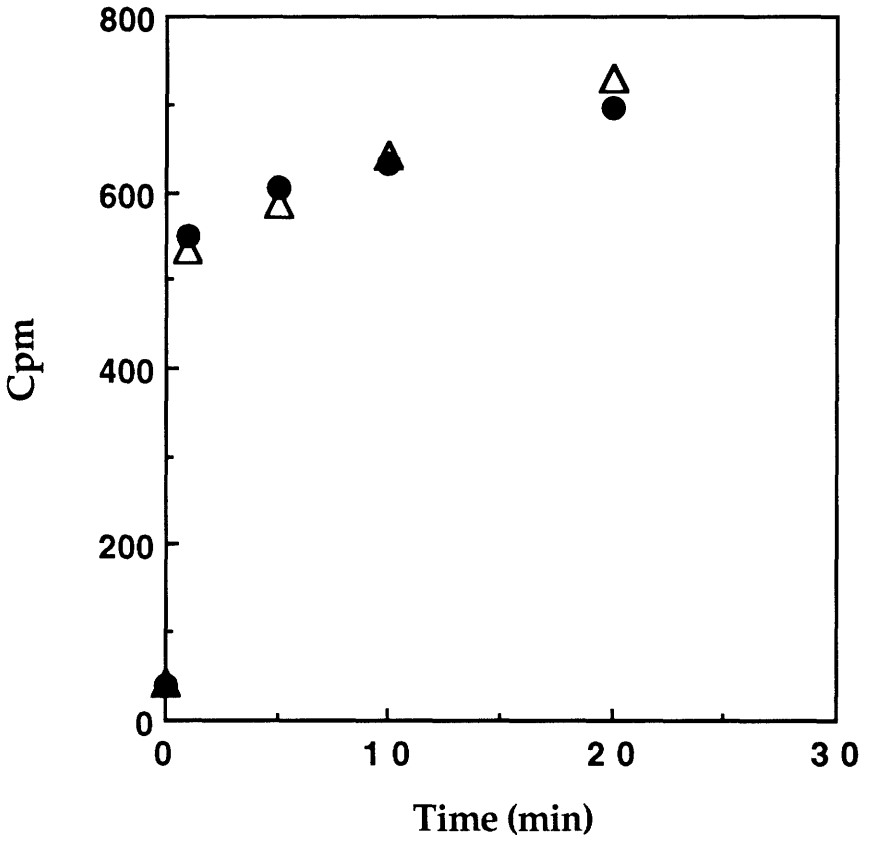


Figure 4.1: Time course for the production of dCTP under single-turnover conditions using wt RTPR. The reaction was conducted as described in the methods using (Δ) NaOAc, or (●) dATP. HPLC analysis for dCTP was performed on the 1 min and 20 min time points.

Table 4.3: Equivalents of dC, cytosine (cyt) and the mystery peak (Z) as a function of time.

	1 min			20 min		
Protein	Eq[a] dC	Eq cyt	Eq Z	Eq dC	Eq cyt	Eq Z
(NaOAc)						
wt	1.5	0.03	<0.01	1.4	0.2	0.2
C731S	0.8	0.02	<0.04	0.8	0.1	0.3
C736S	0.6	0.03	<0.03	0.6	0.2	0.3
C731&736S	0.7	0.02	<0.02	0.7	0.3	0.2
(dATP)						
wt	1.5	0.05	<0.01	1.6	0.4	<0.06
C731S	0.8	0.04	<0.04	0.8	0.2	<0.2
C736S	0.6	0.03	<0.03	0.6	0.2	<0.1
C731&736S	0.7	0.1	<0.04	0.7	0.3	<0.04

[a]Eq=equivalents per mole of RTPR

also catalyzes formation of cytosine from ClUDP at 1/10 the rate of the reduced RDPR (Ator & Stubbe, 1985).

Assays with C119S and C419S RTPR

Cysteines 119 and 419 are proposed to be the cysteines in the active site of RTPR that are directly involved in substrate reduction. If indeed this is the case, then the model presented herein would predict that upon mutating these amino acids to serines and incubating the mutant enzymes with CTP, absolutely no product (dCTP) should be produced. Detailed studies with the mechanism-

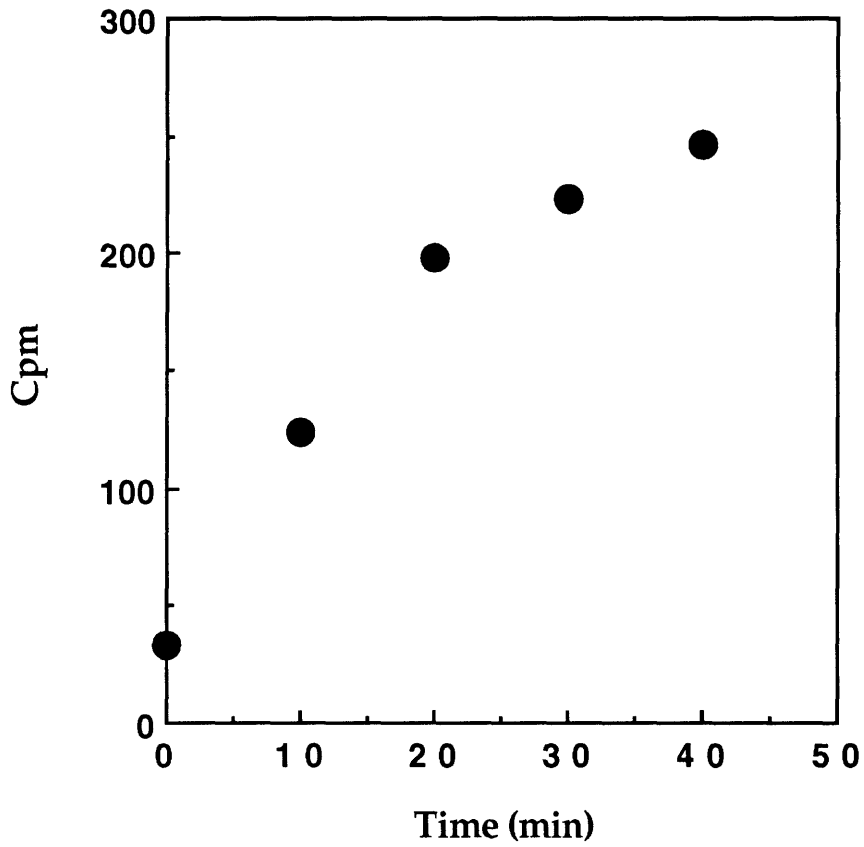


Figure 4.2: Analysis of the production of cytosine and the mystery peak with pre-oxidized wt RTPR. The reaction was carried out as described in the methods section. HPLC analysis for dCTP, cytosine and the mystery peak was carried out on the 40-min time point, and resulted in 0.29 eq of cytosine, 0.28 eq of the mystery peak, and no detectable dCTP.

based inhibitor ClUTP, as well as with the C225S R1 and C462S R1 active site mutants of the *E. coli* RDPR, suggest that in the event that 3′ C-H bond cleavage is uncoupled from the transfer of reducing equivalents to the substrate or substrate analog, the substrate or analog becomes a mechanism-based inhibitor of the enzyme (Harris et al., 1984; Ashley et al., 1988; Mao et al., 1989; Mao et al., 1992a; Mao et al., 1992b; Mao et al., 1992). A set of experiments under various assay conditions was therefore carried out to determine if cytosine or dCTP is produced when these mutants are incubated with RTPR. The results of these experiments are summarized in Table 4.4. As predicted for cysteines providing reducing equivalents required for substrate reduction, no dCTP is produced that is above the experimental limit of detection (0.02-0.08 dCTPs per equivalent of RTPR).

Furthermore, based on the previous studies with ClUTP (Scheme 4.1), removal of the active site reducing equivalents should result in conversion of CTP to 3′ keto-2'-deoxycytidine 5'-triphosphate which would subsequently collapse nonenzymatically to generate cytosine, PPPi, and a 2 methylene-3-(2H) furanone which could alkylate the enzyme. The alkylation has been shown to be accompanied by a ΔA_{320} nm on the protein. As indicated in Table 4.4, cytosine release is observed under all assay conditions. In addition isolation of the protein from the assay mixtures containing the TR reducing system, revealed an absorption feature at 320 nm (data not shown). From the numbers in Table 4.4, it can be seen that the amount of cytosine released with DTT as the reductant is several fold greater than that observed with the TR reducing system under the same set of conditions. Again, based on the previous studies with ClUTP, this difference is attributed to the ability of DTT to trap the highly reactive 2 methylene-3-(2H) furanone before it alkylates the enzyme, allowing further turnovers to proceed.

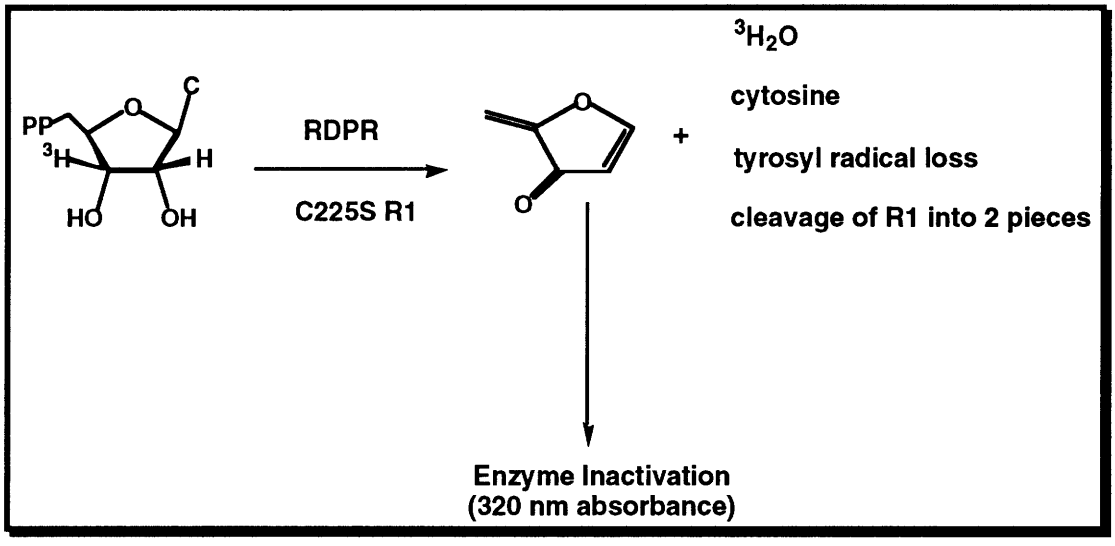
Table 4.4: Phenotypes of the active site C→S mutants: Equivalents of product per equivalent of RTPR.

Mutant (Assay condition)	(equivalents of) Cytosine	(equivalents of) Deoxycytidine
C119S (30 min at 37°C)		
DTT & NaOAc	14	<0.02
DTT & dATP	62	<0.02
TR/TRR & dATP	15	<0.02
TR/TRR & NaOAc	0.6	<0.08
C419S (60 min at 37°C)		
DTT & NaOAc	10	<0.02
DTT & dATP	55	<0.02
TR/TRR & dATP	19.7	<0.02
TR/TRR & NaOAc	2.5	<0.02

Lastly, when either C419S or C119S is treated with CTP in the presence of DTT under anaerobic conditions, AdoCbl is partially destroyed resulting in the production of cob(II)alamin ($B_{12}r$), which can be observed spectrophotometrically at 477 nm (data not shown). The details of the cofactor destruction are currently under investigation; however, it is analogous to loss of the tyrosyl radical with the corresponding *E. coli* active site mutants (Mao et al., 1989; Mao et al., 1992a) (Scheme 4.2). The ability to catalyze cofactor destruction in conjunction with the observation of cytosine release, suggests that each of these mutants is structurally intact, as they are able to bind AdoCbl and CTP, and

catalyze chemistry ascribed to abstraction of the 3′ hydrogen atom of the substrate. The inability of these mutants to catalyze dCTP production, strongly supports their function as the direct providers of reducing equivalents during substrate reduction.

Scheme 4.2: Interaction of mutant C225SR1 of the *E. coli* reductase with cytidine diphosphate.



Characterization of C408S RTPR

Cysteine 408 of RTPR was targeted for mutagenesis on the basis of sequence homology with C439 of the *E. coli* RDPR (Chapter 2). Previous mutagenesis studies on the *E. coli* enzyme have suggested that C439 might be the amino acid residue responsible for initiating the reduction process by abstracting the 3′ hydrogen atom of the substrate (Mao et al., 1992). If C408 of RTPR is the counterpart of C439 of RDPR, then the above model would predict that absolutely no dCTP or cytosine should be produced, since both require 3′ C-H

bond cleavage for their production. The reaction of C408S RTPR with CTP was therefore investigated using both reductants in combination with either the allosteric effector or NaOAc. Under all four sets of conditions, neither dCTP nor significant amounts of cytosine was produced in a 30 min incubation at 37°C. Given the large amounts of enzyme (24 μM) and the use of CTP of high specific activity, this mutant makes product at a rate that is less than $2x10^{-5}$ times that of the wt protein. This is the lower limit of detection in this assay. Thus, C408S RTPR is inactive with respect to nucleotide reduction.

Given that C408S RTPR possesses no detectable activity for the ability to catalyze nucleotide reduction, circular dichroism spectra of both wt RTPR and mutant C408S RTPR were recorded to show that no gross structural perturbations were responsible for the mutant's loss of activity. Both proteins exhibited almost identical ellipticities when normalized for the amount of enzyme used in each determination (Figure 4.3).

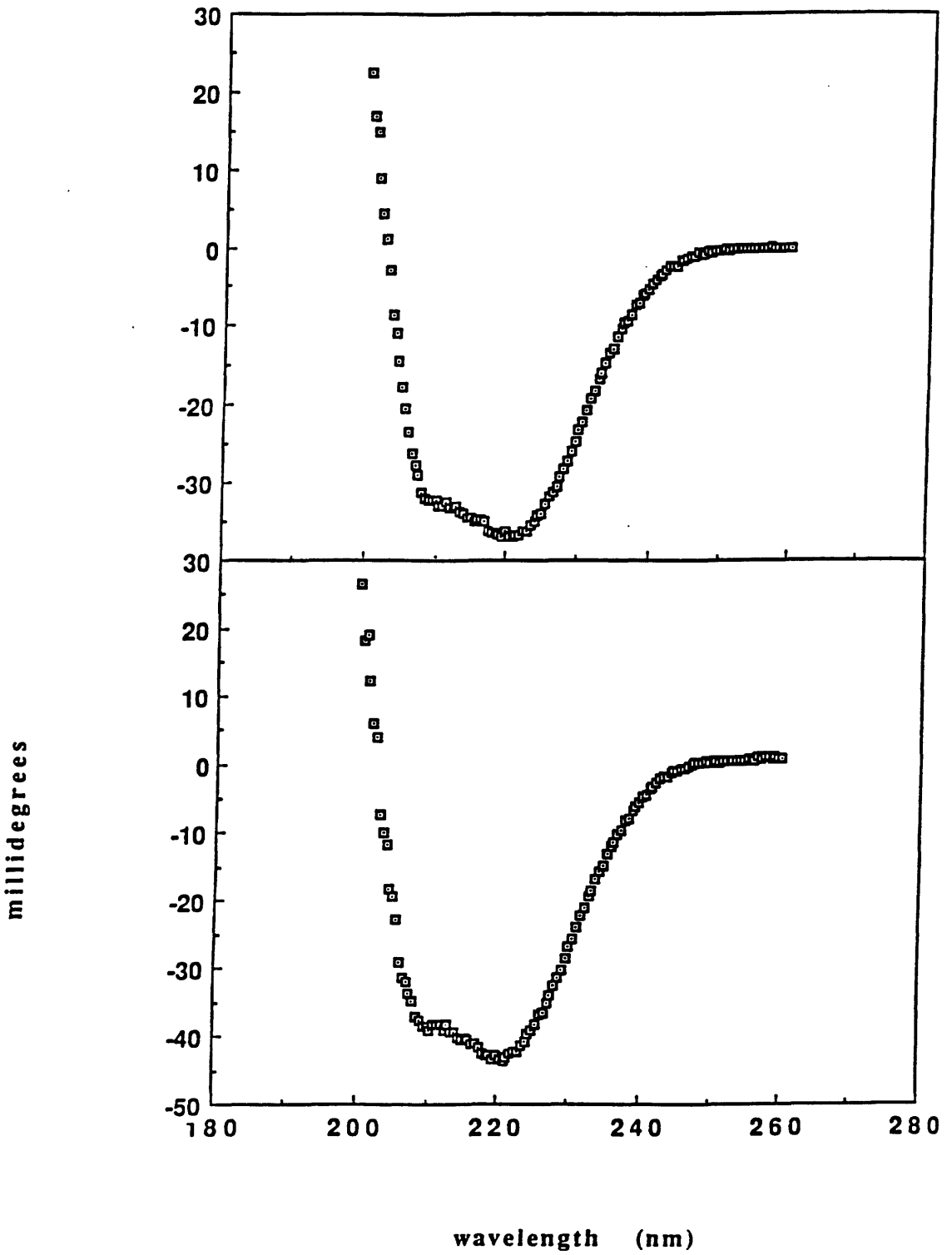
Characterization of C305S RTPR

As a control experiment to insure that the choice of mutants was not fortuitous, C305 was converted to a serine. This mutant was partially purified and was shown to have a specific activity ~60% that of wt RTPR. Furthermore, characterization of the products produced on interaction with CTP revealed only dCTP.

Discussion

The cloning, sequencing, and expression of RTPR (Booker & Stubbe, 1993) (Chapters 2 & 3) has allowed an investigation of the complex role of the cysteines involved in nucleotide reduction using site-directed mutagenesis. The studies presented in this paper, in conjunction with earlier studies on the *E. coli* RDPR,

Figure 4.3: Circular dichroism spectra of mutant C408S RTPR and wt RTPR. Wild-type RTPR (top spectrum) or C408S RTPR (bottom spectrum) were diluted to 9 μM and 12 μM respectively in 10 mM potassium phosphate buffer, pH 7.2 /1 mM DTT. Spectra were recorded from 200-260 nm at 37°C as described in the methods section.


30
20
10
0
-10
-20
-30
30
20
10
0
-10
-20
-30
-40
-50
180
200
220
240
260
280
millidegrees
wavelength (nm)

provide strong support for a model in which five cysteines are required for nucleotide reduction in each of these enzymes (Figure 4.4). The active site thiols of RTPR and their *E. coli* counterparts are proposed to be C408 (C439), C119 (C225), and C419 (C462). Cysteines 119 and 419 are the residues which become oxidized concomitant with substrate reduction. Cysteine 408 is postulated to be oxidized to a thiyl radical by the products resulting from the homolysis of the carbon-cobalt bond of AdoCbl. It is this thiyl radical which is then proposed to initiate substrate reduction by abstracting its 3′ hydrogen atom. Two additional cysteines, C731 and C736 (C754 and C759 in the *E. coli* RDPR) are proposed to shuttle reducing equivalents into the active site disulfide via disulfide interchange from the *in vivo* protein reductant TR.

Both the *E. coli* and *L. leichmannii* reductases have C-terminal tails with similar sequence contexts. The studies with the C→S mutants of these

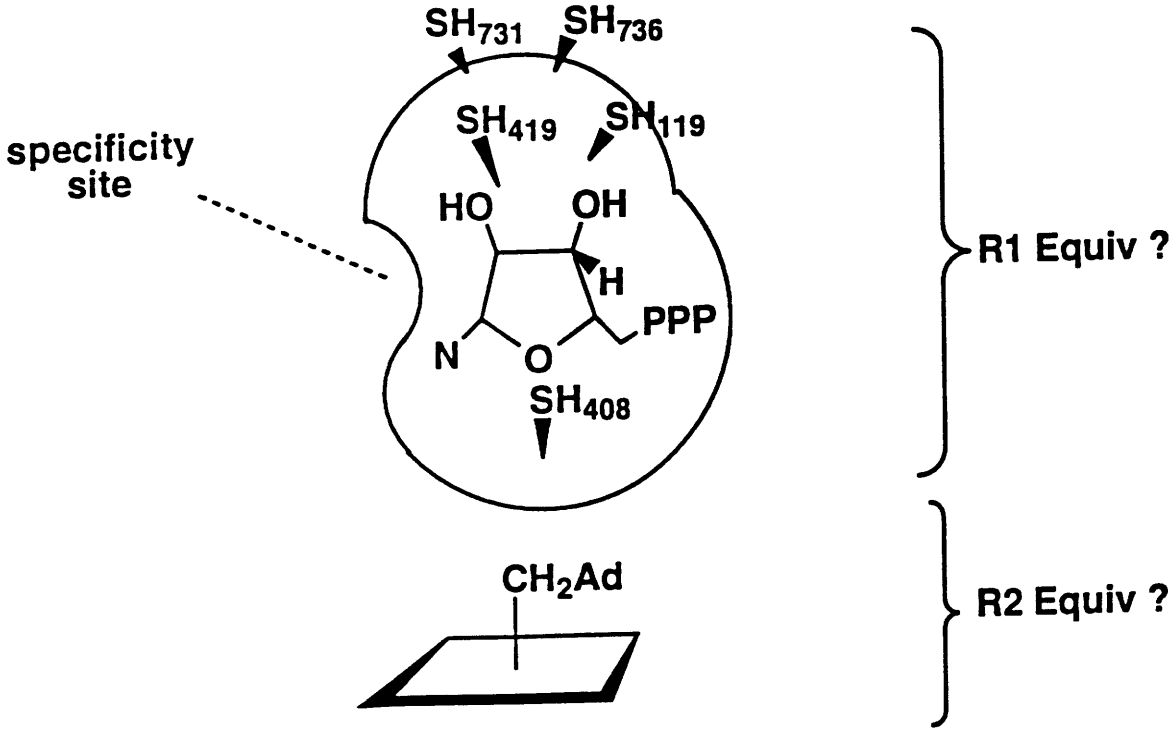
RTPR: DLELVDQTD_C^{731}EGGAC^{736}PIK

RDPR: DLVPSIQDDGC^{754}ESGAC^{759}KI

residues, besides providing strong support for their function, also shed light on the protein's dynamics. Comparison of the results using TR and DTT as site. Second, dATP, the allosteric effector required for CTP reduction, modulates the flexibility of the C-terminal tail as evidenced by a drop in the reduction rate to 1/10 of that observed in the presence of NaOAc. Similar results have previously been reported with the *E. coli* RDPR and these same reductants, again indicating a dynamic C-terminal tail in both of these systems (Mao et al., 1992b).

As indicated earlier, the *L. leichmannii* and *E. coli* enzymes display very little sequence homology with respect to each other (Booker & Stubbe, 1993) (Chapter 2). This hampered the process of deciding which of the ten cysteines in

Figure 4.4. Postulated model for the role of five cysteines in nucleotide reduction. Sequence homology searches with other B_{12}-requiring enzymes suggest that the AdoCbl binding site lies between amino acids 169 and 413.


SH731
SH736
SH419
SH119
specificity site
HO
OH
H
PPP
N
O
SH408
CH2Ad
R1 Equiv ?
R2 Equiv ?

reductants suggest that this C-terminal tail is flexible, and that the flexibility is altered by the presence of allosteric effectors. For example, DTT can provide reducing equivalents directly to the active site cysteines, by-passing the C-terminal tail which must be sufficiently dynamic to allow access to the active RTPR might be directly involved in the reduction of the NTP substrate. An important clue to their assignment came from our earlier biochemical studies which suggested that C119 was able to undergo disulfide interchange with the C-terminal cysteines (Lin et al., 1987). The basis for selecting C419, however, was not as obvious and requires additional comment. C419 was targeted based on its primary sequence relationship relative to C408, 11 amino acids displaced towards the C-terminal end. The corresponding cysteine in the *E. coli* RDPR, C462, is 23 amino acids displaced from C439 in the same sense. A comparison of the dinuclear-iron center reductases from 11 sources suggests that even though C462 is conserved, its spacing relative to C439 is variable (Eriksson & Sjöberg, 1989; Chakrabarti et al., 1993). In human, yeast, and vaccinia virus RDPRs, these cysteines are separated by 15 residues, while in the Epstein Barr virus, they are displaced by 14 residues. The C^{419}NL sequence found in RTPR has also been identified in 9 of 11 non-heme iron-dependent reductases, with the *E. coli* and phage T4 reductases having the sequence CTL. Thus, C419 and C119 were targeted for mutation, the former being considered as the active site equivalent to *E. coli* C462. As a control experiment, an additional cysteine, 305, was also mutated to a serine.

The phenotypes of the serine mutants created from the two cysteines (119, 419) proposed to directly provide reducing equivalents to the nucleotide substrate are very similar to those previously reported for the corresponding *E. coli* RDPR equivalents (225 and 462). In contrast to these previous studies, however, the present studies are unambiguous, as our heterologous expression

system has avoided problems associated with contaminating wt protein. The absence of dCTP and the presence of cytosine when these mutants are incubated with CTP, indicates as our mechanism has proposed, an uncoupling of the reduction step from the initiation step involving hydrogen atom abstraction from the 3′ position of the nucleotide (Chapter 1). The altered chemistry, including cytosine release, which occurs when these steps are uncoupled has been well documented (Mao et al., 1992a). The results in Table 4.4 and the control experiment with C305S RTPR suggest that C419 and C119 are in fact in the active site of RTPR.

The model in Fig. 4.4 also predicts that C408 should be close within three dimensional space to C419 and C119. Its importance has been identified by sequence context and by results with the *E. coli* C439S mutant (Mao et al., 1992). As outlined above, this RTPR mutant is inactive with respect to catalysis of

RTPR: TNPC^{408}GEISLA

RDPR: SNLC^{439}LEIALP

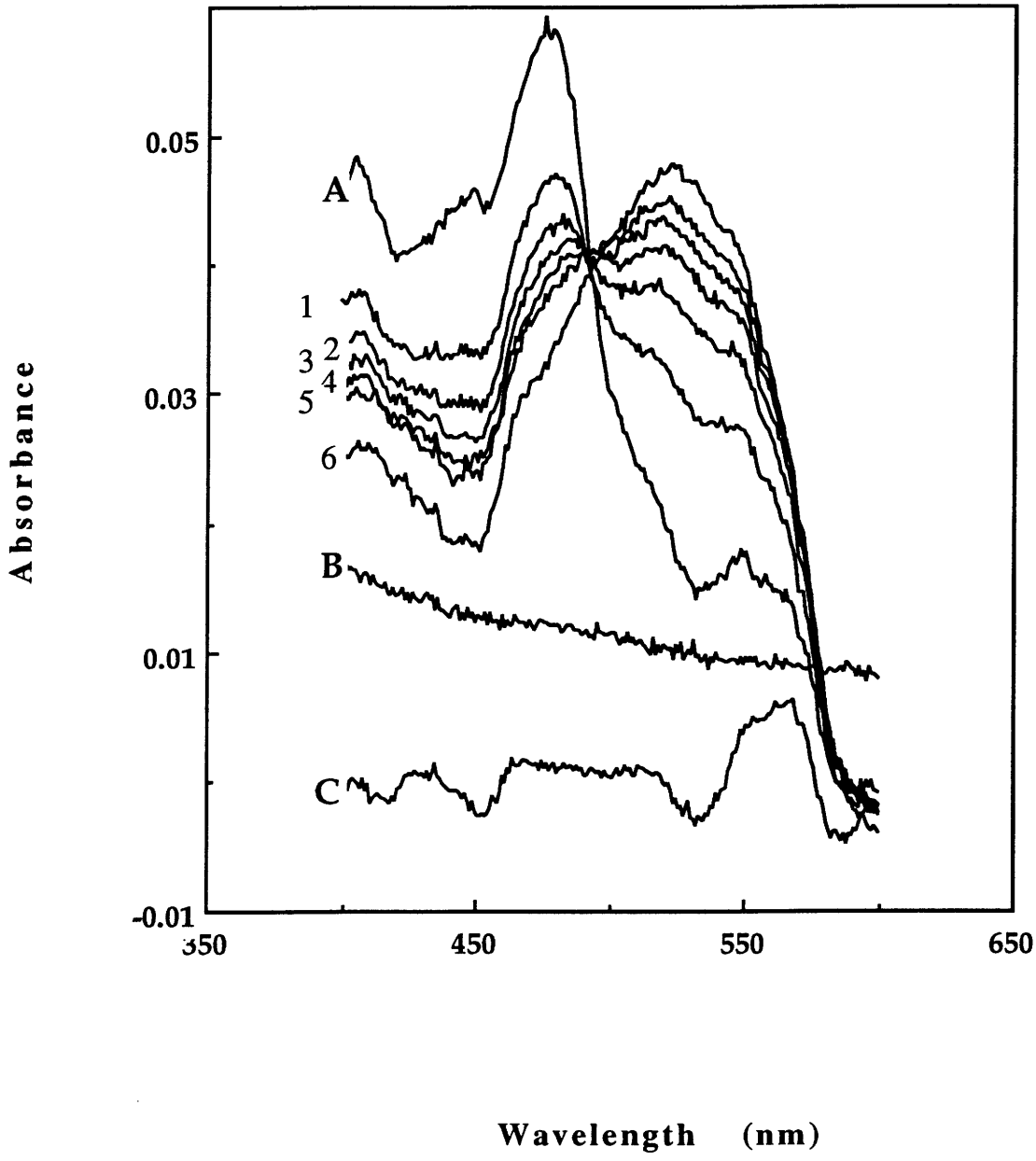
nucleotide reduction. The coenzyme B_{12}-requiring reductases are unique in that they catalyze two additional reactions which can be used as well to investigate the importance of C408. The first reaction, which is the focus of Chapter 5, is the equilibration with water of the 5′ methylene hydrogens of AdoCbl. If the cofactor is syntheiszed with tritium at the 5′ position, the enzyme catalyzes the release of all of the tritium to water (Abeles & Beck, 1967; Hogenkamp et al., 1968). Both wt RTPR and mutant C408S were assayed for their ability to catalyze this exchange (Chapter 5). Wt RTPR (0.06 nmol) catalyzes release of ~5500 cpm of ^3H_2O in a 10-min incubation at 37°C. However, 6 nmol of mutant C408S RTPR releases 60 cpm of ^3H_2O in a 30-min incubation at 37°C, which is virtually

indistinguishable from the results with the control in the absence of enzyme. The results indicate that C408S RTPR has an activity for the exchange reaction that is < 0.006% that of wt RTPR. The second reaction catalyzed by RTPR is a slow decomposition of the cofactor to give 5'-dA and cob(II)alamin ($B_{12}r$) when the experiment is conducted under anaerobic conditions (Yamada et al., 1971). The ability of mutant C408S RTPR to catalyze this slow cob(II)alamin formation was investigated by Stuart Licht in the Stubbe laboratory. In a reaction containing 120-150 μM wt RTPR or mutant C408S RTPR, 100 μM AdoCbl, and the TR/TRR/NADPH reducing system, 0.56 equivalents of $B_{12}r$ are formed with wt RTPR after a 75-min incubation at 37°C, whereas no $B_{12}r$ is detectable with mutant C408S. The limit of detection for this assay is 0.04 equivalents of $B_{12}r$. Thus, C408S RTPR is unable to catalyze two additional reactions characteristic of wt-RTPR.

To conclude from these studies that C408 is essential for catalysis, it is necessary to demonstrate that the mutant protein is properly folded. As indicated above, the circular dichroism spectra of wt RTPR and mutant C408S are virtually indistinguishable. A more definitive experiment to address the folding of C408S RTPR is to examine its ability to bind AdoCbl, allosteric effector (dGTP), and substrate. The ability of wt RTPR to catalyze a slow breakdown of AdoCbl in the presence of dNTP and DTT has previously precluded an accurate determination of a binding constant for the cofactor. The observation that C408S RTPR does not catalyze this decomposition made it a prime candidate to allow a measurement for the first time of a K_d for AdoCbl. Efforts by Stuart Licht to make this determination using filter binding assays (Ormö & Sjöberg, 1990) were, however, unsuccessful. The data were very scattered, suggesting that the K_d is high.

Previous studies of Yamada *et al.* (1971) had indicated that wt-RTPR can bind the putative intermediates in the RTPR catalyzed reaction [cob(II)alamin and 5'-dA] in the presence of dGTP and DTT. These results suggested that the apparent low K_ms for AdoCbl may be a reflection of tight binding of the "intermediates," and not the intact cofactor. C408S RTPR's inability to catalyze formation of cob(II)alamin is perhaps due to the required coupling of this carbon-cobalt bond homolysis to formation of the protein radical (X•), where X is proposed to be C408. This uncoupling would then prevent detection of tight binding of AdoCbl; but if the model is correct, then binding of cob(II)alamin and 5'-dA should be detected. This hypothesis was tested by Stuart Licht. Mutant C408S RTPR was incubated with cob(II)alamin, 5'-dA, and dGTP, and then passed through a Sephadex G-50 column, and the UV-vis spectrum of the protein was recorded (Figure 4.5). The results show that ~0.5 equivalents are bound to the mutant protein and control experiments reveal that both 5'-dA and dGTP are required for this binding (Figure 4.5). A similar experiment with wt-RTPR revealed ~0.25 equivalents of cob(II)alamin bound. The quantitation and structural characterization of the cob(II)alamin was carried out by allowing it to oxidize to aquocobalamin (Figure 4.5). These studies establish the ability of C408S RTPR to bind cofactor in its "active" form as well as the effector dGTP. Binding of the latter was confirmed by Stuart using equilibrium dialysis with [1',2'-^{3}H]dGTP. A Scatchard plot revealed a K_d of 4 ± 1.3 μM and 0.88 ± 0.3 binding sites. These results compare with values of 5.8 ± 0.4 μM and 0.79 ± 0.06 for wt RTPR determined by the same procedure, and 1.73 ± 0.07 μM and 1.07 ± 0.02 previously determined for RTPR isolated from *L. leichmannii* (Singh et al., 1977). It is unclear why less than one site is observed for the wt RTPR, although Chen *et al.* (1974) observed 0.85 sites for dGTP using wt enzyme at 25°C. It is interesting to note that this number is similar to the number of active sites

Figure 4.5. Analysis of cob(II)alamin binding to C408S RTPR. (**A**) Cob(II)alamin binding in the presence of dGTP and 5′-dA. Additional spectra were obtained 2 min (1), 4 min (2), 6 min (3), 8 min (4), 10 min (5) and 12 min (6) after the initial scan. (**B**) Cob(II)alamin binding in the absence of dGTP, and (**C**) in the absence of 5′-dA.


0.05
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0.01
-0.01
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A
1
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3
4
5
6
B
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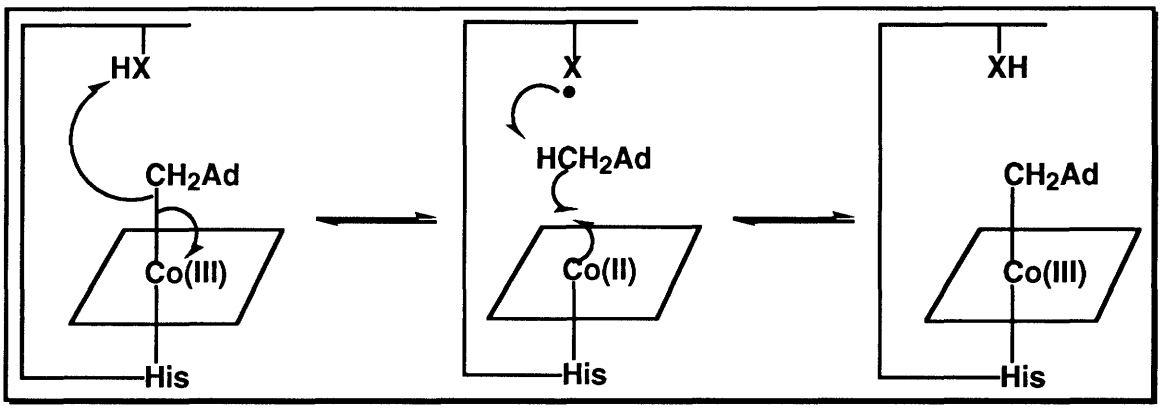
Absorbance
Wavelength (nm)

determined by the single-turnover experiments with the C-terminal mutants of RTPR. Thus, while C408S RTPR possesses no detectable catalytic activity, it is still capable of binding effectors and putative intermediates resulting from AdoCbl. The coupling of carbon-cobalt bond homolysis of AdoCbl to formation of the protein radical (Scheme 4.3) with the wt protein, could explain the observed K_m of 0.3 μM for AdoCbl (Blakley, 1978) even though the K_d for AdoCbl, under similar conditions is too large to measure. While K_ds have not yet been measured for 5'-dA or cob(II)alamin binding to C408S-RTPR due to the unavailability of labeled materials, these binding studies, the ability to bind effector, dGTP, and the inability of C408S RTPR to catalyze any reactions, suggest that C408 plays an essential role in catalysis and is consistent with its role as the X• in our proposed mechanism.

These studies support our hypothesis that AdoCbl required by RTPR for catalysis is equivalent to the R2 subunit of the *E. coli* RDPR. Studies of Tamao and Blakley (1973) have established that subsequent to AdoCbl binding, RTPR catalyzes homolysis of its carbon-cobalt bond resulting in the production of cob(II)alamin and a putative 5'-deoxyadenosyl radical (5'-dA•). This state of RTPR would be equivalent to the tyrosyl radical and dinuclear-iron center of R2 of the*E. coli* RDPR. In neither case, however, is the initially generated radical responsible for hydrogen atom abstraction from the substrate. In the case of RDPR, the x-ray structure and biophysical studies suggest that nucleotide reduction is initiated by long range e^-/H^+ transfer from R1 to R2 to generate a thiyl radical of C439 (Stubbe, 1990b; Mao et al., 1992b; Nordlund & Eklund, 1993; Uhlin & Eklund, 1994). Our studies on RTPR required, as with the *E. coli* RDPR, that the putative 5'-dA• does not directly mediate hydrogen atom abstraction from the substrate (Ashley et al., 1986). The mutagenesis studies reported herein

Scheme 4.3. Model for the generation of a protein radical upon binding AdoCbl. XH is proposed to be C408 of RTPR.



suggest that the putative 5-dA• generates a protein radical, C408, which then initiates catalysis.

What is striking about the studies reported herein is the remarkable similarities of the phenotypes of the five mutants of RTPR in comparison with those of RDPR. Inspite of the facts that the quarternary structures, primary structures, and cofactor requirements for these reductases are unique, they appear to have evolved strikingly similar chemical mechanisms as well as similar roles for the five cysteines in catalysis. These data provide strong support for our original hypothesis that the cofactors act as radical chain initiators, generating a protein radical sufficiently removed from the cofactor binding site such that the redox-active cysteines required for the reduction process would not interfere with the radical-initiator cofactor.

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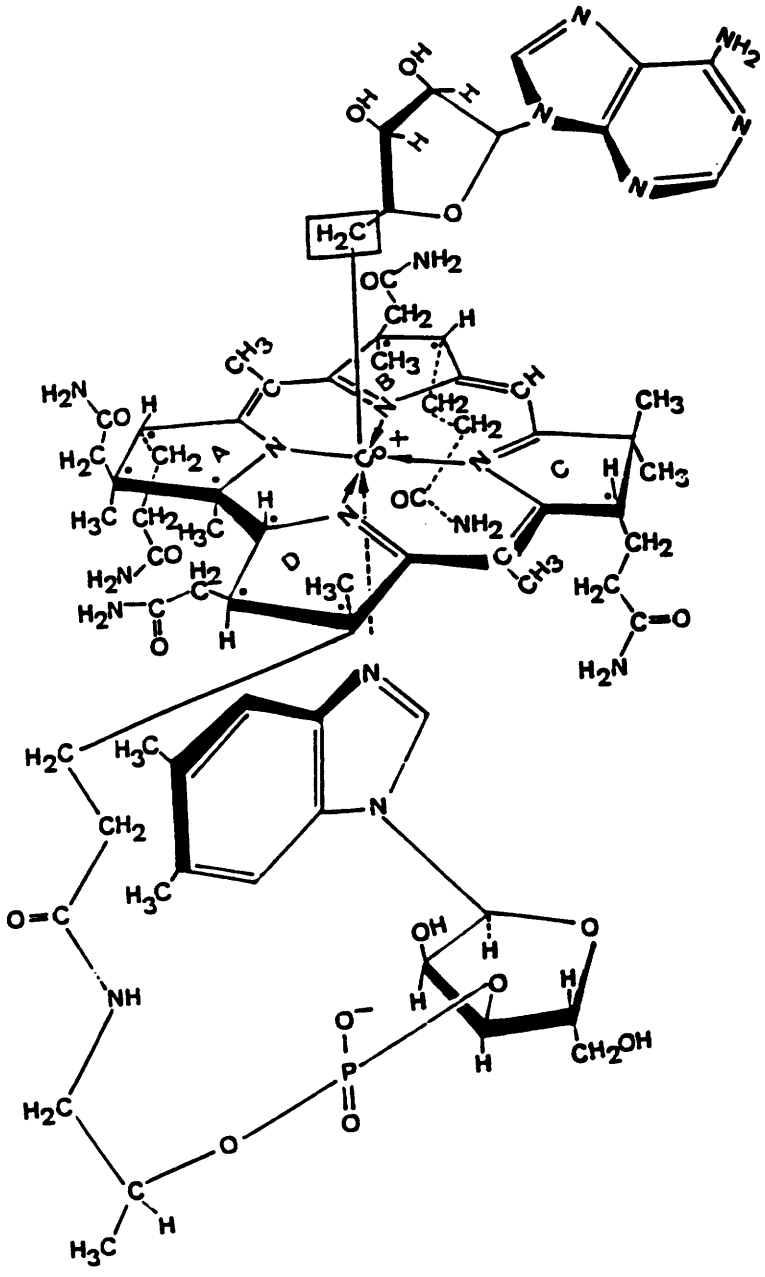
Chapter 5:

A Reinvestigation of the Adenosylcobalamin-dependent Exchange Reaction Catalyzed by the Ribonucleoside Triphosphate Reductase from *Lactobacillus leichmannii*

The studies outlined in Chapter 4 provide a compelling model for the reduction of ribonucleotides to 2'-deoxyribonucletides by the *L. leichmannii* reductase, and lend support to a similar model that is proposed for the ribonucleoside diphosphate reductase (RDPR) isolated from *E. coli* (Mao et al., 1992a; Mao et al., 1992b; Mao et al., 1992c). In both cases, five cysteines are believed to be involved. Two cysteines, which are proposed to be located in the active site, deliver reducing equivalents directly to the substrate molecule that is undergoing reduction and are themselves oxidized to a disulfide bond upon each turnover event. Two additional cysteines are believed to be located on the exterior of both the *E. coli* and *L. leichmannii* reductases, and are proposed to deliver reducing equivalents from the thioredoxin/thioredoxin reductase/NADPH reducing system into the active site disulfide in order that multiple turnovers can be achieved. A fifth cysteine is proposed to initiate substrate turnover by abstracting the 3' hydrogen atom of the substrate. This is hypothesized to occur via a thiyl radical (X•) which is presumably formed upon a one-electron oxidation of the cysteine in a process that is initiated by the cofactor. The mechanism as well as the dynamics of how this reaction takes place are unclear. In the case of the RDPR from *E. coli*, structural information is now becoming available and may offer clues as to how this radical transfer might be accomplished (Nordlund & Eklund, 1993; Uhlin & Eklund, 1994). No such information is presently available or even impending for any of the AdoCbl-dependent reductases. As a tool for trying to understand this process, we have undertaken a study of a second reaction which is unique to the AdoCbl-dependent ribonucleotide reductases, and which may be relevant to the generation of X•. This reaction is the equilibration with H_2O of the 5' methylene hydrogens of coenzyme B_{12} (AdoCbl) (Figure 5.1).

Figure 5.1: Structure of Coenzyme B_{12}. The 5' methylene carbon of 5'-deoxyadenosine is surrounded by a rectangle. The hydrogens on this carbon undergo an enzyme-mediated equilibration with H_2O. The four pyrroline rings are labeled A, B, C, and D, and the chiral centers of the corrin macrocycle are denoted with asterisks. Figure adapted from Vitamin B_{12} (1979) (Zagalak & Friedrich, 1979).



This exchange reaction was first demonstrated in studies by Beck, Abeles, and Robinson, which were designed to show a commonality in function of the cofactor in AdoCbl-dependent enzymes (Beck et al., 1966a). However, unlike the enzymes dioldehydrase and ethanolamine ammonia lyase (Babior, 1968), the *L. leichmannii* ribonucleotide reductase (RTPR) catalyzed an enzyme-dependent transfer of tritium from the 5' methylene hydrogens of [5'-^{3}H]AdoCbl to H_2O, and not to the corresponding product. This reaction was shown to be dependent upon the presence of substrate (CTP) and reductant (dihydrolipoate), and the omission of the allosteric effector (dATP) had little effect on the amount of tritium transferred to H_2O in a given period of time. Subsequent studies by Abeles and Beck (1967) demonstrated that AdoCbl becomes tritiated when the enzymatic reaction is carried out in ^3H_2O, providing evidence for an equilibration between the cofactor and solvent protons. Moreover, the [5'-^{3}H]AdoCbl isolated from the RTPR reaction was shown to label propionaldehyde in the dioldehydrase reaction when 1,2-propanediol is used as substrate, demonstrating that the tritium in [5'-^{3}H]AdoCbl is located in the 5' position of the cofactor.

An extensive characterization of the exchange reaction was carried out by Hogenkamp *et al.* (1968), and revealed that not only is CTP effective in promoting exchange, but most NTPs as well as dNTPs could serve in the same capacity. In fact, dGTP was the most effective nucleotide for promoting this reaction. Additionally, the effect of various thiols on the exchange reaction was studied. Although monothiols were effective, dithiols such as dithiothreitol and dihydrolipoic acid generated the greatest amount of ^3H_2O in a given time period. The thioredoxin (TR)/thioredoxin reductase (TRR)/NADPH reducing system was about twice as effective as monothiols, and 1/2 as effective as dithiols. Conversely, glutathione produced very little

exchange above background (Hogenkamp et al., 1968). Although reductant was shown to be required, the role of the reductant was suggested to be catalytic in nature; it was not consumed during the exchange reaction as it is during substrate turnover. Hogenkamp *et al.* (1968) also studied the reverse reaction - the incorporation of tritium into AdoCbl from ^3H_2O. Interestingly, when the exchange reaction was carried out with unlabeled AdoCbl (0.8 mM) in a volume of 5 mL of ^3H_2O (70 mCi), an average of 1.4 atoms of tritium were shown to be incorporated into each molecule of AdoCbl. In addition, [5'-^{3}H]AdoCbl labeled by chemical methods was shown to transfer all of its label to H_2O. These results demonstrate unequivocally that both of the 5' methylene hydrogens of AdoCbl are capable of participating in the exchange reaction, suggesting that the cofactor is converted into an intermediate in which the 5' methylene hydrogens are equivalent. A model for the exchange reaction that is consistent with this data was put forth by Hogenkamp *et al.* (1968). However, this model predated the seminal experiments of Tamao and Blakley (Tamao & Blakley, 1973) and Orme-Johnson *et al.* (1974), which showed that cob(II)alamin is produced in a kinetically-competent fashion under conditions similar to those of the exchange reaction. This model also predated the now-generally-accepted mechanism of nucleotide reduction advanced by Stubbe and coworkers (Stubbe, 1990b). This original model is thus inconsistent with available information and will therefore not be presented. However, any model proposed for the exchange reaction must accommodate the data of Hogenkamp *et al.* (1968).

The exchange reaction is not unique to the *L. leichmannii* reductase, as several other AdoCbl-dependent reductases have also been shown to catalyze an equilibration between the solvent and the cofactor (Abeles & Beck, 1967; Ong et al., 1992). One such reductase is the ribonucleoside diphosphate

reductase from *Corynebacterium nephridii*. This enzyme uses NDP substrates like the enzyme from aerobically-grown *E. coli*, but the pattern of allosteric regulation is complex, and at present incompletely understood (Tsai & Hogenkamp, 1980). DeoxyGTP stimulates the reduction of ADP, and both dGTP and dTTP stimulate the reduction of UDP. All other dNTPs or NTPs examined were ineffective in stimulating the reduction of GDP or CDP. Very recent work with this enzyme using 3'-C-methyl-ADP and 3'-C-methyl-UDP analogs has led to a new model for the exchange reaction which needs to be addressed (Ong et al., 1992). Both of these analogs would not be expected to be substrates for ribonucleotide reductases due to the absence of a 3' hydrogen atom. Indeed, this was found to be the case when these compounds were incubated with the *C. nephridii* reductase in the presence of AdoCbl. However, their ability to inhibit the reduction of ADP and UDP suggested to the authors that the analogs were binding in the active site of the enzyme. In addition, the inability of the analogs to promote the exchange reaction (as do the corresponding substrates) led to the proposal that cleavage of the 3' C-H bond is a prerequisite for the exchange reaction to occur. Significant to the authors' argument are the results outlined in Table 5.1. The dNDPs, which are the products of normal substrate turnover, are better promoters of the exchange reaction than the corresponding dNTPs, suggesting that exchange is due to an active site-mediated process. From these observations, the authors propose a mechanism (Scheme 5.1) in which two protein radicals are believed to be involved in nucleotide reduction. In their model, one protein radical (X•) is generated upon the homolytic cleavage of AdoCbl and the abstraction of a hydrogen atom of an amino acid residue by the concomitantly-formed 5'-deoxyadenosyl radical. This radical (X•) then generates a second radical (Y•) which during normal substrate turnover is reponsible for initiating the

reduction process by abstracting the 3' hydrogen atom of the substrate. Protein radical X• is accessible to solvent whereas Y• is not. The authors contend (and it is necessary to quote due to an inability to fully follow their logic) "In the absence of a ribonucleotide substrate or 2'-deoxynucleotide product, the equilibria of the homolytic cleavage of the carbon-cobalt bond of the coenzyme and the generation of a protein radical by the 5'-deoxyadenosyl radical lie predominantly to the left (upper reaction). In the presence of a substrate, product, or analog with an abstractable hydrogen at C-3', those equilibria are shifted to the right, generating more protein-based radical X (upper line of Scheme [5.1]) and thereby stimulating the exchange of hydrogen between coenzyme and solvent (second line of Scheme [5.1])."

Table 5.1: dNTP/dNDP stimulation of the tritium exchange reaction between [5'-^{3}H]AdoCbl and solvent[a]. Adapted from Ong *et al* (1992).

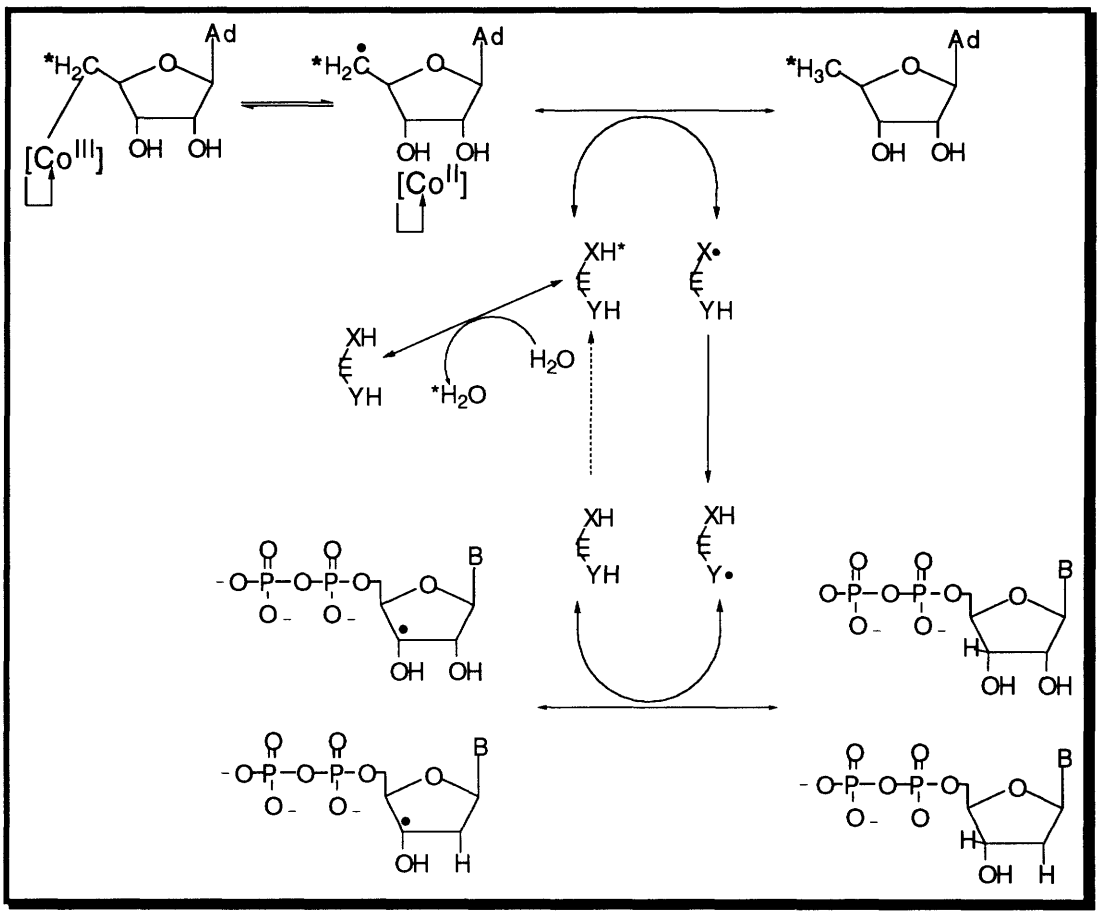
Nucleotide	Conc. (μM)	Activity[b]	Nucleotide	Conc. (μM)	Activity[b]
dGTP	10	14.9	dADP	50	29.2
dGDP	10	54.7	dUTP	500	2.9
dCTP	100	10.9	dUDP	500	4.4
dCDP	100	16.7	dTTP	500	0
dATP	50	0	dTDP	500	4.5

[a]Samples incubated for 10 min at 37°C. Tritium exchange to solvent was kept under 20% of the total tritium in each assay.

[b]cpm(x10^{-5})•mg^{-1} protein.

The above mechanism is not in accord with several previously established experimental facts as well as some of the data presented in the

Scheme 5.1: Model proposed by Ong *et al.* (1992) for the exchange reaction.



paper (shown in Table 5.1). Firstly, studies of dNTP production in the presence of the *L. leichmannii* RTPR in ^3H_2O, indicate that the 3' C-H bond of dGTP is not cleaved. This was investigated by Hogenkamp *et al.* (1968), in an experiment in which dGTP was shown to contain no significant radioactivity after being isolated from an exchange reaction that was conducted in 7 mCi of ^3H_2O. Therefore, at least with the *L. leichmannii* RTPR, the 3' C-H bond

cleavage of dGTP is not effected by the protein, but this nucleotide greatly enhances the exchange reaction. A similar reaction with the *C. nephridii* enzyme would be predicted to give very different results if the mechanism in Scheme 5.1 is valid.

Secondly, and more disturbingly, the data in Table 5.1 show that both dGTP and dCTP can catalyze exchange at appreciable rates. This is contradictory to the premise that exchange is mediated by abstraction of the 3' hydrogen bond (since these are allosteric effectors and not products of substrate turnover), and was not addressed in the work of Ong *et al.* (1992). Therefore, the only experimental results that are consistent with the model that 3' hydrogen-atom abstraction is a prerequisite for exchange are those with the 3'-C-methyl analogs.

As indicated above, the early studies of Hogenkamp *et al.* (1968), as well as those of Tamao and Blakley (1973) and Stubbe and coworkers, can now be meshed into a working hypothesis for the exchange reaction. Tamao and Blakley (1973) provided evidence for a cob(II)alamin intermediate which is generated with a rate constant of 38-46 s^{-1}, and which is dependent upon the presence of a (deoxy)nucleoside triphosphate and reductant. Analogously to the studies of Hogenkamp *et al.* (1968), dGTP was the nucleotide which stimulated formation of the greatest amount of this intermediate. Tamao and Blakley proposed that the exchange reaction is intricately connected to the generation of this cobamide intermediate, and that it is due to the interaction of the cob(II)alamin species with a thiol from the cysteines directly involved in delivering reducing equivalents to the substrate, or perhaps another cysteine on the protein. Stubbe and coworkers have modified this model, proposing that exchange is due to the reversible formation of X•, the protein radical which is proposed to initiate substrate reduction by abstracting the 3' hydrogen

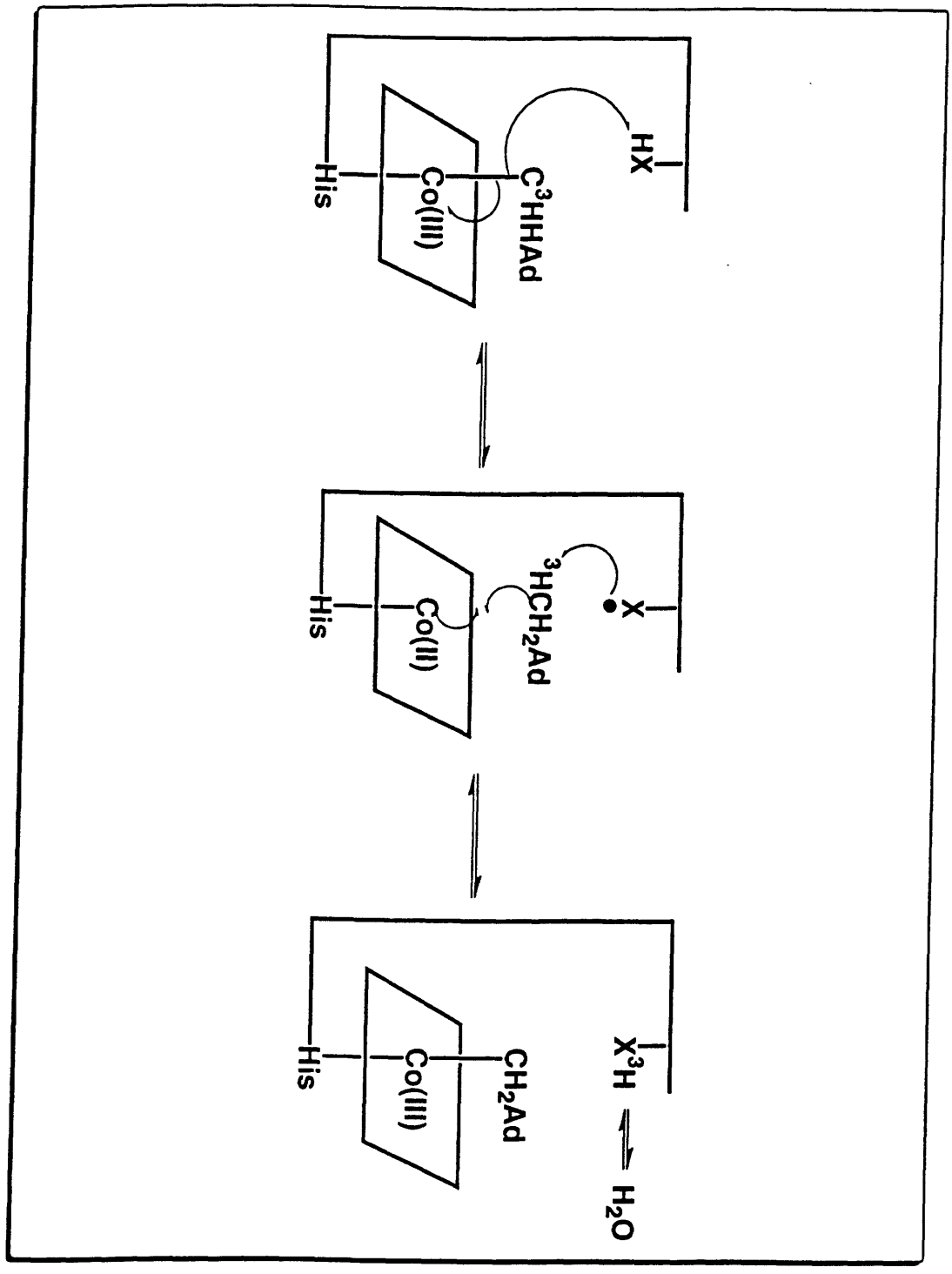
of the substrate (Figure 5.2) (Stubbe, 1989). This mechanism accommodates all available information. The proposal that X• is a thiyl radical is one that has been contested on thermodynamic grounds. The ability of a thiyl radical to abstract a hydrogen atom from 5'-deoxyadenosine is thermodynamically uphill by ~14 kcal. Therefore in order for exchange to occur, it must be coupled to Co-carbon bond reformation (~30 kcal) (Finke, 1984; Halpern et al., 1984). Herein we report our reevaluation of the exchange reaction as a test of our proposed model, with special emphasis on the quantitation of the rates of exchange under varying conditions in order to demonstrate its kinetic competence. We also re-address the role of the reductant and the effector in the exchange process. As discussed subsequently, we believe these studies will provide important new insight into the mechanism of protein radical formation, a key step in ribonucleotide reduction.

Materials and Methods

Materials

Sep-pak C_{18} cartridges were obtained from Millipore. Dithiothreitol was purchased from United States Biochemicals. 2'-Deoxyguanosine 5'-triphosphate (dGTP) was purchased from Pharmacia. Adenosine 5'-triphosphate (ATP), cytosine, 2'-deoxycytidine (dC), β-nicotinamide adenine dinucleotide phosphate (reduced form) (β-NADPH), phenylmethanesulfonyl fluoride (PMSF), tosyllysine chloromethylketone (TLCK), tosylphenylalanine chloromethylketone (TPCK), and coenzyme B_{12} were purchased from Sigma. [5'-^{3}H]AdoCbl (specific activity 1-10x10^7 cpm/μmol) was a much-appreciated gift from Professor H.P.C.

Figure 5.2: Working hypothesis for the RTPR-catalyzed exchange reaction.


His
Co(III)
C3HHAd
HX
His
Co(II)
3HCH2Ad
X
His
Co(III)
CH2Ad
X3H
H2O

Hogenkamp of the University of Minnesota (Minneapolis), or was provided by Stuart Licht. Alkaline phosphatase (specific activity 3143 units/mg) from calf intestine (CIP) was purchased from Boehringer Mannheim. Anion-exchange resin AG1-X2 (50-100 mesh) was purchased from Bio-Rad. [2-^{14}C]Cytidine 5'-triphosphate was prepared as described in Chapter 4.

Wild-type (specific activity 1.2 U/mg) and mutant RTPRs were purified from *E. coli* strains HB101/pSquire and JM101/pSquire as described in Chapters 3 and 4. *E. coli* thioredoxin (TR) and thioredoxin reductase (TRR) were isolated from overproducing strains SK3981 (specific activity 50 U/mg) and K91/pMR14 (specific activity 800 U/mg) (Lunn et al., 1984; Russel & Model, 1985).

UV-vis absorption spectra were recorded on a Hewlett-Packard 8452A diode-array spectrophotometer. All scintillation counting was performed on a Packard 1500 liquid scintillation analyzer using 8 mL of Sc¢INT-A XF scintillation cocktail (Packard) per 1 mL of aqueous reaction. High Pressure Liquid Chromatography (HPLC) was carried out using a Beckman 110 Solvent Delivery Module, 421A Controller, and a 163 Variable Wavelength Detector, in combination with an Alltech Econosil C_{18} column.

Purification of [5'-^{3}H]AdoCbl

[5'-^{3}H]AdoCbl (0.17 μmol; 1x10^{7} cpm/μmol) was loaded onto an Alltech C_{18} column equilibrated in 20% CH_3OH/80% H_2O. The column was washed in the above solvent (flow rate 1 mL/min) for 10 min, followed by a gradient from 20-100% CH_3OH over a period of 20 min. The column was subsequently washed for an additional 10 min with 100% CH_3OH. [5'-^{3}H]AdoCbl eluted at 35 min (75% CH_3OH), with aquocobalamin and hydroxocobalamin eluting at 32 min and 44 min (65 and 100% CH_3OH). [5'-^{3}H]AdoCbl was transferred to a

pear-shaped flask, and the solvent was removed by rotary evaporation in the dark. The product was redissolved in 0.5-1.0 mL of H_2O, and a portion of it was used to determine its concentration ($\varepsilon=8x10^3$ at 522 nm) and specific activity. The radioactive coenzyme B_{12} was diluted with cold material to a concentration of 1-2 mM, and a specific activity of 7-100x10^5 cpm/μmol.

Assay for Tritium Exchange from [5'-^{3}H]AdoCbl

RTPR was exchanged into 2 mM potassium phosphate (pH 7.5) using a Sephadex G-50 column (0.75x10 cm), and its concentration was determined by UV-vis absorption spectroscopy ($E^{1\%}$=13.3 at 280 nm) (Blakley, 1978). A typical assay contained in a volume of 305 μL: 50 mM potassium phosphate (pH 7.5), 300 μM dGTP, 4 mM EDTA, 50-300 nM wt RTPR, 50-200 μM [5'-^{3}H]AdoCbl (7-100x10^5 cpm/μmol), 0.2 mM NADPH, 65 μM TR, and 0.5 μM TRR. All assays were conducted using the TR/TRR/NADPH reducing system except where specifically specified. In these cases, either reductant was omitted, or the TR/TRR/NADPH reducing system was replaced with 30 mM DTT. All reagents except [5'-^{3}H]AdoCbl and RTPR were pre-incubated at 37°C for 3-5 min. Subsequent to this pre-incubation, all manipulations except for scintillation counting were carried out in the dark under dim red light. [5'-^{3}H]AdoCbl was added, and a 50-μL aliquot of the reaction mixture was removed and loaded onto a Sep-pak C_{18} cartridge which had been previously washed with 10 mL of CH_3CN followed by 10 mL of H_2O. The cartridge was washed with 3 mL of H_2O, and a 1-mL aliquot of the eluate was subjected to scintillation counting. The reaction was initiated by the addition of RTPR (5-10 μL) to the reaction mixture. At 1-2-min time intervals, 50-μL aliquots were removed and treated in the same manner as the zero time point. Rates of exchange were calculated from least-squares fits of plots of the amount of

radioactivity (cpm) released to water vs time. As an internal check, the cofactor was isolated by washing each of the columns with 3 mL of CH_3CN, and a 1-mL aliquot was analyzed by scintillation counting.

Determination of a K_m for AdoCbl in the Exchange Reaction with wt RTPR

The assay conditions for the K_m determination for AdoCbl are as described above. The concentrations of [5'-^{3}H]AdoCbl were varied from 21 to 427 μM, and the concentration of RTPR was 63 nM. All components of the reaction mixture except RTPR and [5'-^{3}H]AdoCbl were premixed into a 2.1-mL stock solution which was sufficient for 10 determinations. A 210-μL aliquot of the stock solution was removed and diluted to a final volume of 300 μL with the appropriate amount of [5'-^{3}H]AdoCbl (specific activity 1.1×10^7 cpm/μmol) and H_2O. Subsequent to the removal of a 50-μL aliquot from the assay mixture (t=0), the reaction was initiated with the addition of RTPR (2μL, 0.02 nmol), and the production of ^3H_2O was monitored at various time points as described above. The kinetic constants V_{max} and K_m were determined from a fit to Eq 1 using a FORTRAN version of the program HYPER (Cleland, 1979), where v is the initial rate, V_{max} is the maximum velocity, and K_m is the Michealis-Menton constant.

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad \text{Eq 1}$$

Determination of a K_m for dGTP in the RTPR-Catalyzed Exchange Reaction

The reaction mixture contained in a final volume of 300 µL: 50 mM potassium phosphate, pH 7.5, 4 mM EDTA, 65 µM TR, 0.5 µM TRR, 200 µM NADPH, 200 µM [5'-^{3}H]AdoCbl, 0.17 µM RTPR, and dGTP (3 µM, 9 µM, 15 µM, 30 µM, 59 µM, 119 µM, or 222 µM). All components of the reaction mixture except dGTP, [5'-^{3}H]AdoCbl, and RTPR, were combined into a stock solution which was sufficient for 10 assays. [5'-^{3}H]AdoCbl and dGTP were added, and the reaction mixture was incubated at 37°C for 5 min. Subsequent to the removal of a 50-µL aliquot (t=0), RTPR (10 µL, 0.04 nmol) was added to initiate the reaction. Additional aliquots were removed at 2, 4, 6, and 8 min, and ^3H_2O was separated from [5'-^{3}H]AdoCbl using Sep-pak C_{18} columns as described previously. The kinetic constants were determined as described above.

Analysis of the Ability of NaOAc to Replace dGTP in the Exchange Reaction

Wild-type RTPR was pre-reduced with 30 mM DTT in 50 mM potassium phosphate, pH 7.5. The protein was separated from the DTT and exchanged into 2 mM potassium phosphate, pH 7.5, using a Sephadex G-50 column as described above, and then placed on ice. The reaction mixture was as described above except that 1 M NaOAc replaced dGTP. A 50-µL aliquot of the reaction mixture containing all components except RTPR (t=0) was removed and incubated at 37°C for the duration of the assay. RTPR (10 µL, 9.4 µM) was added to initiate the reaction, and additional aliquots were removed at 2, 4, 6, and 8 min, and analyzed for the production of ^3H_2O as described previously.

Analysis for Consumption of Reductant during the Exchange Reaction

The reaction mixture was as described previously except that the reaction volume was 500 μL, and unlabeled AdoCbl (120 μM) was employed. All components of the assay mixture, except enzyme, were placed in a 0.75 mL cuvette, and incubated for 5 min at 37°C. The cuvette was placed in the cell holder of a Cary 210 spectrophotometer, and the background rate of NADPH oxidation was recorded at 340 nm (range, 2; chart speed, 50 s/cm). RTPR (1 μM) was added to the cuvette, and the enzyme-dependent rate of NADPH oxidation was subsequently measured. The net rate of NADPH oxidation was calculated as the difference between the observed rate and the background rate. A control reaction using [5'-^{3}H]AdoCbl was carried out with the same batch of enzyme to show that the enzyme was indeed active. ^3H_2O was monitored using Sep-pak C_{18} columns as described above.

Analysis of the Ability of Mutants C119S and C419S to Catalyze the Exchange Reaction

The reaction mixture contained in a final volume of 300μL: 50 mM HEPES (pH 7.5), 300 μM dGTP, 4 mM EDTA, 200 μM [5'-^{3}H]AdoCbl (6.9×10^6 cpm/μmol), 0.2 mM NADPH, 65 μM TR, 0.5 μM TRR, and C119S (12 μM), or C419S (23 μM). All reagents except [5'-^{3}H]AdoCbl and the mutant protein were incubated for 3-5 min at 37°C. [5'-^{3}H]AdoCbl was added as described above, and a 100-μL aliquot (t=0) was removed and incubated at 37°C for 20 min. The appropriate mutant RTPR (10 μL, 2 nmol C119S or 5 nmol C419S) was added to the remaining 200 μL to initiate the reaction, and 100-μL aliquots were removed at 10 min and 20 min, placed in a 10-mL pear-shaped flask, and shell frozen on dry ice. The above control which lacked enzyme was treated in the same fashion, and the volatile tritium from each of the three

samples was separated from [5'-^{3}H]AdoCbl by bulb-to-bulb distillation. The distillate containing the ^3H_2O and the residue containing [5'-^{3}H]AdoCbl were brought to a final volume of 1 mL with H_2O and analyzed by scintillation counting. Plots of the release of ^3H_2O as a function of time, as well as the decrease in the radioactivity of [5'-^{3}H]AdoCbl as a function of time were constructed. Exchange rates of both mutants were calculated as the average of these two rates.

Analysis of the Ability of Mutant C408S RTPR to Catalyze the Exchange Reaction

The reaction mixture contained in a final volume of 210 µL: 50 mM HEPES (pH 7.5), 0.3 mM dGTP, 4 mM EDTA, 65 µM TR, 0.21 µM TRR, 0.2 mM NADPH, 200 µM [5'-^{3}H]AdoCbl (specific activity $7.5x10^5$ cpm/µmol), and 28 µM mutant C408S RTPR. All reaction components except dGTP were pre-incubated for 5 min at 37°C. A 100-µL aliquot (used as the control) was removed and incubated at 37°C for the duration of the assay. DeoxyGTP was added to initiate the reaction, and subsequent to a 30-min incubation at 37°C, a 100-µL aliquot was removed, placed in a 10-mL pear-shaped flask, and shell-frozen in a dry ice/acetone bath. The control reaction was treated in the same fashion, and both reactions were analyzed by bulb-to-bulb distillation as described above for mutants C119S and C419S RTPRs.

Analysis of the Ability of Pre-reduced RTPR to Catalyze the Exchange Reaction

Wild-type or double mutant C731&736S RTPR (30 nmol) was pre-reduced (in a total volume of 100 µL) for 20 min at 37°C with 30 mM DTT in 50 mM HEPES (pH 7.5). This mixture was loaded onto a Sephadex G-50 column (0.75x7 cm) equilibrated in 2 mM HEPES (pH 7.5) to remove the DTT.

Fractions containing protein (A_{280}) were pooled, and the protein concentration was determined from the UV-vis spectrum as described above. A 10-μL aliquot (0.16 nmol) was used in the exchange assay which contained (in a volume of 300 μL) 50 mM HEPES (pH 7.5), 4 mM EDTA, 300 μM dGTP, and 200 μM [5'-^{3}H]AdoCbl (6.9×10^{6} cpm/μmol). The reaction mixture was incubated at 37°C for 3-5 min, and subsequent to the removal of a 50-μL aliquot (t=0), the assay was initiated with the addition of the pre-reduced RTPR.

The remaining pre-reduced RTPR was concentrated using a Centricon 30 ultrafiltration device and used to show that RTPR had been fully reduced. A 50-μL aliquot (6.6 nmol) was added to a reaction mixture which contained in a final volume of 200 μL: 50 mM HEPES (pH 7.5), 4 mM EDTA, 1 M NaOAc, 1 mM [2-^{14}C]CTP (2.3×10^{6} cpm/μmol), and 60 μM AdoCbl. The reaction mixture was incubated for 3-5 min at 37°C, and an aliquot (100 μL) containing everything except AdoCbl was removed at the zero time point. The reaction was initiated with the addition of AdoCbl (2 μL) and subsequent to a 3 min incubation, it was quenched by a 2-min incubation in a boiling water bath. A 10-μL aliquot of 0.5 M Tris-HCl, pH 8.5 / 1 mM EDTA, and 10 U of CIP were added, and the reaction mixture was incubated at 37°C for 1.5 h. Carrier cytosine and dC (120 nmol each) were added, and the reaction mixture was loaded onto 0.75x7 cm AG1-X2 columns (borate form 50-100 mesh). The production of [2-^{14}C]dCTP was analyzed by the method of Steeper and Steuart (1970) as described in Chapter 4.

Analysis of the Ability of Pre-oxidized RTPR to Catalyze the Exchange Reaction

A portion (9 nmol) of the pre-reduced RTPR from the above experiment was added to a reaction mixture containing in a volume of 200 µL: 50 mM HEPES (pH 7.5), 4 mM EDTA, 1 M NaOAc, 1 mM CTP, and 60 µM AdoCbl. The reaction was initiated with AdoCbl (5 µL), and subsequent to a 3 min incubation at 37°C, was loaded onto a Sephadex G-50 column equilibrated in 2 mM HEPES (pH 7.5). Fractions containing protein were pooled (A_{280}) and the protein concentration was determined from the UV-vis spectrum as described above. A 20-µL aliquot (0.27 nmol) was used in the exchange assay, which was carried out in a fashion that was identical to that described for the exchange reaction with pre-reduced RTPR. A control reaction with [2-^{14}C]CTP was also carried out under single-turnover conditions to show that the enzyme had undergone a full turnover. The production of [2-^{14}C]dCTP was analyzed for as described above.

Isolation of Mutant C32S TR

E. coli strain A291 was streaked onto LB agar plates supplemented with ampicillin (50 µg/mL), and grown overnight at 37°C. A single colony was used to inoculate 3 mL of the same liquid media, and the resulting culture was allowed to grow to saturation at 37°C. This culture was used to inoculate 4x2 L of KD media [per liter: 10 g of case amino acids, 5 g of Bacto yeast extract, 20 mL of balanced salt solution (40.6 mM $MgSO_4 \bullet 7H_2O$, 476 mM citric acid, 2.9 M K_2HPO_4, 840 mM $Na(NH_4)HPO_4 \bullet 4H_2O$)] supplemented with ampicillin (50 µg/mL). The bacteria were grown for 20 h at 37°C, and then pelleted by centrifugation for 30 min at 4°C and 10,000xg. Each of the 4 pellets was resuspended in 40 mL of TE (50 mM Tris-HCl, pH 7.4 / 3 mM EDTA)

containing 1 mM each of PMSF, TPCK, and TLCK, and the bacteria were lysed by passage through a French pressure cell at 16,000 psi. The solution was centrifuged, and streptomycin sulfate (5% in TE buffer) was added to the supernatant (over a period of 20 min) to a final concentration of 1%. The solution was stirred for an additional 20 min, and the precipitate was subsequently removed by centrifugation. The resulting solution (182 mL) was diluted with an equal volume of buffer A (20 mM potassium phosphate, pH 7.3 / 3 mM EDTA), and then loaded onto a DE-52 anion-exchange column (5x22 cm) equilibrated in the same buffer. The protein was eluted with a 2-L linear gradient from 20 mM to 150 mM potassium phosphate, pH 7.3 / 3 mM EDTA. Under these conditions, two major protein peaks (A_{280}) eluted. The second peak (~90 mM buffer) was pooled and concentrated to ~20 mL using an Amicon ultrafiltration apparatus fitted with a YM-5 membrane. This protein solution was loaded onto a Sephadex G-75 column (2.5x110 cm) which was equilibrated in 50 mM potassium phosphate pH 7.3 / 3 mM EDTA. Again, two peaks were observed (A_{280}). The second peak, which contained TR (by analysis of its elution pattern with wild-type TR), was pooled and concentrated to 7 mg/mL (ε_{280}=0.95 mL/mg), and stored in aliquots at -80°C.

Results

Assay for Exchange: Isolation and Quantitation of ^3H_2O

The successful measurement of rates of ^3H_2O production requires the establishment of a convenient method for separating [5'-^{3}H]AdoCbl from ^3H_2O. In the initial experiments, volatile tritium (^3H_2O) was isolated by bulb-to-bulb distillations. Although this procedure was reliable, it was not conducive to analyzing the large numbers of samples required for time

courses of reactions. A method using a Sep-pak C_{18} cartridge was therefore developed as a means of rapidly separating [5'-^{3}H]AdoCbl from ^3H_2O. The reactions were terminated at various times by the addition of the reaction mixture to the Sep-pak cartridge, and the resulting ^3H_2O was isolated by subsequently washing the column with 3 mL of H_2O. Table 5.2 shows a typical profile of ^3H_2O per mL of eluate. Greater than 80% of the total tritium added elutes in the first fraction, with the overall yield as calculated from the first 3 fractions being greater than 90%. Control experiments in which [5'-^{3}H]AdoCbl is applied to the cartridge, show that no leak through of the radioactive cofactor occurs. For the calculation of initial rates, time courses were

Table 5.2: Profile of ^3H_2O elution from Sep-pak C_{18} cartridge.

Fraction # (1 mL)	^3H_2O (cpm)[a]
1	889.2
2	98.6
3	32
4	26
5	26

[a]Total of 2100 cpm of ^3H_2O added to column.
Counted 1/2 of each fraction.

employed in which no more than 10% of the tritium initially present as [5'-^{3}H]AdoCbl had exchanged to H_2O. The time dependent loss of tritium from [5'-^{3}H]AdoCbl could also be measured by washing the column with 3 mL

of 100% CH_3CN subsequent to the removal of the ^3H_2O. Figure 5.3 depicts an exchange reaction in which Sep-pak columns were used to separate ^3H_2O from [5'-^{3}H]AdoCbl. This reaction was allowed to proceed past the extent of reaction used to calculate rates of exchange in order to show that the increase in ^3H_2O is concomitant with the decrease of tritium from [5'-^{3}H]AdoCbl. As expected the rates of these two processes are very similar, with the rate of ^3H_2O production being 0.13 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, and the rate of tritium loss from [5'-^{3}H]AdoCbl being 0.11 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. These experiments show that this rapid method of separating [5'-^{3}H]AdoCbl from ^3H_2O is effective, and as a consequence, it was used to determine the rate constants for the exchange reaction in most of the subsequent experiments described.

Deleterious Effects of DTT on the Exchange Reaction

Hogenkamp *et al.* (1968) previously showed that a variety of thiols are effective in stimulating the exchange reaction. The initial characterization of the exchange reaction reported herein was carried out using DTT as the reductant. During the course of these experiments however, it was observed that DTT catalyzed a time-dependent loss of tritium from [5'-^{3}H]AdoCbl in the absence of RTPR. The rate of this non-enzymatic reaction, as monitored by the Sep-pak method, was dependent upon the concentration of [5'-^{3}H]AdoCbl in the reaction mixture. The cause of this background rate of exchange in the presence of DTT is presently not understood. It is difficult to understand from a chemical standpoint how DTT alone could catalyze tritium exchange. A more reasonable explanation for the observed tritium in the H_2O wash might be that DTT can convert AdoCbl into a species that is not retained on the C_{18} columns. This species would have to be considerably

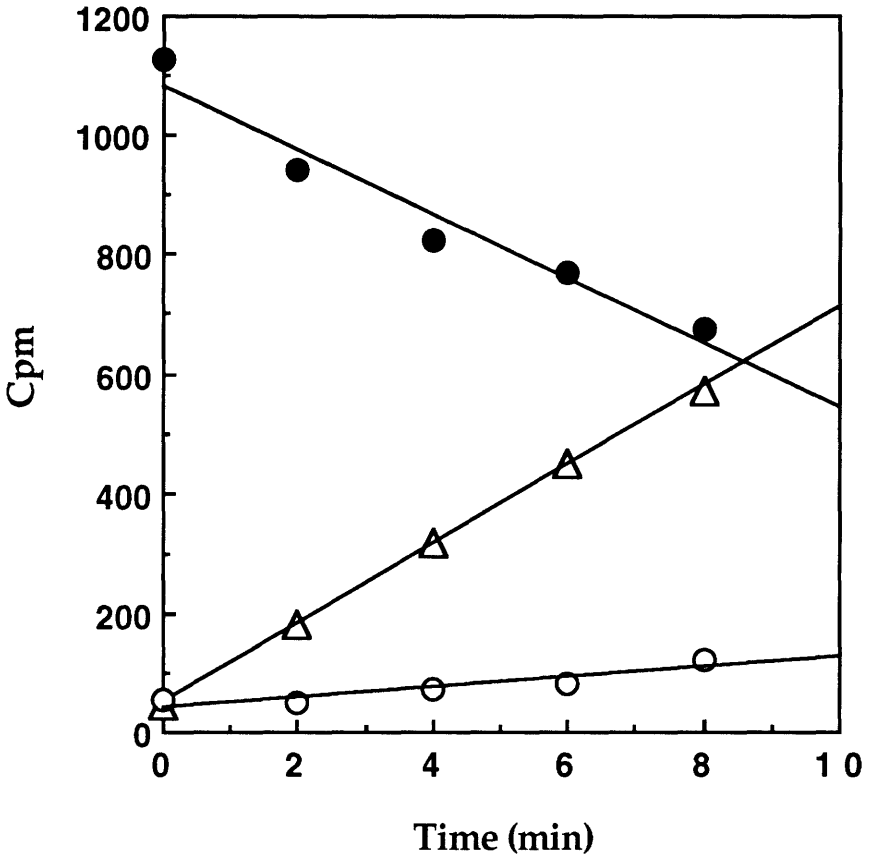


Figure 5.3: Time course for the exchange of tritium from [5'-^{3}H]AdoCbl. The reaction contained 46 μM [5'-^{3}H]AdoCbl (specific activity 9.4x10^5 cpm/μmol), 30 mM DTT, 150 μM dGTP and 0.2 μM RTPR. (●) [5'-^{3}H]AdoCbl. (Δ) ^3H_2O. (O) Background ^3H_2O.

more polar than AdoCbl to be eluted with H_2O. In order to test this hypothesis, exchange reactions were carried out in the presence of 30 mM DTT but in the absence of RTPR, and volatile tritium was quantified using the bulb-to-bulb distillation method. In this case, no significant volatile tritium over background (-30 cpm) was isolated in a period of 40 min. The material eluting from the Sep-pak cartridge with H_2O is therefore not ^3H_2O.

Thiol-mediated decomposition of AdoCbl in the presence of 200 mM 2-mercaptoethanol has been previously reported (Hogenkamp et al., 1987). The pseudo-first-order rate constant for this decomposition at pH 9.5 and 70°C is reported to be 0.14×10^{-4} s^{-1} (Hogenkamp et al., 1987). Studies using ^{13}C NMR identified the breakdown product of AdoCbl as S-adenosylmercaptoethanol. Although the thiol-mediated decomposition of AdoCbl is slow in the presence of 2-mercaptoethanol, it is several-fold faster in the presence of dithiols such as dithioerythritol and dithiothreitol (Schrauzer et al., 1972). The details of the reaction between DTT and AdoCbl to produce a polar compound which elutes with H_2O remains to be elucidated. However, as indicated in Figure 5.4, this reaction is problematic in analyzing any exchange reactions carried out in the presence of DTT. Table 5.3 shows how the background rate of exchange compares to the actual enzyme-dependent rate at various concentrations of [5'-^{3}H]AdoCbl. Because of this uncharacterized reaction, the TR/TRR/NADPH reducing system was substituted for DTT in most of the quantitative experiments reported herein. The concentration of thioredoxin was maintained at 65 μM (the concentration that is saturating for substrate turnover) throughout the characterization of the exchange reaction. The rate constants for exchange at 13 μM and 65 μM TR were identical, indicating that this concentration is also saturating for the exchange reaction.

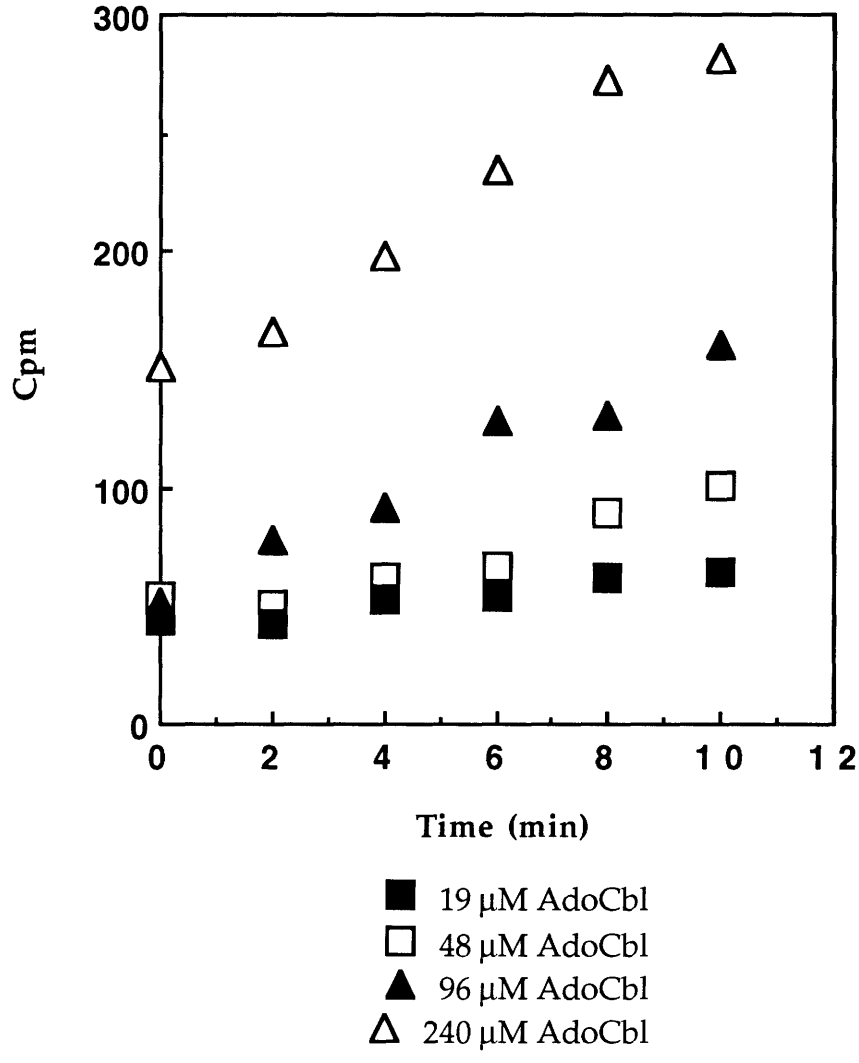


Figure 5.4: The background rate of exchange at varying concentrations of [5'-^{3}H]AdoCbl. Each reaction included 50 mM potassium phosphate buffer, pH 7.5, 0.3 mM dGTP, 30 mM DTT, 4 mM EDTA, and the indicated amounts of [5'-^{3}H]AdoCbl.

Dependence of the Exchange Reaction on the Choice of Buffer

Another aspect of the exchange reaction which deserves comment is the choice of buffers. Initially, exchange studies were carried out in potassium phosphate buffer, pH 7.5; however, the buffer was changed (due to the interference of phosphate in the assay for nucleotide reduction) to HEPES, pH 7.5, since this was the buffer used to characterize substrate turnover with the various C→S mutants (Chapter 4). In general, rates of exchange in potassium phosphate buffer are 1.5-2 times faster than rates of exchange in HEPES. Figure 5.5 depicts the results of exchange reactions carried out in parallel using phosphate and HEPES buffers at identical pHs. From least-

Table 5.3: DTT-dependent background rate of exchange as a function of [5'-^{3}H]AdoCbl concentration.

[Substrate]	Observed Rate (x10^6) [a](μmol•min^{-1})	Background Rate (x10^6) (μmol•min^{-1})	Net Rate (x10^6) (μmol•min^{-1})
9 μM	132	~0	132
19 μM	270	38	232
48 μM	570	81	489
96 μM	885	165	720
240 μM	1440	225	1215

[a]Exchange carried out in potassium phosphate buffer, pH 7.5, containing 150 μM dGTP and 30 mM DTT.
RTPR concentration was 0.2 μM

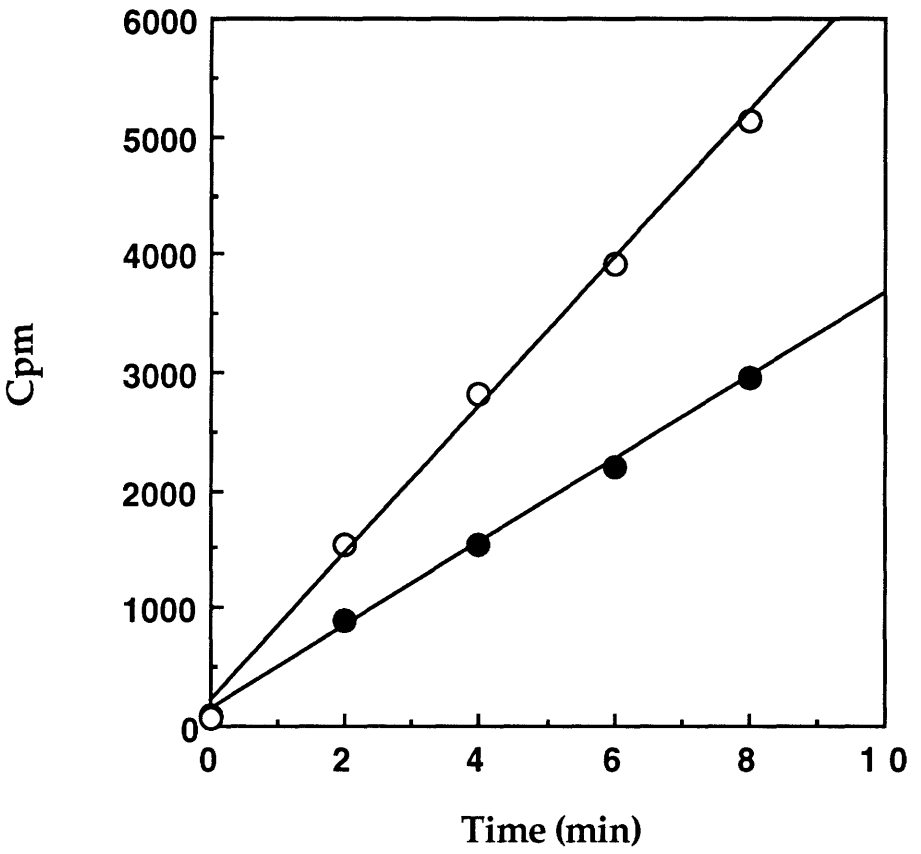


Figure 5.5: Effect of buffer on the exchange reaction. Exchange was carried out in (●) 50 mM HEPES, pH 7.5, or (O) 50 mM potassium phosphate, pH 7.5, in a reaction mixture containing 200 μM [5'-^{3}H]AdoCbl, 300 μM dGTP, 150 nM RTPR, and the TR/TRR/NADPH reducing system.

squares fits of the data, the rate of exchange for wt RTPR in phosphate buffer is calculated to be 0.28 μmol•min^{-1}•mg^{-1} while the rate of exchange in HEPES is calculated to be 0.16 μmol•min^{-1}•mg^{-1}.

Determination of an Apparent K_m for dGTP in the Exchange Reaction

Experiments by Hogenkamp *et al.* (1968) showed a very interesting dependence of the exchange reaction on the presence of nucleotides and deoxynucleotides. Under a defined set of conditions the largest rate constant for the formation of ^3H_2O was observed when RTPR, reductant, and [5'-^{3}H]AdoCbl were incubated with 2 mM dGTP. Interestingly, the addition of any other NTPs or dNTPs to an exchange reaction containing dGTP decreased the amount of exchange observed relative to dGTP alone. The NTP substrates generally conferred a greater degree of inhibition than the dNTP products and allosteric effectors. These results suggested that a unique opportunity was at hand to observe the exchange reaction without the complicating effects of nucleotide reduction. As a first step in characterizing the exchange reaction, we set out to determine the kinetic constants for dGTP and [5'-^{3}H]AdoCbl. Initially, a wide range of dGTP concentrations were investigated in order to determine the range of dGTP concentrations required for determination of its K_m. A dGTP concentration of 150 μM was chosen as a starting point since this concentration affords the maximal rate of turnover of ATP (Chapter 3). As shown in Figure 5.6, higher concentrations of dGTP produced very little change in the rate of exchange. Actual rates under these conditions were 0.11, 0.13, and 0.13 μmol•min^{-1}•mg^{-1}, for 150 μM, 530 μM, and 1 mM dGTP. Assuming that 150 μM dGTP barely saturates the reaction, a K_m determination for dGTP in exchange was therefore carried out at concentrations ranging from 3-119 μM. Figure 5.7 shows the Lineweaver-Burk for the initial rate data as a

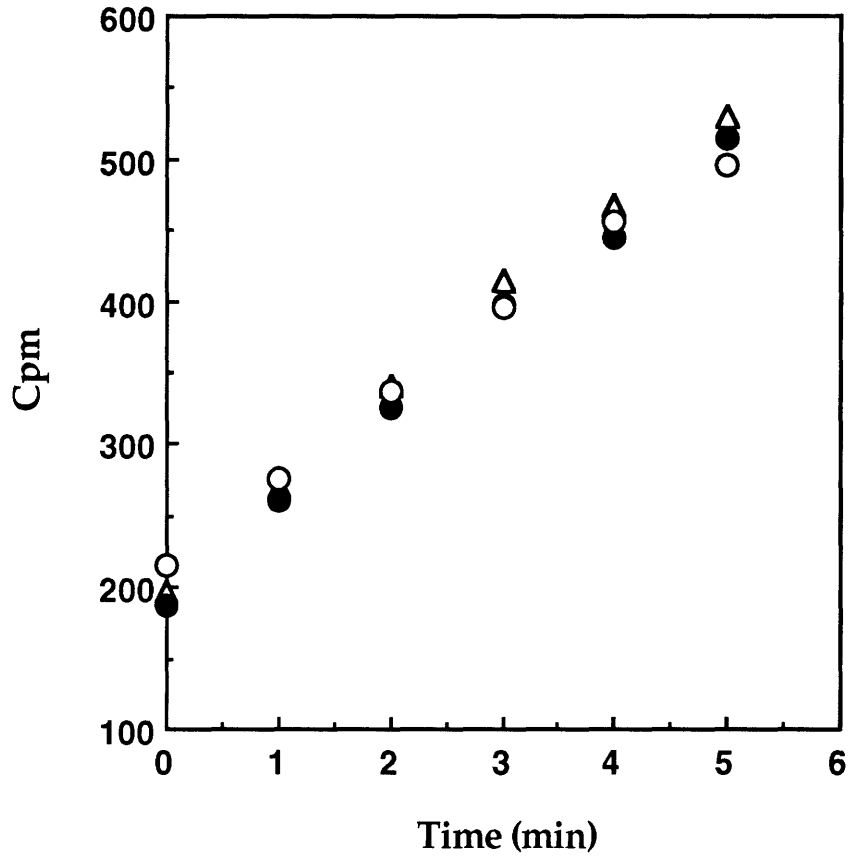


Figure 5.6: Initial rate of exchange as a function of [dGTP]. The exchange reaction was carried out in the presence of 0.2 μM RTPR, 30 mM DTT, 50 μM [5'-^{3}H]AdoCbl (specific activity 9.5x10^{5} cpm/μmol), and (O) 150 μM dGTP, (Δ) 530 μM dGTP, or (●) 1 mM dGTP.

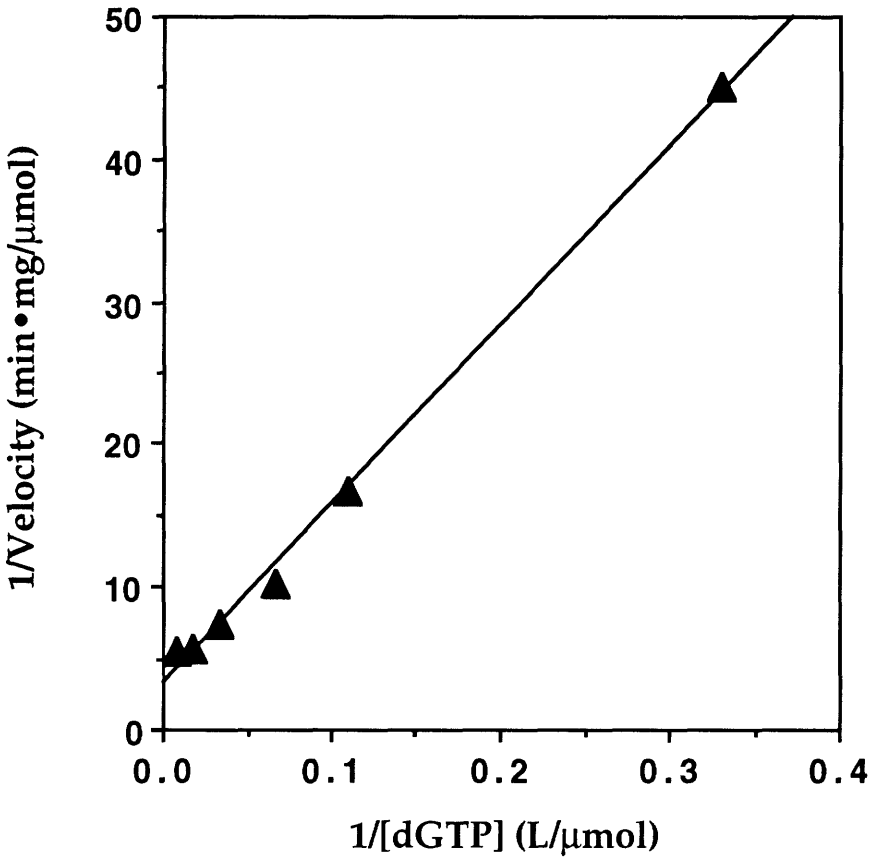


Figure 5.7: Determination of an apparent K_m for dGTP in the presence of the TR/TRR/NADPH reducing system. Initial rates of exchange were obtained with dGTP concentrations of 3 μM, 9 μM, 15 μM, 30 μM, 59 μM, and 119 μM.

function of dGTP concentration. From a fit to the program HYPER, the K_m and V_{max} were determined to be 17.28±3.41 μM and 0.21±0.01 μmol•min^{-1}•mg^{-1}. The K_m for dGTP as determined in this study is not in accordance with that previously obtained by Hogenkamp *et al.* (1968) which was reported to be 100 μM. Whether this discrepancy is buffer dependent (3,3-dimethylglutarate vs potassium phosphate) or whether it is dependent on the concentration of AdoCbl (150 μM AdoCbl was used in the Hogenkamp study), is presently not known. It must be mentioned however, that the rates reported by Hogenkamp *et al.* were calculated from single fixed time points rather than time courses, and thus are unreliable for determining actual kinetic parameters. It is important to re-emphasize that the K_m value determined herein, as well as the K_m value determined by Hogenkamp *et al.* (1968) are apparent K_ms, as the concentration of [5'-^{3}H]AdoCbl is not saturating.

Determination of a K_m for AdoCbl in the Exchange Reaction

The exchange reaction was also characterized with respect to the Michaelis-Menton constant for AdoCbl. Initial determinations were carried out in the presence of 30 mM DTT and suggested that the K_m for AdoCbl was between 70 and 150 μM. The K_m value was determined using the TR/TRR/NADPH reducing system as reductant. Using the values from the previous determinations with DTT as reductant as a guide, concentrations of [5'-^{3}H]AdoCbl were varied from 21-427 μM. The kinetic constants K_m and V_{max} were obtained from a fit to HYPER and determined to be 60±19 μM and 0.31±0.03 μmol•min^{-1}•mg^{-1}. A Lineweaver-Burk plot of the data is shown in Figure 5.8.

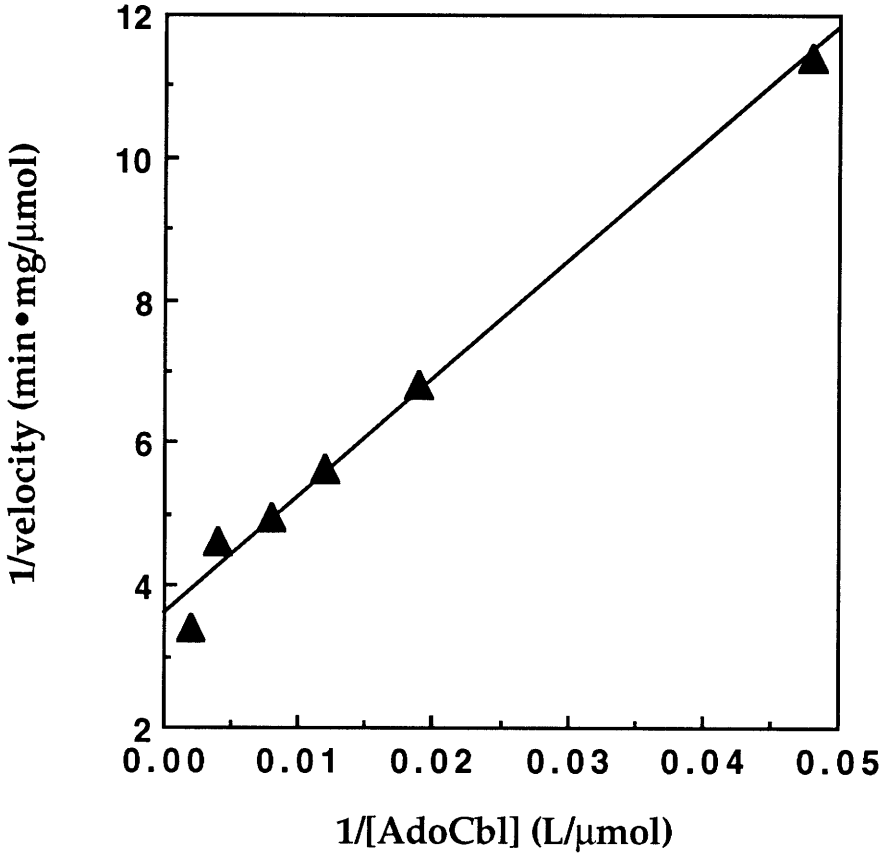


Figure 5.8: Determination of a K_m for AdoCbl using the TR/TRR/NADPH reducing system. [5'-^{3}H]AdoCbl concentrations were varied from 21 μM to 427 μM, and ^3H_2O was isolated by the Sep-pak method as described in the methods section.

Analysis of the Ability of NaOAc to Substitute for dGTP in the Exchange Reaction

As already mentioned in Chapter 4, RTPR possesses a very unique system of allosteric regulation. Specific dNTPs can stimulate the reduction of specific NTP substrates (Beck et al., 1966b). In the presence of high concentrations of certain salts (e.g. NaOAc), the enzyme can turnover all substrates at maximal rates without the allosteric effectors, although the K_m for each substrate as well as the K_m for AdoCbl is increased (Jacobsen & Huennekens, 1969; Blakley, 1978). Since substrate is not needed to effect exchange, the question arises as to whether the allosteric effector can be replaced by NaOAc (as in substrate reduction), thereby circumventing any need for a nucleotide in this reaction. Preliminary experiments carried out in the presence of DTT suggested that the rate of exchange with NaOAc was very slow, and too close to an artifactual background rate that DTT affords. Efforts were therefore made to increase the sensitivity of the assay by using [5'-^{3}H]AdoCbl of high specific activity, higher concentrations of RTPR (9.4 μM), and conducting the assay in the presence of the TR/TRR/NADPH reducing system. The results of this experiment are displayed in Table 5.4. Although the radioactivity in ^3H_2O increases in the first 6 min of the reaction, the low level of ^3H_2O observed, coupled with the 8 min time point being lower than the 6 min time point, suggests that substantial error is associated with this experiment. A least-squares fit of the data however, results in a specific activity of 0.19×10^{-3} cpm•μmol^{-1}•min^{-1}, which is approximately a factor of 1000 lower than that of wt-RTPR in the presence of dGTP. This experiment was repeated, in an attempt to show that the rate of exchange is dependent upon the concentration of RTPR. Again, as shown in Table 5.5, exchange appears to occur. However, it also appears that the

Table 5.4: [a]Time course for exchange in a reaction in which 1 M NaOAc is substituted for dGTP.

Time (min)	Radioactivity (cpm) in H_2O
0	142
2	240
4	242
6	394
8	339

[a]Exchange was carried out in potassium phosphate buffer, pH 7.5, containing 200 μM [5'-^{3}H]AdoCbl ($1.1x10^7$ cpm•μmol^{-1}), 9.4 μM RTPR, and the TR/TRR/NADPH reducing system.

Table 5.5: [a]Effort to show enzyme dependence on an exchange reaction in which 1 M NaOAc is substituted for dGTP.

	Radioactivity (cpm) in H_2O		
Time (min)	9 μM RTPR	18 μM RTPR	27 μM RTPR
0	139	160	130
5	748	1191	1131
10	963	1274	1286
20	1181	1210	1186

[a]The conditions are as described above in Table 5.3 unless otherwise specified.

enzyme is inactivated during the process, with most of the inactivation occurring within the first time point. The number of equivalents of ^3H_2O released in the first 5 min per equivalent of RTPR is 0.19 for the reaction containing 18 μM RTPR, and 0.13 for the reaction containing 27 μM RTPR. For the reaction containing 9 μM RTPR, an estimate of the rate of exchange was calculated using the 5 min time point. The rate of 0.52×10^{-3} μmol•min^{-1}•mg^{-1} is in fair agreement with the rate calculated from the previous experiment (0.19×10^{-3} cpm•μmol^{-1}•mg^{-1}).

Characterization of the Reductant Dependency on the Exchange Reaction

Previous studies of Hogenkamp *et al.* (1968) suggested that thiols are required for the exchange of tritium from [5'-^{3}H]AdoCbl (Table 5.6). The experiments were carried out using a fixed time point assay under arbitrarily determined sets of conditions. These results can serve as a starting point to ask two questions: firstly, is reductant required, and secondly, if it is required, what is its function? The studies of Hogenkamp *et al.* (1968) suggested that the role of the reductant was catalytic in nature. This was demonstrated by showing that a 100 μM solution of dihydrolipoate could catalyze the washout of all of the tritium from 470 μM [5'-^{3}H]AdoCbl in 2 h at 37°C under anaerobic conditions. DTT catalyzes the break down of [5'-^{3}H]AdoCbl in an as yet uncharacterized reaction, and dihydrolipoate would be expected to effect a similar transformation. We have therefore re-addressed the question of the catalytic nature of the reductant in the exchange reaction using the TR/TRR/NADPH reducing system. In a reaction containing 200 μM NADPH, 150 μM dGTP, and 100 μM AdoCbl, there is no change in the rate of oxidation of NADPH upon the addition of RTPR to a final concentration of 1 μM (data not shown). A conservative limit of detection of NADPH oxidation in this

Table 5.6: [a]Effect of various thiols on the transfer of tritium from [5'-^{3}H]AdoCbl to H_2O. Table adapted from Hogenkamp *et al.* (1968).

Thiol Added	Radioactivity in ^3H_2O (cpm)	Specific Activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)
None	30	0
Dihydrolipoic acid	12,200	0.24
2-mercaptoethanol	2520	0.049
Cysteine	2410	0.047
Glutathione	160	0.002
Dithiothreitol	12,670	0.25
Dithioerythritol	11,675	0.23
Dihydrolipoate + lipoate	5355	0.10
TR/TRR/NADPH	5595	0.11

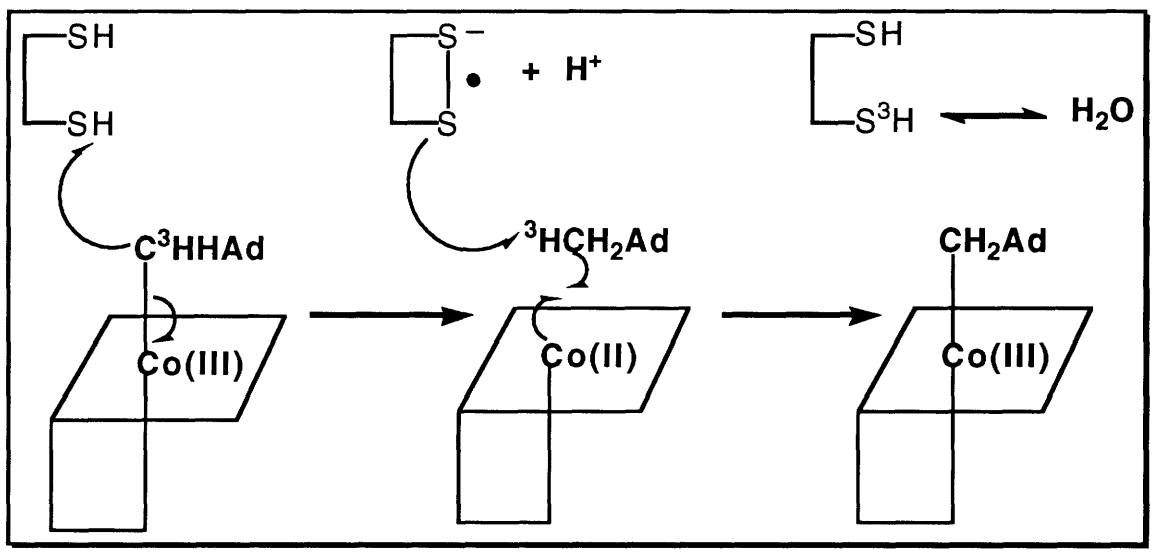
[a]The concentration of all thiols was 30 mM. Thioredoxin isolated from *E. coli* was present at 1.64 μM, dGTP at 2 mM, RTPR at 5.4 μg, and [5'-^{3}H]AdoCbl (630,000 $cpm \cdot \mu mol^{-1}$) at 150 μM.

experiment is 0.06 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, whereas the rate of tritium exchange is ~0.2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ under these conditions. The rate of exchange of hydrogen would be expected to be 10-20 times faster given a typical tritium selection effect. Therefore if reductant were necessary for each exchange event, the rate of oxidation of NADPH might be expected to be 2-4 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. These results support the proposal that the reductant acts in an "allosteric" capacity.

The allosteric nature of the reductant in the exchange reaction begs the question as to its actual function. One conceivable scenario is that the

reductant is directly involved in this process - whether it be TR or DTT. Subsequent to cleavage of the Co-carbon bond of AdoCbl, a hydrogen atom from one of the two cysteines on TR or one of the two sulfhydryls of DTT is transferred to the 5'-deoxyadenosyl radical to afford 5'-deoxyadenosine and a disulfide-radical anion. This disulfide-radical anion is then left with the painstaking task of abstracting a hydrogen atom from 5'-deoxyadenosine to form the 5'-deoxyadenosyl radical, which subsequently recombines with $B_{12}r$ (Scheme 5.2). Alternatively, the data also support the model outlined in Figure 5.2, in which reductant would play an allosteric role in which either

Scheme 5.2: A model for exchange which suggests that it might be mediated through the reductant.



the binding of AdoCbl to RTPR or the cleavage of the Co-carbon bond of AdoCbl is accelerated when the redox active cysteines are in the reduced state. To distinguish between these two models, exchange studies were carried out in the absence of reductant, with enzyme that had been pre-reduced. As

described in detail in the methods, pre-reduction of wt RTPR involves incubation with DTT, followed by its removal from the enzyme by gel filtration. At the end of the exchange reaction, a control reaction, in which the pre-reduced enzyme was treated with [2-^{14}C]CTP, indicated that 1.5 dCTPs were produced. This number is identical to that which was previously determined in Chapter 4 for wt RTPR, and is consistent with the enzyme being maintained in the reduced form during the course of the reaction. Figure 5.9 shows the time courses for exchange under several conditions with pre-reduced wt RTPR. From these time courses, rates of exchange were calculated, and are presented in Table 5.7. The rate of exchange for pre-reduced enzyme is 0.078 $\mu mol \bullet min^{-1} \bullet mg^{-1}$. This number is approximately a factor of 2 lower than the rate of exchange in the presence of the TR/TRR/NADPH reducing system, which is 0.14 $\mu mol \bullet min^{-1} \bullet mg^{-1}$ under similar conditions. We postulated that the dependence of the reducing system on the rate of exchange might be due to the binding of reductant

Table 5.7: Characterization of the ability of pre-reduced RTPR to catalyze the exchange reaction under several conditions.

[a]Exchange Conditions	Rate ($\mu mol \bullet min^{-1} \bullet mg^{-1}$)
Pre-reduced	0.078
Pre-reduced TR (no TRR/NADPH)	0.03
Pre-reduced TR Mutant	0.081

[a]Exchange carried out in 50 mM HEPES pH 7.5, 200 μM [5'-^{3}H]AdoCbl, and 300 μM dGTP.

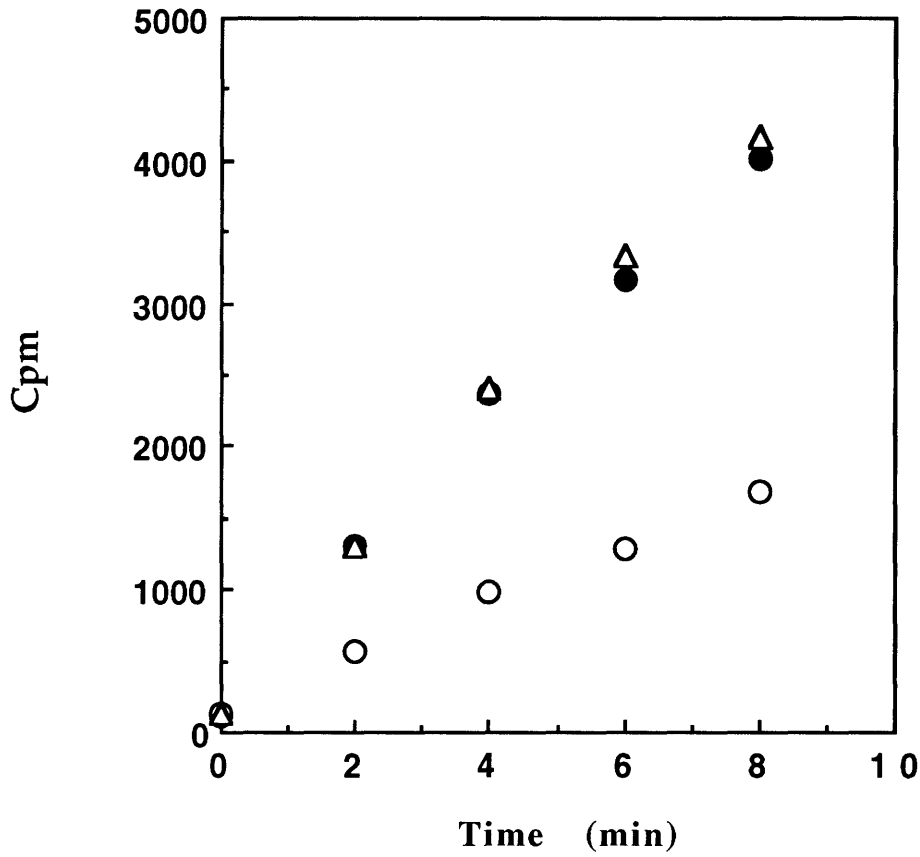


Figure 5.9: Exchange of tritium from [5'-^{3}H]AdoCbl with pre-reduced wt RTPR. Exchange was carried out with 200 μM [5'-^{3}H]AdoCbl ($1x10^7$ cpm•μmol^{-1}), 50 mM HEPES, pH 7.5, and 300 μM dGTP (O) in the presence of TR,, (Δ) in the presence of the mutant TR C32S, and (●) in the absence of any added reductant.

and its action as an allosteric effector. This hypothesis was investigated using a mutant of TR in which one of the two redox-active cysteines is replaced by a serine. This mutation destroys the ability of TR to act as a reductant during substrate turnover, although its 3° structure is maintained, and presumably its ability to act as an allosteric effector as well. However, when the exchange reaction is performed with pre-reduced RTPR in the presence of the mutant TR, no significant enhancement in the rate of ^3H_2O production is observed (Table 5.7).

An unexpected result which is also shown in Table 5.7, is that the exchange reaction is inhibited when performed in the presence of wt TR without the accompanying TRR and NADPH. This same phenomenon was reproduced using a much lower concentration of [5'-^{3}H]AdoCbl (22 μM, specific activity $6.6x10^6$ cpm•μmol^{-1}) in a reaction containing TR and TRR, but no NADPH (Table 5.8) (Figure 5.10). These results suggest that if TR is acting as an allosteric effector, then it is the reduced form that is essential for this effect to occur, and that the C32S TR does not function as a good model for the reduced wt TR.

These experiments show that reductant is clearly not essential for the exchange reaction, thus ruling out the model shown in Scheme 5.2. However, reduced wt RTPR is not sufficient for maximal exchange, and TR plays a role which is over and above keeping RTPR in the reduced state.

Characterization of the Ability of Mutant C731&736S RTPR to Catalyze the Exchange Reaction

An extension of the dependency of the exchange reaction on reductant was also carried out with mutant C731&736S RTPR. A detailed characterization of this mutant (Chapter 4), showed that the function of these

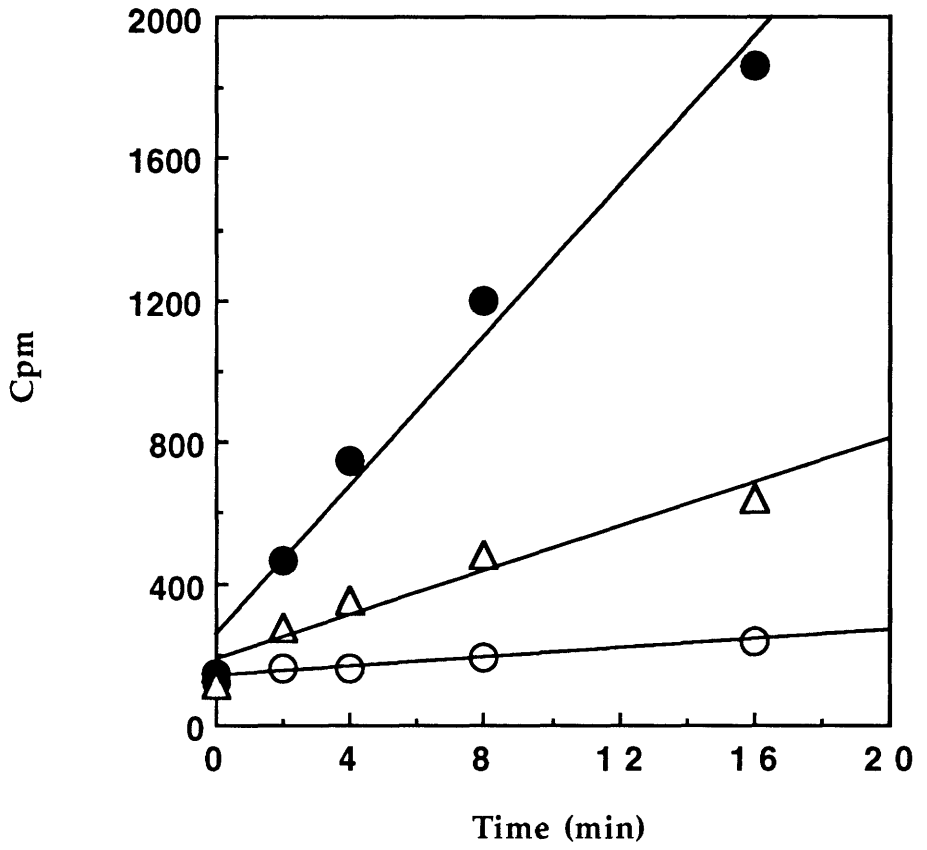


Figure 5.10: Re-characterization of the ability of TR to inhibit the exchange reaction. The reaction was carried out with 0.2 μM pre-reduced RTPR, 22 μM [5'-^{3}H]AdoCbl (6.6×10^{6} cpm•μmol^{-1}), and 150 μM dGTP in the (●), presence of TR/TRR/NADPH, (○) presence of TR/TRR, and (Δ) with no added reductant.

two C-terminal cysteines is to transfer reducing equivalents from TR into the active-site disulfide of RTPR so that multiple turnovers can be achieved.

Table 5.8: Re-characterization of the ability of TR to inhibit the exchange reaction in the absence of NADPH.

[a]Exchange Conditions	Rate ($\mu mol \bullet min^{-1} \bullet mg^{-1}$)
Pre-reduced	0.006
Pre-reduced TR/TRR (no NADPH)	0.001
Pre-reduced TR/TRR/NADPH	0.02

[a]Exchange carried out in 50 mM potassium phosphate buffer, pH 7.5, 22 μM [5'-^{3}H]AdoCbl, and 150 μM dGTP.

Based on the previous studies with the pre-reduced wt RTPR in conjunction with our model for the exchange reaction, we anticipated that pre-reduced C731&736S RTPR would catalyze exchange at the same rate as the pre-reduced wt RTPR. Figure 5.11 displays the time course for the exchange reaction catalyzed by the pre-reduced mutant C731&736S RTPR. The results of this experiment are summarized in Table 5.9. As predicted, C731&736S RTPR shows the expected behavior. The inability of the TR/TRR/NADPH reducing system to increase the rate constant for exchange to that of wt RTPR suggests that either this RTPR mutant is not able to bind to TR, or that the enhancement of exchange by the TR/TRR/NADPH reducing system reflects the thiol/disulfide equilibrium on RTPR, with NADPH being able to shift it to the all-reduced state via TR and TRR. The specific activity of the mutant in the absence of reductant is 0.18 $\mu mol \bullet min^{-1} \bullet mg^{-1}$, while in the presence of

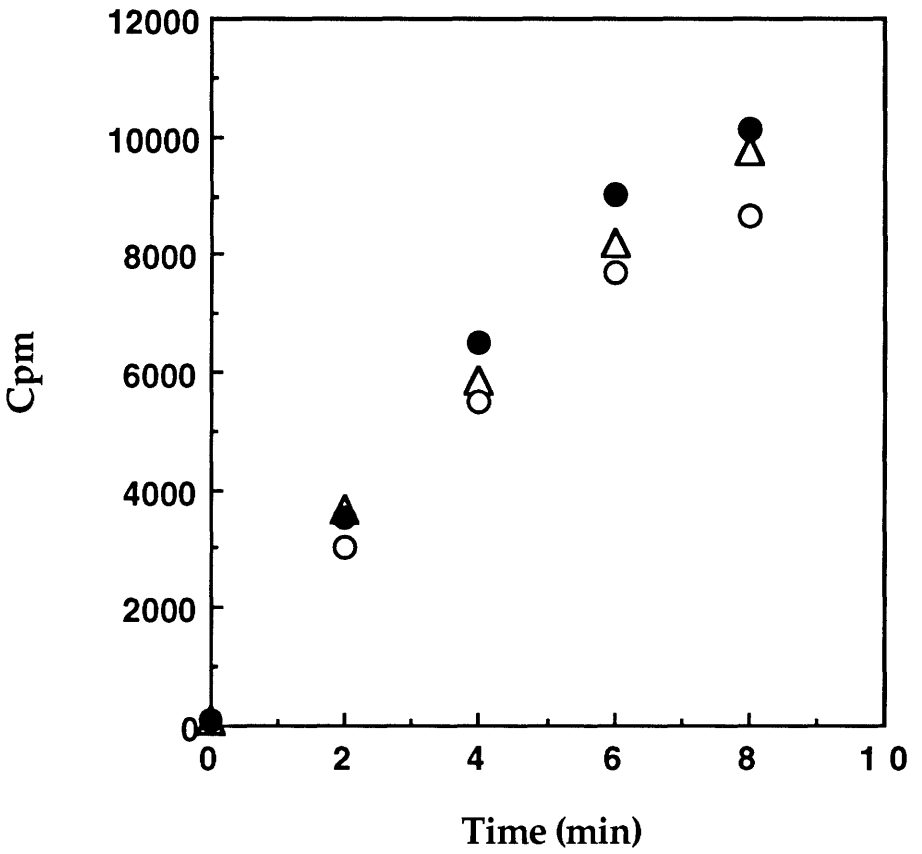


Figure 5.11: Exchange studies carried out with pre-reduced mutant C731&736S RTPR. Exchange reactions were performed in the (O) presence of the TR/TRR/NADPH reducing system, (●) presence of C32S TR/TRR/NADPH, and (Δ) absence of any added reductant.

TR mutant/TRR/NADPH it is 0.19 μmol•min^{-1}•mg^{-1}. In the presence of wt TR/TRR/NADPH, the rate is slightly lower (0.16 μmol•min^{-1}•mg^{-1}). A control reaction was run under single turnover conditions upon completion of the exchange reactions, and showed the production of 0.7 equiv of dCTP - the quantity previously shown to be produced under single-turnover conditions with mutant C731&736S-RTPR (Chapter 4). At first glance, these rates appear to be higher than the rates for the pre-reduced wt-RTPR.

Table 5.9: Characterization of mutant C731&736S RTPR with respect to the exchange reaction under pre-reduced conditions.

[a]Exchange Conditions	Rate (μmol•min^{-1}•mg^{-1})
Pre-reduced	0.18
Pre-reduced + TR/TRR/NADPH	0.16
Pre-reduced + TR mutant/TRR/NADPH	0.19

[a]Exchange carried out in 50 mM potassium phosphate buffer, pH 7.5, with 200 μM [5'-^{3}H]AdoCbl, and 300 μM dGTP.

However, the characterization of this mutant was conducted in 50 mM potassium phosphate buffer, pH 7.5. As described earlier, rates in this buffer tend to be 1.5-2 times faster than rates in 50 mM HEPES, pH 7.5, under identical conditions.

Characterization of the Ability of the Active-Site C→S Mutants to Catalyze the Exchange Reaction

Chapter 4 presents a detailed analysis of the roles that five cysteines in RTPR play during catalysis, which has allowed these residues to be classified into three categories. The first category, which has already been touched upon above, comprises cysteines 731 and 736. These cysteines are presumed to be on the surface of RTPR, and function to transfer reducing equivalents from TR into the active site disulfide. The next two categories contain cysteines that are in the active site and which play a more intimate role in nucleotide reduction. Cysteines 119 and 419 transfer reducing equivalents directly to the substrate, and are the residues which form the disulfide bond that is reduced by cysteines 731 and 736. Cysteine 408 is postulated to be the amino acid residue that is converted into a protein radical, and which initiates catalysis by abstracting the 3' hydrogen of the substrate. Our model, which is outlined in Figure 5.2, proposes that C408 is essential for nucleotide reduction as well as exchange. In the event that this amino acid is altered, our model predicts that the exchange reaction would be abolished. As described in Chapter 4, our initial characterization of these mutants revealed that wt RTPR was present in each of our mutant preparations. This contamination was also reflected in the exchange reaction for C408S, as a rate which was 0.33% that of wt RTPR was observed. Upon removal of the contamination, the rate of exchange for C408S was indistinguishable from background. In order to set a good lower limit for mutant C408S RTPR-catalyzed exchange, 240 μg of mutant was incubated for 30 min with 200 μM [5'-^{3}H]AdoCbl (7.5×10^5 cpm•μmol^{-1}) in HEPES, pH 7.5, and the ^3H_2O was isolated by bulb-to-bulb distillation. The primary data for this experiment are displayed in Table 5.10. Very little ^3H_2O is observed in the case of mutant C408S RTPR. The few counts above background that are

detected are within the error of this technique, which is ± 100 cpm with the amount of radioactivity employed. The limit of detection in this particular experiment is $0.9x10^{-5}$ μmol•min^{-1}•mg^{-1}, which is 0.006% the rate of wt-RTPR when assayed under identical conditions (Table 5.11). Our model in its simplest form also predicts that mutant C119S and C419S-RTPRs should have

Table 5.10: [a]Characterization of the ability of mutant C408S to catalyze the exchange reaction.

RTPR	Concentration	Background (Cpms)	Actual (Cpms)	Incubation Time
C408S	28 μM	223	281.6	30 min
Wild-type	0.28 μM	160	5892	10 min

[a]In addition to the amounts of protein indicated in Table 5.10, the exchange reaction included 200 μM [5'-^{3}H]AdoCbl ($7.5x10^5$ cpm•μmol^{-1}), the TR/TRR/NADPH reducing system, 300 μM dGTP, and 50 mM HEPES, pH 7.5.

Table 5.11: Characterization of the ability of the active site RTPR mutants to catalyze the exchange reaction.

Mutant RTPR	Rate (μmol•min^{-1}•mg^{-1})	% Wild-type RTPR
[a]C408S-RTPR	$0.9x10^{-5}$	<0.006
[b]C119S-RTPR	0.002	1.3
[b]C419S-RTPR	0.001	0.7

[a]See Table 5.10 for experimental conditions.

[b]The experimental conditions are as described in Table 5.10 except that [5'-^{3}H]AdoCbl, $6.9x10^6$ cpm•μmol^{-1} was used. Concentrations of C119S and C419S-RTPRs are 12 μM and 23 μM.

no effect on the exchange reaction. Unexpectedly however, C119S and C419S RTPRs catalyze rates of exchange that are 1.3 and 0.7% the rate of wt RTPR in the presence of the TR/TRR/NADPH reducing system. The primary data for the characterization of mutant C119S and C419S-RTPRs are listed in Table 5.12. Plots of the appearance of ^3H_2O vs time, as well as the loss of radioactivity from [5'-^{3}H]AdoCbl vs time were constructed. Rates of exchange for each

Table 5.12: [a]Primary data for the characterization of mutants C119S and C419S RTPRs.

	Mutant C119S-RTPR		Mutant C419S-RTPR	
Time (min)	(Cpm) ^3H_2O	(Cpm) [5'-^{3}H]AdoCbl	(Cpm) ^3H_2O	(Cpm) [5'-^{3}H]AdoCbl
0	481	129647	1664	128509
10	21348	118819	23609	118205
20	35698	103372	33283	94342

[a]The experimental conditions are described in Table 5.10

mutant were calculated as an average of least-squares fits for both of these processes, resulting in rates of 0.002 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for mutant C119S RTPR, and 0.001 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for mutant C419S RTPR.

Characterization of the Ability of Oxidized RTPR to Catalyze the Exchange Reaction

The results reported for exchange with mutants C119S and C419S RTPR are interesting, and suggest in combination with our model, that perhaps

active site conformation - which might be mediated through these cysteines - is important in the exchange process. In addition the results reported for pre-reduced wt RTPR as well as pre-reduced mutant C731&736S RTPR, suggest that RTPR in the completely reduced state is required to observe maximal exchange rates. These results would predict that oxidized RTPR would have a limited capacity to catalyze this exchange reaction. This prediction was investigated by allowing both pre-reduced wt RTPR and pre-reduced mutant C731&736S RTPR to undergo a single turnover, and then separating the oxidized proteins from the other constituents of the reaction by gel filtration. The pre-oxidized proteins were then concentrated by ultrafiltration, and subsequently analyzed for their ability to catalyze exchange. The results of this experiment are shown in Figures 5.12 and 5.13. From least-squares fits of the plots, rates of exchange of 0.006 and 0.008 $\mu mol \bullet min^{-1} \bullet mg^{-1}$ were calculated for pre-oxidized RTPR in the absence and presence of the TR mutant (Table 5.13). This rate is a factor of 10 lower than the rate of exchange for pre-reduced RTPR, and a factor of 27 lower than the rate of exchange in the presence of reductant. The same trend is observed for the pre-oxidized mutant protein (Table 5.13). The rates of exchange for the pre-oxidized mutant proteins are ~15 times lower than the rates for the pre-reduced C731&736S RTPR. Whether the difference in rates between the pre-oxidized mutant and the pre-oxidized wt proteins is significant is not presently known. Again, this difference may reflect the fact that the mutant protein was characterized in potassium phosphate buffer, while the wt protein was characterized in HEPES.

The low level of exchange that is observed in the presence of pre-oxidized enzyme suggests an explanation for the inhibition of exchange of pre-reduced wt-RTPR when TR is present without TRR and NADPH. TR is isolated in the absence of reductant and has been shown to be fully oxidized to

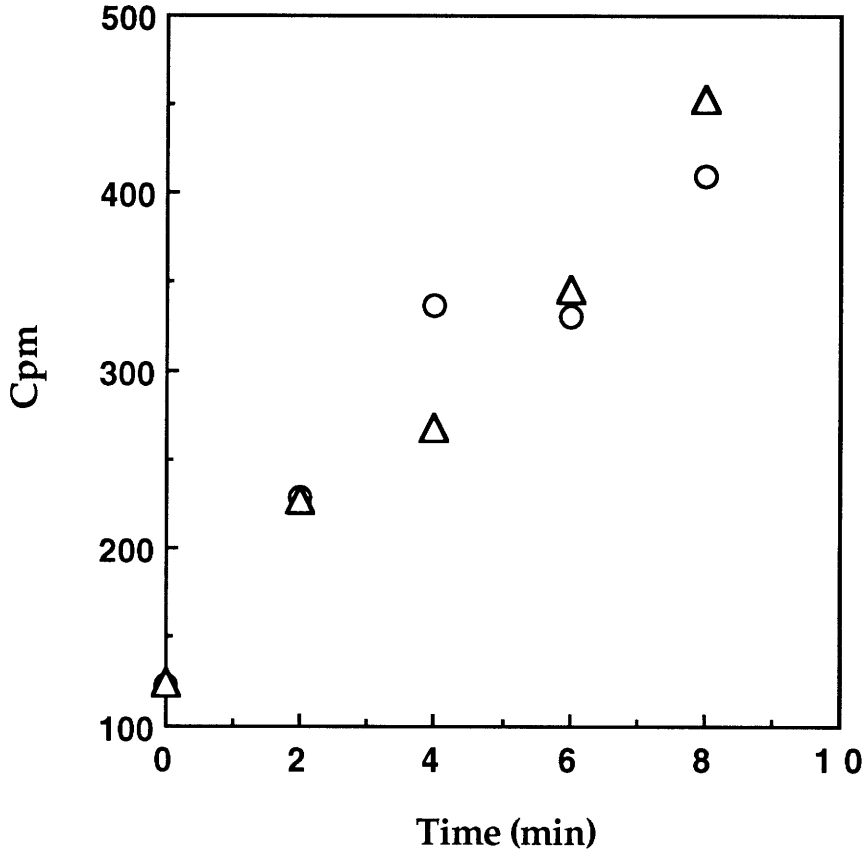


Figure 5.12: Characterization of exchange using pre-oxidized wt RTPR. Exchange was carried out in 50 mM HEPES, pH 7.5, containing 200 μM [5'-^{3}H]AdoCbl 1x10^{7} cpm•μmol^{-1}), and 300 μM dGTP, (Δ) in the presence of C32S-TR, and (O) in the absence of C32S-TR.

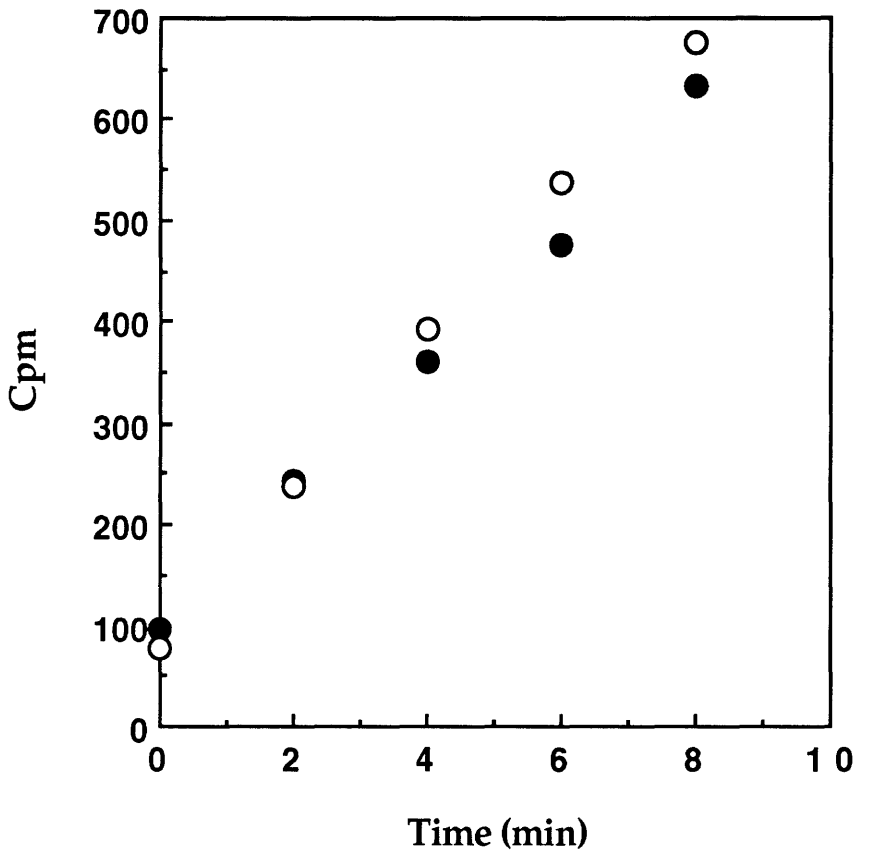


Figure 5.13 Exchange studies with pre-oxidized mutant C731&736S RTPR. Reactions were performed in 50 mM potassium phosphate buffer, pH 7.5, containing 200 μM [5'-^{3}H]AdoCbl ($1x10^{7}$ cpm•μmol^{-1}) in the (●) absence of reductant or (O) presence of the TR/TRR/NADPH reducing system.

the disulfide under these conditions (Laurent et al., 1964). When this oxidized TR is added to the exchange reaction, it is conceivable that it can oxidize RTPR via disulfide interchange with the C-terminal cysteines. As shown above, the

Table 5.13: Characterization of the ability of pre-oxidized wt RTPR and pre-oxidized mutant C731&736S RTPR to catalyze the exchange reaction.

[a]Exchange Conditions	Rate ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)
Pre-oxidized (wt)	0.006
Pre-oxidized (wt)+ TR mutant	0.008
Pre-oxidized (C731&736S)	0.011
Pre-oxidized (C731&736S) + TR/TRR/NADPH	0.013

[a]Wt-RTPR was characterized in 50 mM potassium phosphate buffer, pH 7.5, while mutant C731&736S-RTPR was characterized in 50 mM HEPES, pH 7.5. The other components of the reaction mixtures were identical, and included 200 μM [5'-^{3}H]AdoCbl (1x107 cpm•μmol), and 300 μM dGTP.

rate of exchange of fully oxidized RTPR is ~10-fold lower than when RTPR is fully pre-reduced. The observed 2-3-fold lower rate with TR may reflect the amount of RTPR that was oxidized by TR at the beginning of the assay. Where the equilibrium lies in the absence of electrons from the TR/TRR/NADPH reducing system is presently unknown. These results however provide strong evidence that although reductant is not needed, maximal exchange occurs in the presence of TR/TRR/NADPH with wt RTPR. In addition, subtle modifications within the active site - even those which might mimic pre-reduced RTPR - reduce the level of RTPR-catalyzed exchange.

Discussion

The reduction of ribonucleotides to 2'-deoxyribonucleotides is a very complex process. Essential to this reduction is the cleavage of the 3' C-H bond of the substrate in a reaction that is postulated to be mediated by a thiyl radical (X•)(Stubbe, 1990a). In the RDPR from *E. coli*, this thiyl radical has been proposed to be generated by long range electron transfer from C439 on the R1 subunit to Y122 on the R2 subunit (Mao et al., 1992b; Mao et al., 1992c; Nordlund & Eklund, 1993). Recent crystallographic studies by Uhlin and Eklund suggest however, that the generation of a protein radical on R1 may involve both electron transfer as well as hydrogen atom transfer, or perhaps a coupled e^-/H^+ transfer (Uhlin & Eklund, 1994). In the RTPR from *L. leichmannii*, the thiyl radical is proposed to be generated via the abstraction of a hydrogen atom from C408 by the 5'-deoxyadenosyl radical which is generated upon homolysis of the Co-carbon bond of AdoCbl. In an ongoing effort to elucidate the detailed mechanism of ribonucleotide reduction, as well as the more general question of how enzymes generate and utilize protein radicals to carry out difficult reactions with amazing stereospecificity, we have recently focused much attention on the generation of X• by AdoCbl. We have chosen the exchange reaction as one avenue for addressing this issue. Our model proposes that the exchange reaction is intimately connected to the reversible formation of X•, and is therefore an integral part of the pathway leading to substrate reduction.

In our initial efforts to characterize the exchange reaction, dGTP was chosen to satisfy the (deoxy)nucleotide requirement, and a K_m value of 17 μM was subsequently determined for this deoxynucleotide. The advantages afforded by this deoxynucleotide are (1) it promotes the greatest rate of

exchange of all (deoxy)nucleotides tested, and (2) because it is not a substrate for the enzyme, the kinetic scheme for the exchange reaction is much less complex. One interesting observation that can be gleaned from the work of Hogenkamp *et al.* (1968) is that the effect of (deoxy)nucleotides on the exchange reaction closely parallels their effect on the amount of cob(II)alamin detected at steady state by UV-vis stopped-flow spectroscopy (Tamao & Blakley, 1973). As summarized in Table 5.14, the rate of exchange follows the following pattern (dGTP>GTP>ATP>CTP>ITP>UTP), which with the exception of ITP,

Table 5.14: Similarity of the nucleotide requirement for exchange and the rapid production of cob(II)alamin.

(deoxy)nucleotide	[a]Radioactivity of H_2O (cpm)	[b]ΔA at 525 nm
dGTP	11665	0.07
GTP	5880	0.03
ATP	3545	0.025
CTP	1968	0.018
ITP	5455	0.018
UTP	1278	0.012

[a]Hogenkamp *et al.* (1968). Assay included 30 mM dihydrolipoic acid, 2 mM each (deoxy)nucleotide, 0.15 mM [5'-^{3}H]AdoCbl (630,000 cpm•μmol^{-1}), and 5.4 μg of RTPR. Single time points of the radioactivity released to H_2O in a 15 min incubation at 37°C.

[b]Tamao and Blakley (1973). Assay included 1 mM each (deoxy)nucleotide, 11.3 μM RTPR, 25 mM dihydrolipoic acid, and 100 μM AdoCbl. Cob(II)alamin production at steady state was measured as the decrease in absorbance of AdoCbl at 525 nm.

parallels the amount of cob(II)alamin observed at steady state. These observations are consistent with the proposal that exchange occurs via the

intermediate produced upon homolysis of the Co-carbon bond of AdoCbl, and that the rate of exchange is dependent upon the concentration of this intermediate.

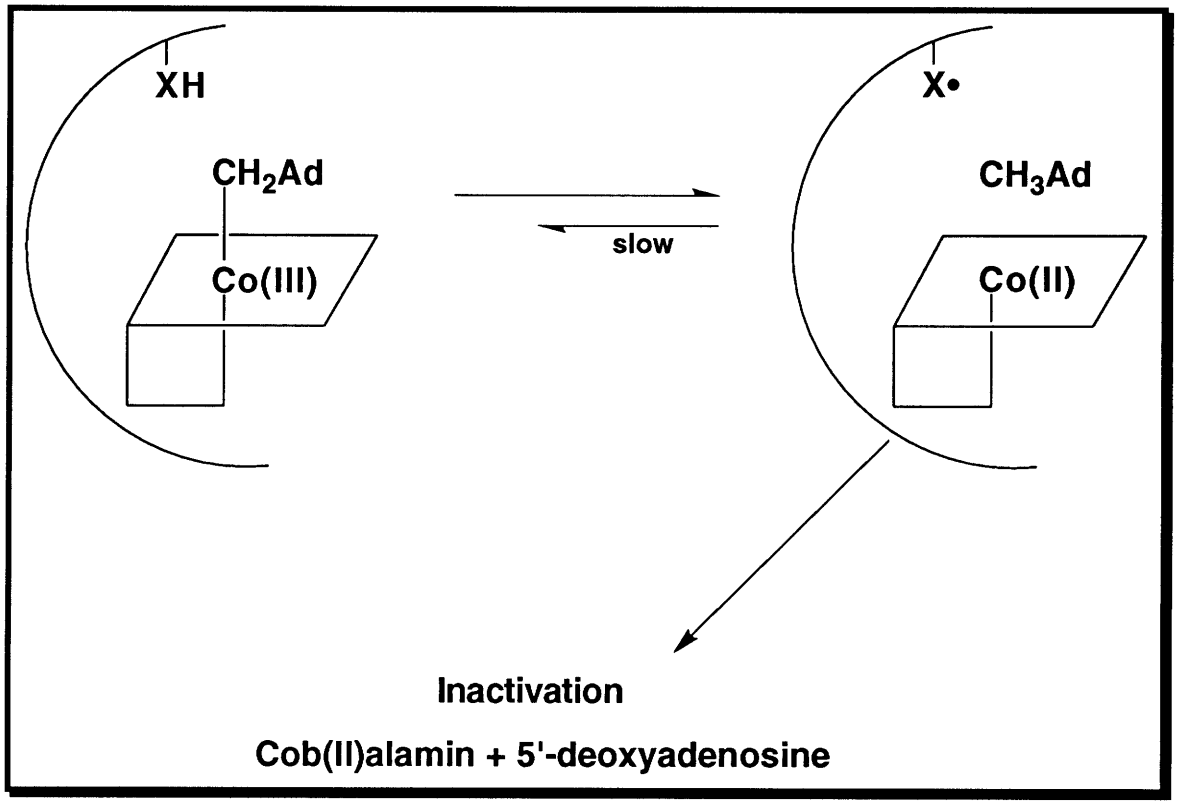
Experiments carried out with NaOAc suggest that the (deoxy)nucleotide requirement for exchange is fairly stringent. In the absence of (deoxy)nucleotide and in the presence of NaOAc, the rate of exchange is extremely slow ($0.2\text{-}0.5 \times 10^{-3}$ $\mu mol \bullet min^{-1} \bullet mg^{-1}$) and is 1/1000 that of wt RTPR. The role of the (deoxy)nucleotide may therefore be to enhance the binding of the cofactor to the enzyme, and/or induce homolysis of AdoCbl. Although 1 M NaOAc allows all of the four common ribonucleotides to be reduced at maximal rates in the absence of a deoxynucleotide effector, the above result suggests that NaOAc is not playing the role of an effector. What appears more likely is that it permits each of the ribonucleotides to stimulate its own reduction when present at sufficiently high concentrations. The inactivation of the enzyme during exchange in the absence of (deoxy)nucleotide is very interesting. Although this reaction has not yet been studied in detail, it may provide clues to understanding the intricacies of the generation of X•. One scenario to explain this inactivation is outlined in Scheme 5.3. Although a (deoxy)nucleotide is needed in general to promote the exchange reaction, a small amount of AdoCbl (which perhaps reflects the dissociation constant in the absence of nucleotide) binds to RTPR and homolyses, generating X• in the process. In the absence of nucleotide however, cob(II)alamin and 5'-deoxyadenosine do not bind to the enzyme very well (Yamada et al., 1971), and can easily dissociate, leaving a protein radical in the active site. The amount of exchange perhaps reflects the partitioning between the back reaction (regenerating cofactor), and dissociation of cob(II)alamin and 5'-deoxyadenosine from the enzyme. This scenario has precendent in the

studies of Yamada *et al.* (1971), in which they show that the incubation of AdoCbl with RTPR in the presence of dGTP and reductant results in a slow, non-reversible formation of cob(II)alamin and 5'-deoxyadenosine.

A K_m value of 60 μM was determined for [5'-^{3}H]AdoCbl in the exchange reaction using TR/TRR/NADPH as the reductant. Although this number is high with respect to the K_m value of AdoCbl during substrate turnover (2 μM, Allison Walsh, unpulished results 1992) it is probably on the order of, or much lower than the K_d for AdoCbl, which is unknown at this time. As described in Chapter 4, efforts to obtain this number using various techniques were unsuccessful. The data were very scattered, suggesting that the K_d is high. Studies by Sando *et al.* (1975) in which the exchange reaction as well as substrate turnover were investigated with various AdoCbl analogs, report that the K_m for AdoCbl for exchange as well as nucleotide reduction are similar (4.7 μM, exchange; 4.0 μM, reduction) in the presence of 2 and 10 mM ATP.

The requirement for reductant in the exchange reaction deserves special attention, as our finding, that reductant is not required for exchange, is contradictory to all previously reported findings concerning this aspect of the reaction (Beck et al., 1966a; Abeles & Beck, 1967; Vitols et al., 1967b; Hogenkamp et al., 1968). Our model predicts that if exchange is indeed related to the generation of X•, then it should proceed in the absence of reductant. Although the formation of cob(II)alamin has not been studied with pre-reduced enzyme in the absence of reductant, the ability to carry out turnover with pre-reduced RTPR - as in the single-turnover experiments described in detail in Chapter 4 - suggests that X• can be formed in the absence of reductant. We have approached this question with a battery of techniques, using pre-reduced RTPR, pre-oxidized RTPR, and four RTPR analogs containing C→S mutations of cysteines that have been determined to undergo redox cycling

Scheme 5.3: Hypothesis for the inactivation of RTPR during the exchange reaction in the presence of NaOAc.



during normal substrate turnover. Our results suggest strongly that in the presence of dGTP exchange does occur in the absence of reductant. The rate of exchange for pre-reduced wt RTPR (0.078 $\mu mol \bullet min^{-1} \bullet mg^{-1}$) is only a factor of 2 lower than exchange in the presence of the TR/TRR/NADPH reducing system (0.16 $\mu mol \bullet min^{-1} \bullet mg^{-1}$). In addition, pre-reduced mutant C731&736S RTPR afforded a rate of exchange (0.18 $\mu mol \bullet min^{-1} \bullet mg^{-1}$) that is only slightly lower than that of wt RTPR under similar conditions (0.28 $\mu mol \bullet min^{-1} \bullet mg^{-1}$). The addition of the TR/TRR/NADPH reducing system to the mutant reaction resulted in no increase in the rate of exchange,

and actually decreased the rate slightly (0.16 $\mu mol \bullet min^{-1} \bullet mg^{-1}$). This result shows unambiguously that exchange is not occurring through the reductant as proposed by Hogenkamp *et al.* (1968).

Why did Hogenkamp *et al.* not see exchange in the absence of reductant? A clue to the answer of this question may be provided by our study with oxidized RTPR. Pre-oxidized wt RTPR affords a rate of exchange that is ~1/27 that of wt RTPR in the presence of the TR/TRR/NADPH reducing system. Table 5.6 summarizes the results of a study carried out by Hogenkamp *et al.* (1968) of the effect of various thiols on the rate of dGTP-mediated exchange. From this table, TR/TRR/NADPH (the reducing system used in these studies) mediates the washout of 5595 cpm compared to 30 cpm in the absence of thiol. In the Hogenkamp *et al.* study, RTPR was purified by the procedures of Vitols *et al.* (1967a), in which reductant is not added during the enzyme preparation, and the RTPR is thus probably oxidized. From our quantitative analysis, the amount of radioactivity detected in H_2O should have been ~200 cpm. The reason for the discrepancy between our work and theirs is not clear.

The characterization of the exchange reaction with RTPR analogs containing C→S mutations of cysteines presumed to be in the active site, supports our model outlined in Figure 5.2, and further refines it. Firstly, C408S affords a rate of exchange that is less than 0.006% (our limit of detection) that of wt RTPR. Based on our model, this result was actually predicted due to the inability of this mutant to generate cob(II)alamin under various sets of conditions (Stuart Licht, unpublished results). Unexpectedly, however, the rate of exchange with each of the other active site mutant proteins is only 1% that of wt RTPR. These reactions have not yet been studied in detail, and various experiments need to be performed to see what step is rate-limiting. In

particular, the rate of cob(II)alamin formation as well as its steady-state level need to be addressed. The fact that pre-oxidized RTPR affords a rate that is ~4% that of wt RTPR, in conjunction with the results of these mutant proteins, suggests that active site conformation may play an important role in the exchange reaction. These results may offer an explanation for the failure of the 3'-methyl analogs of Ong *et al.* (1992) to catalyze exchange. The recent crystal structure of the R1 subunit of the *E. coli* RDPR solved by Uhlin and Eklund (1994), shows that the active site redox-active cysteines are only 6 Å away from C439 - the proposed X•. By extrapolating to the AdoCbl systems, a substitution of a methyl group on the nucleotide ring for a hydrogen atom could sterically perturb the generation of X•. Cob(II)alamin formation needs to be addressed in the *L. leichmannii* system with the 3'-methyl analogs.

So what have we learned about the exchange reaction and the generation of X•? Firstly, the rate of exchange is not slow. The rate of 0.16 $\mu mol \bullet min^{-1} \bullet mg^{-1}$ is only a factor of 10 lower than the rate of nucleotide reduction. In fact, normalizing for the statistical factor in that three 5' hydrogens on 5'-deoxyadenosine are accessible for removal, brings the rate of exchange within a factor of 3.5 of the rate of turnover. Given a normal tritium selection effect of 10-20 on the exchange reaction, the rate of exchange (7-14 s^{-1}) becomes greater than the rate of turnover (2-3 s^{-1}), and only a factor of 3-6 less than cob(II)alamin production (38-48 s^{-1}) (Tamao & Blakley, 1973). If AdoCbl were particularly "sticky," the true rate of exchange would be masked due to failure of AdoCbl to promptly dissociate so that the enzyme could pick up another molecule of [5'-^{3}H]AdoCbl. This has recently been addressed by Stuart Licht in our laboratory using rapid quench technology. In a reaction containing 1 mM dGTP, and equimolar amounts of [5'-^{3}H]AdoCbl and RTPR, he demonstrated that the rate constants for exchange in the pre-steady state are

very similar to those reported here under steady-state conditions. This suggests that dissociation of AdoCbl is not rate-limiting. In addition, for the first time, the other homolysis product - 5'-deoxyadenosine - has been observed. The rate constant for the formation of this species is approximately the same as the rate constant for the formation of cob(II)alamin. Moreover, no other species (including 5',8-cycloadenosine) is observed under these conditions. These studies by Stuart Licht in combination with the exchange studies presented herein and our model, suggest that the formation of X• may be a concerted process. Consistent with this proposal is the inability of mutant C408S RTPR to generate cob(II)alamin, even though it binds the homolysis products of AdoCbl [cob(II)alamin and 5'-deoxyadenosine] in the presence of dGTP and reductant equally well as the wt RTPR.

The cloning, sequencing, and expression of RTPR has allowed us to take a detailed look at the intricate workings of a very complex enzyme using several techniques. Firstly, using site-directed mutagenesis, we have been able to offer a compelling model for the roles in which five cysteines on RTPR play during catalysis. In addition, we have been able to use these same mutants to better clarify the role of the reductant in the exchange reaction, as well as appreciate how subtleties in the active site conformation of RTPR can produce fairly large effects on the rate of exchange. Lastly, due to the quantities of enzyme that are now available, we are better equipped to characterize some of the intermediates on the reaction coordinate of this enzyme using pre-steady state techniques.

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