On the Chemical Mechanism of Assembly of the
Tyrosyl Radical-Dinuclear Iron Cluster Cofactor
of *E. coli* Ribonucleotide Reductase

by

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Signature of Author

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December 1, 1992

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To my parents, who made me what I am

and

to Wendy, who loves me for (and in spite of) it
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By far the most valuable and enjoyable aspect of my graduate school experience was the interaction that I had with my tremendously interesting and talented coworkers. Many of these people made important scientific contributions to this work. Many others contributed less directly by helping me to maintain my sanity.

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Abstract

The R2 subunit of ribonucleotide reductase from *E. coli* contains the tyrosyl radical-diiron(III) cluster cofactor which is required for the enzyme's catalytic activity. Assembly of this cofactor occurs spontaneously *in vitro*: upon treatment of iron-free R2 (apo R2) with aqueous Fe²⁺ and O₂, a protein-bound diiron(II) cluster reductively activates O₂ to effect the one electron oxidation of tyrosine 122, concomitantly forming the diiron(III) cluster. In this work, the chemical mechanism of the cofactor assembly reaction was probed by a combination of rapid kinetic methods (including the stopped-flow and rapid freeze-quench techniques) spectroscopic methods (including light absorption, electron paramagnetic resonance and Mössbauer spectroscopies) and site-directed mutagenesis. The results of these studies indicate 1) that assembly of the cofactor proceeds by two distinct reaction pathways, 2) that the partition ratio between the pathways is exquisitely sensitive to the availability of exogenous reducing equivalents, and 3) that a different reactive species generates the tyrosyl radical in each pathway. On the basis of its light absorption and EPR spectra, the first intermediate responsible for tyrosyl radical production is proposed to be a tryptophan radical cation, which the crystal structure of R2 suggests may reside on tryptophan 48. EPR and Mössbauer spectroscopic characterization of the second tyrosyl radical-generating intermediate indicates that it is a heretofore unprecedented iron cluster, which consists of two high-spin ferric ions coupled to a ligand radical. The results of this work thus indicate, in contrast to several mechanisms which were previously proposed for assembly of the R2 cofactor, that high valent iron (Fe(IV) or Fe(V)) intermediates are not responsible for production of the tyrosyl radical.

Thesis Supervisor: Prof. JoAnne Stubbe
Title: John C. Sheehan Professor of Chemistry and Biology
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Chapter 1: The R2 Subunit of E. coli Ribonucleotide Reductase:

Structure, Function and Reactivity
Oxo- or Hydroxo-Bridged Dinuclear Iron Clusters in Biology

The prevalence and biological importance of proteins which contain oxo- or hydroxo-bridged dinuclear iron clusters is now widely recognized (for recent reviews, see Lippard, 1988; Que & Scarow, 1988; Sanders-Loehr, 1989; Kurtz, 1990; Que & True, 1990; Vincent et al., 1990). To date, the list of members of this class includes the R2 subunit of ribonucleotide reductase (RNR) from *E. coli*, mammals and viruses (Lammers & Follmann, 1983; Sjöberg & Gräslund, 1983; Thelander et al., 1985; Reichard, 1988; Stubbe, 1990); protein A of methane monooxygenase (MMO) from methanotrophic bacteria (Woodland et al., 1986; Ericson et al., 1988; Fox et al., 1988; DeWitt et al., 1991); hemerythrin from certain marine worms (Wilkins & Wilkins, 1987; Sanders-Loehr, 1988); purple acid phosphatases from a variety of microbial, plant and animal sources (Antanaitis & Aisen, 1983; Doi et al., 1988); and ruberythrin from *Desulfovibrio vulgaris* (LeGall et al., 1988). Several of these dinuclear iron proteins perform critical functions in the organisms in which they are found: ribonucleotide reductase (RNR) converts ribonucleotides to deoxyribonucleotides, thus providing the precursors for DNA biosynthesis (Thelander & Reichard, 1979); methane monooxygenase converts methane to methanol, thereby allowing methanotrophic bacteria to use methane as their sole carbon source (Dalton, 1980; Anthony, 1982); and hemerythrin carries O₂ in the blood of certain marine worms, allowing these organisms to respire (Wilkins and Wilkins, 1987). In each of the three aforementioned proteins, hemerythrin, R2 of RNR, and protein A of MMO, the function of the iron cluster involves the reaction of its reduced (diferric) state with molecular oxygen. The mode of reactivity with O₂, however, is distinct for each protein. In hemerythrin, the diferric cluster reacts reversibly with O₂ to form a diferric hydroperoxide species (Scheme 1.1A) (Shiemke et al., 1984; Stenkamp et al., 1985; Shiemke et al., 1986). In R2, the diferric cluster reductively activates O₂ to effect the one electron oxidation of an endogenous tyrosine residue, in the process forming the catalytically essential, stable tyrosyl radical.
Scheme 1.1: Three distinct modes of O₂ reactivity in proteins which contain dinuclear iron clusters.

A. Hemerythrin - reversible binding of O₂

B. R2 of Ribonucleotide Reductase - one electron oxidation of Y122

C. Methane Monooxygenase - two electron oxidation of methane
and oxo-bridged diiron(III) cluster of the native protein (Scheme 1.1B) (Atkin et al., 1973; Sahlin et al., 1989; Bollinger et al., 1991a; Fontecave et al., 1992). And in MMO, the diferrous cluster activates O$_2$ to effect the two electron oxidation (hydroxylation) of methane (Scheme 1.1C) (Dalton, 1980; Woodland & Dalton, 1984; Green & Dalton, 1985; Prior & Dalton, 1985; Fox et al., 1989). An objective which is central to the study of these three proteins is to define the structural features of each protein and iron cluster which determine its mode of reactivity (reversible binding, one electron oxidation, or two electron oxidation) toward O$_2$. Achieving this objective will require both a knowledge of the structure of the proteins and their iron clusters and an understanding of the chemical mechanisms of their reactions with O$_2$. The former is emerging: the three dimensional structures of hemerythrin (Stenkamp et al., 1976; Hendrickson, 1978; Stenkamp et al., 1984; Sheriff et al., 1987) and of the R2 subunit of E. coli RNR (Nordlund et al., 1990) have been determined, and preliminary x-ray diffraction data on protein A of M. capsulata MMO have been reported (Rosenzweig et al., 1992). The studies which are described herein contributes to the latter. In this work, the mechanism by which the diferrous cluster in the R2 subunit of E. coli RNR activates O$_2$ to effect the one electron oxidation of an endogenous tyrosine residue has been probed by rapid-kinetic and spectroscopic methods, including stopped-flow light absorption, rapid freeze-quench EPR and rapid freeze-quench Mössbauer spectroscopies. Application of these techniques to the R2 reaction has allowed for 1) the identification of two species which are responsible for tyrosine oxidation, 2) the preliminary characterization of one of these species as a unique iron cluster which consists of two high-spin ferric ions coupled to a ligand radical, and 3) the proposal of a coherent mechanism for the reaction. Thus, the results of this work, together with the crystal structure of R2 (Nordlund, et al., 1990), provide a basis for assessing the relationship between the structure of R2 (especially of its iron cluster) and its oxygen reactivity. When similar information becomes available for MMO, it may
be possible to deduce general structure-reactivity relationships for these dinuclear iron proteins which function by virtue of their reactivity with O₂.

_E. coli_ Ribonucleotide Reductase

The enzymes ribonucleotide reductase (RNR) catalyze the conversion of all four ribonucleotides to the corresponding deoxyribonucleotides (Thelander and Reichard, 1979; Eriksson & Sjöberg, 1989; Stubbe, 1990). Reducing equivalents are delivered by enzyme cysteine residues which become oxidized to disulfides upon turnover (Scheme 1.2). This reaction is the only means by which cells obtain the deoxynucleotide precursors for DNA biosynthesis, and is the first committed and rate limiting step in DNA biosynthesis. For these reasons, RNRs are critical to all known organisms and cell types. Because of their universal importance to DNA and cell replication, RNRs are justifiably considered to be attractive targets for the rational design of anticancer and antiviral drugs. Consequently, considerable effort has been devoted to understanding the structures and catalytic mechanisms of these important enzymes.

The most extensively studied of the RNRs is that from _E. coli_. In both its structure (Thelander and Reichard, 1979; Lammers and Follmann, 1983) and its catalytic mechanism (Stubbe, 1990), the _E. coli_ enzyme is prototypical of the known mammalian and viral RNRs. _E. coli_ RNR is made up of two homodimeric subunits, R1 and R2. Both subunits are required for catalytic activity. The R1 subunit (a homodimer of Mr = 171,000) contains the binding sites for the nucleoside diphosphate substrates and the nucleoside triphosphate and deoxynucleoside triphosphate allosteric effectors. R1 also contains the cysteine residues which reduce the substrate. The R2 subunit (a homodimer of Mr = 87,000) contains the enzyme's unique and catalytically essential cofactor, a stable tyrosyl free radical adjacent to an oxo-bridged dinuclear iron(III) cluster. The active enzyme is thought to be a one-to-one complex between R1 and R2. Historically, it has been assumed that the active site is located at the interface between the two subunits

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(Reichard & Ehrenberg, 1983; Sjöberg and Gräslund, 1983; Sjöberg, 1986). The recently determined 3-dimensional structure of R2 (discussed below) (Nordlund, et al., 1990) and subsequent new ideas concerning the catalytic mechanism of the enzyme have led to the view that the active site may be completely contained within R1, with R2 acting as a catalytic cofactor (Mao et al., 1992).

**Scheme 1.2:** Reaction catalyzed by ribonucleotide reductases. In *E. coli* RNR, NADPH provides the reducing equivalents for the reaction via thioredoxin and thioredoxin reductase or via glutaredoxin, glutathione, and glutathione reductase.

**R2 Subunit of *E. coli* RNR**

*Structure and Physical Properties of R2 and its Cofactor*

The discoveries of the iron cluster and tyrosyl radical in R2 were facilitated by their UV-visible absorption features (Brown et al., 1969; Atkin, et al., 1973) and by the EPR signal of the radical (Ehrenberg & Reichard, 1972). The absorption spectrum of R2 (Fig. 1.1) has a broad band at 365 nm and a shoulder at 320 nm, which are due to the iron cluster (specifically, oxo- to iron charge transfer) (Sanders-Loehr, 1989), and a very sharp feature at 410 nm, which is due to the tyrosyl radical. Weaker bands at lower energy (not
Fig. 1.1: UV-Visible light absorption spectra of the diferric cluster (A) and of the tyrosyl radical (B). Spectra were obtained by subtraction, as described in the Materials and Methods section of Chapter 3. Spectrum C is the sum of A and B.
shown in Fig. 1.1) arise from both the iron cluster and the radical. The low temperature EPR spectrum of the tyrosyl radical of R2 (Fig. 1.2) exhibits a large doublet hyperfine coupling, which is due to one of the β-hydrogens, and a smaller triplet splitting, which arises from the ring hydrogens in positions 3 and 5 (Sjöberg et al., 1977).

Since the report of the presence of iron and a free radical in R2, a battery of physical and biochemical methods has been applied in an attempt to define the structure of the cofactor and its location within the dimeric protein. EPR and ENDOR spectroscopy on R2 subunits containing uniformly or specifically deuterated tyrosine residues demonstrated that the radical arises from tyrosine, allowed assignment of spin densities on the carbon and oxygen nuclei of the tyrosine side chain, and permitted calculation of the dihedral angle between the β-C-H bonds and the plane of the tyrosine ring (Sjöberg, et al., 1977; Bender et al., 1989). These experiments also suggested that the radical is deprotonated, a conclusion supported by resonance Raman results (Backes et al., 1989). Additional EPR experiments demonstrated that electronic relaxation of the radical is enhanced by magnetic interaction with the iron cluster (Sahlin et al., 1987; Hirsh et al., 1992), indicating that the components are spatially close to one another. Alignment of R2 sequences from different organisms (Sjöberg et al., 1985) showed conservation of the tyrosine corresponding to position 122 in E. coli R2, and site-directed mutagenesis of tyrosine 122 showed that the radical is produced from this residue (Larsson & Sjöberg, 1986). Selected data concerning the tyrosyl radical are listed in Table 1.1.

Characterization of the dinuclear iron cluster of R2 was greatly facilitated by its spectroscopic similarities to the structurally well characterized cluster found in metazidohemerythrin (shown schematically in Fig. 1.3) (Lippard, 1988). UV-visible absorption (Atkin, et al., 1973), resonance Raman (Sjöberg et al., 1980; Sjöberg et al., 1982; Backes, et al., 1989), Mössbauer (Atkin, et al., 1973; Lynch et al., 1989), and EXAFS spectroscopies (Bunker et al., 1987; Scarrow et al., 1987) applied to R2 all
Fig. 1.2: X-band EPR spectrum of the tyrosyl radical of R2. The spectrum was acquired at 100 K with a microwave power of 1 mW, a modulation amplitude of 4 G, a sweep time of 200 s, a time constant of 200 ms, and a receiver gain of $1 \times 10^5$. 
Fig. 1.3: Schematic representation of the oxo-bridged diiron(III) cluster in metazidohemerythin (Stenkamp, et al., 1984).
Table 1.1: Selected physical data on the tyrosyl radical of R2. Adapted from (Nordlund, 1990). For a recent review of biophysical investigations on R2, see (Sanders-Loehr, 1989).

**UV-Visible Absorption** (Brown, et al., 1969; Atkin, et al., 1973)
- Sharp band at 410 nm, broader bands at 390 nm and 600 nm

**Resonance Raman** (Backes, et al., 1989)
- Resonance enhanced band at 1498 cm\(^{-1}\) assigned to neutral radical (i.e. radical is not protonated at Oη)

**EPR and ENDOR** (Ehrenberg and Reichard, 1972; Sahlin, et al., 1987; Bender, et al., 1989; Hirsh, et al., 1992)
- Doublet with gav = 2.0047
- Interacts weakly with diferric cluster: |J| = 0.0047 ± 0.0003 cm\(^{-1}\)
  - EPR spectrum broadened at temperatures above 75 K due to relaxation enhancement by the nearby iron cluster
  - Mössbauer spectrum of iron cluster unaffected by presence of radical and EPR spectrum of radical unaffected by presence of \(^{57}\)Fe in cluster

**on R2 with specifically deuterated tyrosine residues**
- 19 G doublet splitting due to β-proton, 7 G triplet due to ring protons in 3 (ε) and 5 (ε') positions
- Seven-member odd-alternate species - spin densities: Cγ = 0.49, Cδ = -0.07, Cε = 0.26, Cζ = -0.03, Oη = 0.16
- Dihedral angles between Cγpz orbital and β-protons = 30° and 90°
- Radical is charge neutral

indicated that the iron cluster is composed of two inequivalent, high-spin ferric ions antiferromagnetically coupled through an oxo-bridge. The resonance Raman data allowed estimation of the Fe-O-Fe angle as 130° and of the Fe-O distance as 1.78 Å, giving an Fe-Fe distance of 3.2 Å (Sjöberg, et al., 1982; Backes, et al., 1989). These dimensions agree well with results obtained by EXAFS spectroscopy, from which an
Fe-O distance of 1.78 Å and an Fe-Fe distance of 3.22 Å (Scarrow, et al., 1987) or 3.24-3.48 Å (Bunker, et al., 1987) were calculated. The EXAFS data also revealed a first shell distance somewhat shorter than in hemerythrin, suggesting that more oxygen and fewer nitrogen ligands are coordinated in R2 (no more than one histidine ligand per Fe(III) ion) (Bunker, et al., 1987; Scarrow, et al., 1987). Data of importance to current understanding of the structure and physical properties of the R2 Fe-cluster are summarized in Table 1.2.

An issue which remained controversial long after the discovery of the iron and free radical in R2 was the stoichiometry of the two components. As originally isolated from E. coli B, R2 was found by a colorimetric iron assay to contain 2 Fe$^{3+}$ ions per dimeric subunit (Brown, et al., 1969; Thelander, 1973). This quantitation led to the hypothesis that a single iron cluster is coordinated between the monomers of R2, and that each dimer contains a single tyrosyl radical in one of its monomers. This proposal gained wide acceptance, despite the fact that it is apparently inconsistent with the observation by Mössbauer spectroscopy that the Fe$^{3+}$ ions are inequivalent (Atkin, et al., 1973).

The cloning, sequencing, and overproduction of R2 (Eriksson et al., 1977; Platz & Sjöberg, 1980; Carlson et al., 1984; Salowe & Stubbe, 1986; Sjöberg et al., 1986) markedly affected the observed Fe$^{3+}$ and tyrosyl radical stoichiometries in two ways. Firstly, the molecular weight of the protein was corrected from the previously reported value of 78 kD, which had been determined by sedimentation methods (Thelander, 1973), to a value of 87 kD, which is predicted by the nrdB (the designation for the gene which encodes R2) sequence (Carlson, et al., 1984). Correction of the molecular weight of R2 required correction of the molar absorptivity at 280 nm ($\varepsilon_{280}$) of the protein from 117,000 M$^{-1}$cm$^{-1}$ (Thelander, 1973) to 131,000 M$^{-1}$cm$^{-1}$, which in turn increased the calculated Fe$^{3+}$ and tyrosyl radical content of R2. Secondly, the overproduction of R2 greatly simplified its purification, thereby minimizing loss of Fe$^{3+}$ and tyrosyl radical which may have occurred during the more arduous purification. As a result of these two factors, recombinant R2, isolated following addition of Fe$^{2+}$ and ascorbate to crude cell
Table 1.2: Selected physical data on the dinuclear iron cluster of R2. Adapted from (Nordlund, 1990).

**UV-Vis Absorption** (Atkin, et al., 1973; Sanders-Loehr, 1989)
- Bands at 325 nm and 365 nm assigned as oxo-to-iron charge transfer transitions
- Bands at 500 and 600 nm assigned as ligand field transitions

**Resonance Raman** (Sjöberg, et al., 1980; Sjöberg, et al., 1982; Backes, et al., 1989)
- Band at 493 cm⁻¹ assigned as Fe-O-Fe symmetric stretch (shifts to 480 cm⁻¹ in H₂¹⁸O)
- Band at 756 cm⁻¹ assigned as Fe-O-Fe asymmetric stretch
- Data give Fe-O-Fe angle of 130°, Fe-O distance of 1.78 Å, and Fe-Fe distance of 3.2 Å

- Two magnetically inequivalent, antiferromagnetically coupled high-spin ferric ions
- One iron cluster in each polypeptide chain (four Fe sites per R2 subunit)

**Magnetic Susceptibility** (Petersson et al., 1980)
- Fe⁴⁺-Fe⁴⁺ exchange coupling constant (J) of -108 ± 25 cm⁻¹

**EXAFS** (Bunker, et al., 1987; Scarow, et al., 1987)
- First shell distance of 1.78 Å assigned to Fe-O bond
- Major first shell distance of 2.06 Å
- Fe-Fe distance of 3.22 Å or 3.24-3.48 Å

**EPR** (Hirsh, et al., 1992)
- Fe⁴⁺-Fe⁴⁺ exchange coupling contant (J) of -94 ± 7 cm⁻¹
extracts, was found to contain $2.7 \pm 0.3 \text{Fe}^{3+}$ and $1.1 \pm 0.1$ tyrosyl radicals per R2 subunit (Sjöberg et al., 1987).

The fact that the measured Fe$^{3+}$ stoichiometry of R2 exceeded the theoretical value of 2 (assuming one iron cluster bound between the two monomers) prompted Lynch and coworkers to reevaluate this quantity (Lynch, et al., 1989). These authors redetermined ε$_{280}$ of R2 (isolated from N6405/pSPS2, specific activity 4700-5700 U/mg) to be 141,000 M$^{-1}$cm$^{-1}$. In addition, they determined the tyrosyl radical content of R2 by EPR spectroscopy (by comparing the double integrals of R2 spectra to those of a DPPH standard). They also determined the Fe$^{3+}$ content of R2 by a new procedure. Fe$^{3+}$ was extracted from R2 by incubation of the protein at 100 °C in 2 N HCl, followed by repeated HCl extraction of the protein pellet. The Fe$^{3+}$ was then quantified by inductively coupled plasma emission spectroscopy. Using these methods, the authors determined that 3.9 Fe$^{3+}$ and 1.4 tyrosyl radicals were present per R2 dinner. The authors then examined the protein by Mössbauer and EPR spectroscopies and found that >97% of the $^{57}$Fe present was in the form of diferric clusters. From these data, they concluded that R2 must contain a diferric cluster and tyrosyl radical in each of its monomers. As discussed below, the location of the diferric cluster within each monomer, rather than between the monomers, was later confirmed by the crystal structure of R2 (Nordlund, et al., 1990).

While the work of Lynch and coworkers was in progress, we also undertook to determine the Fe$^{3+}$ and tyrosyl radical content of R2. (The R2 used in our experiments was also isolated from N6405/pSPS2, and had a specific activity of 5800-8000 U/mg.) Three different methods of extracting the Fe$^{3+}$ from the protein and three different methods of Fe$^{3+}$ quantitation were employed. The results of these experiments are summarized in Table 1.3. Using R2 isolated from the same source and by the same procedure as that of Lynch et al. (1989), we have consistently found significantly less Fe$^{3+}$ than these authors. The reason for this discrepancy is unknown.
Table 1.3: Summary of quantitation of Fe$^{3+}$ in R2.

<table>
<thead>
<tr>
<th>Method of Fe$^{3+}$ Extraction</th>
<th>Method of Quantitation</th>
<th>No. Trials</th>
<th>Average Fe$^{3+}$/R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>add protein to 1M HNO$_3$ and heat at 80 °C until dryness, redissolve residue in 0.1 M HNO$_3$</td>
<td>atomic absorption</td>
<td>7</td>
<td>2.8$^a$ (3.0)$^b$</td>
</tr>
<tr>
<td>incubate protein at 100 °C for 15 min in 2 M HCl, re-extract pellet 3x with 2 M HCl$^d$</td>
<td>inductively coupled plasma emission$^c$</td>
<td>2</td>
<td>3.2$^a$ (3.4)$^b$</td>
</tr>
<tr>
<td>precipitate protein by bringing to 4.5% in trichloroacetic acid$^e$</td>
<td>assay with ascorbic acid and ferrozine</td>
<td>9</td>
<td>3.0$^a$ (3.2)$^b$</td>
</tr>
<tr>
<td></td>
<td>assay with ascorbic acid and ferrozine</td>
<td>4</td>
<td>3.0$^a$ (3.2)$^b$</td>
</tr>
</tbody>
</table>

$^a$ calculated assuming $\varepsilon_{280}$ for R2 = 131 mM$^{-1}$cm$^{-1}$
$^b$ calculated assuming $\varepsilon_{280} = 141$ mM$^{-1}$cm$^{-1}$
$^c$ done in collaboration with Dr. J. Lynch and Prof. L. Que at the U. of Minnesota
$^d$ method from (Lynch, et al., 1989)
$^e$ method from (Salowe, 1987)

We have also determined the tyrosyl radical content of several preparations of R2 by EPR and by using the dropline corrected absorbance at 412 nm (method described in Chapter 2). In agreement with the results of Lynch et al. (1989), we consistently find less than two radicals per R2 subunit. Our best preparations contain 1.3-1.4 or 1.4-1.5 tyrosyl radicals per R2, assuming $\varepsilon_{280} = 131,000$ M$^{-1}$cm$^{-1}$ or $\varepsilon_{280} = 141,000$ M$^{-1}$cm$^{-1}$, respectively. A plausible reason for the lower than theoretical tyrosyl radical content is put forth in Chapter 2.

While our work was in progress, structural studies on R2 and its cofactor culminated with the report of the 3-dimensional structure of the protein, determined by x-ray crystallography (Nordlund et al., 1989; Nordlund, et al., 1990). The crystal structure of R2 confirmed much of what had been deduced from less direct methods about the
tyrosyl radical-diferric cluster cofactor, and provided several new insights. As predicted (Lynch, et al., 1989), R2 contains one Fe-cluster in each of its monomers. The cofactor is held deep within the monomer, such that Y122 is buried 10 Å from the nearest surface. The implication of this finding for the catalytic mechanism of RNR is discussed below.

A schematic structural representation of the cofactor is shown in Fig. 1.4 (Nordlund, et al., 1990). Y122, which harbors the radical, is 5.3 Å from the nearest ferric ion, and the Fe-Fe distance is 3.3 Å. The cluster is bridged by an oxo- ligand, derived either from solvent or from O2, and a glutamate carboxylate ligand (E115). Each Fe3+ coordinates a single histidine ligand (H118 and H241) at the δ-nitrogen, and the remaining ligands are side-chain carboxylates and H2O. The oxo- bridge is cis to the histidine ligands. A tryptophan residue (W48) near the putative R1-binding domain of the subunit is linked to Fe1 by a hydrogen bonding network involving D237 and the iron ligand H118 (Fig. 1.5). The authors suggest that W48 may be important in the interaction of R2 with R1, as discussed below.

Function of R2 in Catalysis

In the last decade, much progress has been made toward elucidating the catalytic mechanism of E. coli RNR. The available evidence supports the working hypothesis shown in Scheme 1.3 (Stubbe, 1990). In this mechanism, a protein radical (X•) initiates turnover by abstracting the 3'-H atom from the ribose ring of the substrate. Subsequent to this 3' C-H bond homolysis, loss of the 2'-OH group (probably as H2O), multiple electron and proton transfers from the active-site cysteine residues to the substrate, and return of the 3'-H atom produce the deoxynucleoside diphosphate product and regenerate the protein radical. The participation of a protein radical in the reaction mechanism provides an explanation for the enzyme's requirement for the tyrosyl radical in R2. Direct participation of the tyrosyl radical in 3'-H atom abstraction is unlikely, however, as a large conformational change would be required to bring the buried Y122 radical into
Fig. 1.4: Schematic representation of the oxo-bridged diiron(III) cluster in the R2 subunit of *E. coli* ribonucleotide reductase. Adapted from (Nordlund, et al., 1990).
Fig. 1.5: Hydrogen bonding network in R2 which connects W48 to Fe1. The atomic coordinates used in this illustration come from the structure of Mn$^{2+}$-substituted R2 (Atta et al., 1992). These coordinates were generously provided by Dr. Pär Nordlund.
Scheme 1.3: Working hypothesis for the catalytic mechanism of *E. coli* ribonucleotide reductase.

proximity with the substrate (Nordlund, et al., 1990). The fact that no observable change in the spectroscopic properties of the R2 cofactor occurs upon binding of R1 nor upon turnover provides argument against such a conformational change (Ehrenberg, 1988). It has been proposed, therefore, that the first step in the catalytic mechanism is electron transfer from the R1 subunit to the tyrosyl radical in R2, a step which generates an as yet
undetected, transient R1 radical and a reduced Y122 (Mao, et al., 1992). At the end of a catalytic cycle, electron transfer in the reverse direction regenerates the tyrosyl radical and the resting (radical-free) form of R1.

The recognition that electron transfer between R1 and R2 may be important in the catalytic mechanism of RNR has fueled efforts to identify residues near the R1 binding domain of R2 that might be involved in an electron transfer pathway between the subunits. Interpreting the crystal structure of R2, Nordlund and coworkers have implicated W48 as a candidate to be involved in such a pathway (Nordlund, et al., 1990; Nordlund, 1990). This residue is linked to the iron cluster by a hydrogen bonding network involving H118 and D237 (see Fig. 1.5), is near the putative R1-binding domain, and is completely conserved among all known R2 sequences. Nordlund and coworkers have suggested that, in the catalytic mechanism of RNR, W48 may cycle between an oxidized, radical form and its normal, reduced form. In support of this possibility, the authors point out the analogy of the hydrogen bonding network involving W48 of R2 to a similar network found in cytochrome c peroxidase (Finzel et al., 1984), in which the analogous tryptophan residue (W191) becomes oxidized to a radical during catalysis (Mauro et al., 1988; Erman et al., 1989) (discussed in more detail below). In our study, evidence has been found that a tryptophan radical may form during assembly of the cofactor of R2. It is interesting to speculate that this radical may be produced by the oxidation of W48 (an hypothesis which is currently being tested). If this speculation is correct, our work would provide direct evidence that W48 can transfer an electron into the assembling cofactor. This finding would, in turn, lend credence to the suggestion that W48 is involved in electron transfer between R1 and R2.

Reactivity of R2 and its Cofactor

In addition to its structure and role in catalysis, the chemical reactivity of E. coli R2 also has been extensively probed (see Fontecave, et al., 1992 for a recent review). To
date, five interconvertible forms of the subunit have been prepared, of which at least four are physiologically relevant. (The semimet form, which contains no tyrosyl radical and a mixed-valence (ferrous, ferric) cluster, may be the only exception. It seems to be unstable with respect to disproportionation, and has been observed only upon reduction of the native protein with diimide or hydrazine (Gerez et al., 1991; Gerez & Fontecave, 1992), and upon x-irradiation of the native protein (Hendrich et al., 1991). Nevertheless, it has been proposed as an intermediate in the conversion of apo R2 to native R2 (Elgren et al., 1991).) Treatment of the native protein with one electron reductants such as the clinically used anticancer agent, hydroxyurea, reduces the tyrosyl radical without affecting the diferric cluster (Brown, et al., 1969). This radical reduced form of R2 has been designated met R2, in analogy to the nomenclature used for hemerythrin. Treatment of met R2 with H₂O₂ (Sahlin et al., 1990) or with oxygen atom donors such as 2iodosobenzoate (Fontecave et al., 1990a) regenerates the native protein, although the yield of tyrosyl radical from this process is poor. Treatment of met R2 or of the native protein with a ferric ion chelator (historically, 8-hydroxyquinoline-5-sulfonic acid) in the presence of a mild denaturant (1M imidazole) removes the iron, producing apo R2, which lacks both iron and radical (Atkin, et al., 1973). Incubation of apo R2 with ferrous ion in the absence of oxygen, produces ferrous R2 (Sahlin, et al., 1989), which reacts upon exposure to O₂ to give the native protein (also called reconstituted R2) (Brown, et al., 1969; Atkin, et al., 1973). Addition of ferrous ion to R2 in the presence of O₂ leads directly to the native protein presumably with ferrous R2 as an intermediate. Ferrous R2 can also be prepared from native R2 or from met R2 by in situ reduction of the cofactor. This reduction can be carried out (in the absence of O₂) with dithionite and methyl- or benzylviologen (Sahlin, et al., 1989), with reduced flavins and Fe²⁺ (Fontecave et al., 1989), with DTT at elevated pH (Fontecave et al., 1990b), or with diimide (Gerez, et al., 1991). In addition, a flavin reductase enzyme activity has been identified in E. coli which can regenerate native R2 from met R2 by reducing the diferric center to diferrous (which
then reacts with O₂) (Fontecave, et al., 1989). Scheme 1.4 summarizes the different forms of R2, and the manner in which they are interconverted. The ability to convert native R2 to apo R2 (1), and then to reconstitute the native protein by incubation of apo R2 with Fe²⁺ and O₂ (2), has made possible the in vitro study of the cofactor assembly reaction which is described herein.

**Dinuclear Iron Cluster-Heme Iron Center Parallel**

Over the past decade, a parallel has emerged among the functions of the dinuclear iron clusters in hemerythrin, R2 and MMO and the functions of heme iron centers in related proteins (Table 1.4). Each of these dinuclear iron proteins has a counterpart in which a heme iron center serves the same function as the dinuclear iron cluster. Thus, hemerythrin has its counterpart in the mammalian oxygen carrying protein, hemoglobin, which reversibly binds O₂ at a heme iron center. Likewise, R2 has its functional analogue in the peroxidases, which use a heme iron center and a peroxide co-substrate to catalyze the one electron oxidation of a variety of substrates (including tyrosine and other phenols) (Marnett et al., 1986). Finally, MMO has its counterpart in cytochrome P-450, which activates O₂ at a heme iron center to effect the hydroxylation of unactivated hydrocarbons (Ortiz de Montellano, 1986). The analogy between MMO and cytochrome P-450 and between R2 and the peroxidases extends beyond the normal functions of the iron centers. For example, both MMO and P-450 have extremely broad substrate specificities: they catalyze the hydroxylation of a wide variety of unsaturated hydrocarbons, the epoxidation of olefins, and several other oxidation reactions (Colby et al., 1977; Ortiz de Montellano, 1986). Both MMO and P-450, in addition to activating O₂ at their reduced (ferrous) active sites, can use hydrogen peroxide at their oxidized (ferric) active centers to carry out the same reactions (Ortiz de Montellano, 1986; Andersson et al., 1991). In the case of R2 and the peroxidases, the functional analogy of the iron centers also appears to include reactivity with alternative substrates. Incubation
Scheme 1.4: Different states of R2 and methods for their interconversion.

1. mild denaturant (e.g. 1 M imidazole) and chelator (e.g. 8-hydroxyquinoline-5-sulfonic acid) (Atkin, et al., 1973)

2. Fe$^{2+}$, O$_2$ (Brown, et al., 1969; Atkin, et al., 1973)

3. one electron donors (e.g. hydroxyurea, hydroxylamine, reduced flavin) (Brown, et al., 1969; Fontecave, et al., 1992)

4. oxo- donors (e.g. H$_2$O$_2$, iodosobenzoate) (Fontecave, et al., 1990a; Sahlin, et al., 1990)

5. chelator (e.g. EDTA) (Salowe, 1987)

6. Fe$^{2+}$ (Sahlin, et al., 1989)

7. dithionite and viologen mediator, flavin reductase and Fe$^{2+}$, reduced flavin and Fe$^{2+}$, DTT at pH > 8, or diimide (all in absence of O$_2$) (Fontecave, et al., 1989; Sahlin, et al., 1989; Fontecave, et al., 1990b; Gerez, et al., 1991)

8. O$_2$

9. H$_2$O$_2$ (Gerez and Fontecave, 1992)

10. diimide, hydrazine, or x-irradiation and annealing (Gerez, et al., 1991; Hendrich, et al., 1991; Gerez and Fontecave, 1992)
of met R2 with hydrogen peroxide results in slow production of tyrosyl radical (Sahlin, et al., 1990). This result has been interpreted as an indication that \( \text{H}_2\text{O}_2 \) can react at the diferric cluster of R2 to effect the normal one electron oxidation reaction (although the reaction is extremely slow and produces only sub-stoichiometric quantities of tyrosyl radical, and although it has not yet been demonstrated that \( \text{H}_2\text{O}_2 \) reacts specifically at the diferric cluster). Furthermore, incubation of met R2 with oxo- donors such as iodosobenzoate results in generation of tyrosyl radical (Fontecave, et al., 1990a). (Again, this reaction is very slow, and the participation of the diferric cluster has not been established.) The peroxidases likewise accept oxo- donors as alternative co-substrates to peroxides (Marnett, et al., 1986). Thus, the extensive analogy between the dinuclear iron reactions and the heme iron reactions would seem to suggest that they employ similar mechanisms.

**Table 1.4: Parallel functions among the dinuclear iron proteins and the heme iron proteins.**

<table>
<thead>
<tr>
<th>Reaction Mediated at Fe center</th>
<th>Dinuclear Iron Protein</th>
<th>Heme Iron Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>reversible binding</td>
<td>hemerythrin</td>
<td>hemoglobin, myoglobin</td>
</tr>
<tr>
<td>one-electron oxidation</td>
<td>R2 of RNR</td>
<td>peroxidases</td>
</tr>
<tr>
<td>two electron oxidation</td>
<td>methane monooxygenase</td>
<td>cytochrome P-450</td>
</tr>
</tbody>
</table>

**Heme Iron Peroxidase Mechanism**

Much is known about the mechanisms of the heme iron peroxidases and oxygenases, both from studies on the proteins themselves (Marnett, et al., 1986; Ortiz de Montellano, 1986) and from studies on heme iron model complexes (McMurry & Groves, 1986). In the case of the peroxidases (Marnett, et al., 1986), chemically
competent intermediates can be generated in the absence of reducing substrates, and these intermediates are sufficiently long-lived to be characterized. Two oxidized, reactive intermediates, designated compound I and compound II, are common to most of the peroxidases and have been extensively characterized. Both of these are competent to oxidize the relevant substrate by one electron. Compound I is formed by the heterolytic decomposition of an enzyme bound peroxide equivalent (Scheme 1.5). In this intermediate, which is oxidized by two electrons relative to the resting (ferric) enzyme, one oxidizing equivalent is stored as an oxyferryl (Fe(IV)) heme species, and a second is stored either as a porphyrin π-cation radical (as in the case of horseradish peroxidase) or as an amino acid radical (as in the case of yeast cytochrome c peroxidase). Compound II, which is formed by the one electron reduction of compound I, stores a single oxidizing equivalent as oxyferryl heme. (Although it is somewhat controversial (Coulson et al., 1971; Ho et al., 1983), some evidence suggests that the site of reduction of yeast cytochrome c peroxidase compound I may be the oxyferryl heme rather than the amino acid radical (Summers & Erman, 1988; Hazzard et al., 1987). In this case compound II would contain ferric heme and an amino acid radical. Nevertheless, cytochrome c peroxidase containing only oxyferryl heme has been prepared by reaction of the reduced (ferrous) enzyme with H₂O₂ (Ho, et al., 1983).

Cytochrome C Peroxidase Mechanism

During the course of this study, it has become apparent (in part due to information from the crystal structure of R2) that R2 and its reaction with O₂ may have particular analogy with yeast cytochrome c peroxidase (CCP) and its catalytic reaction. CCP is a monomeric heme enzyme which catalyzes the peroxide-dependent oxidation of ferrous cytochrome c (Marnett, et al., 1986). As alluded to above, the enzyme is unique among the peroxidases in the identity of one of the oxidizing equivalents of its compound I.
**Scheme 1.5:** General catalytic mechanism for the heme iron peroxidases.

![Mechanism Diagram]

 intermediate. Like compound I in other heme peroxidases, CCP compound I contains oxyferryl heme (Lang et al., 1976; Marnett, et al., 1986). In contrast to the other heme peroxidases (most notably horseradish peroxidase) (Schonbaum & Chance, 1976), CCP compound I does not contain a porphyrin π-cation radical, but instead stores its second oxidizing equivalent as an amino acid radical (Yonetani et al., 1966). This radical is
responsible for the axially symmetric, \( g = 2.0 \) EPR signal exhibited by CCP compound I. The radical species also exhibits a broad visible absorption band centered near 570 nm (Coulson, et al., 1971; Ho, et al., 1983). The magnetic behavior of CCP compound I indicates that the ferryl heme and radical interact weakly (Lang, et al., 1976; Hoffman et al., 1981; Lerch et al., 1981), which suggests that the sites are well separated. Site-directed mutagenesis applied in conjunction with crystallographic, spectroscopic and rapid kinetic methods have provided convincing evidence that the radical of CCP compound I results from one electron oxidation of W191. The crystal structure of CCP reveals that this residue is linked by hydrogen bonding with D235 to the proximal histidine (H175) of the heme (Fig. 1.6), suggesting that it might be capable of electron transfer to the heme (Finzel, et al., 1984). (As mentioned above, a similar hydrogen bonding network is found in R2. See Fig. 1.5.) The crystal structure of CCP compound I reveals small structural perturbations (relative to the resting enzyme) in the region of W191 (Edwards et al., 1987). A mutant CCP in which this residue is replaced by a less easily oxidized residue, F, fails to exhibit the axial \( g = 2.0 \) EPR signal upon reaction with \( H_2O_2 \), but instead exhibits a shorter-lived isotropic \( g = 2.0 \) signal (Scholes et al., 1989). The CCP(F191) mutant also forms a transient porphyrin \( \pi \)-cation radical which is not observed in the unaltered protein (Erman, et al., 1989). The W191→F mutation, while having only a small effect on the overall structure of CCP, greatly reduces the steady state rate at which ferrocytochrome c is oxidized by the enzyme and the rate at which the Fe(IV)=O form of the enzyme is reduced by ferrocytochrome c (Mauro, et al., 1988). These and other data have been interpreted as an indication that W191 is the radical site of CCP compound I, and that the residue is essential for electron transfer from cytochrome c to the heme site of CCP (Erman, et al., 1989).

Another aspect of CCP which is unique among the peroxidases (and is analogous to the R2 reaction) is the reactivity of its ferrous form with \( O_2 \). Whereas the reduced forms of other heme iron peroxidases react with \( O_2 \) to form metastable \( Fe^{2+}-O_2 \)
Fig. 1.6: Hydrogen bonding network in cytochrome c peroxidase which connects W191 to the heme (Finzel, et al., 1984).
Hydrogen Bonding Network in Cytochrome C Peroxidase
complexes (Wittenberg et al., 1967; Renganathan et al., 1985) which decay slowly to the ferric enzymes (Tamura & Yamazaki, 1972; Phelps et al., 1974), the reduced form of CCP reacts with O₂ to generate catalytically competent intermediates (Wittenberg et al., 1968; Anni et al., 1985). The reaction of ferrous CCP with O₂ in the presence of excess ferrocytochrome c results in oxidation of 3 equivalents of substrate, indicating that an intermediate forms which retains 3 of the oxidizing equivalents of O₂ (Miller et al., 1992). In the absence of substrate, an intermediate forms which is spectroscopically indistinguishable from compound I (Anni, et al., 1985), and the reaction results in cross-linking of the enzyme (Miller, et al., 1992). It has been proposed that the cross-linking results from formation of one or more tyrosyl radicals at the surface of the protein (Miller, et al., 1992). W191 of CCP is essential to the reactivity of its ferrous form with O₂, as CCP(F191), CCP(Q191), and CCP(H191) mutants of the enzyme, like other peroxidases and in contrast to the CCP(W191) protein, react with O₂ only to form Fe²⁺-O₂ complexes, which decay slowly and without detectable accumulation of intermediates to give back the ferric proteins (Miller, et al., 1992).

**Cytochrome P-450 Mechanism**

For cytochrome P-450, although the proposed activated intermediates have not been identified, a mechanism also involving high valent iron-oxo intermediates is currently accepted (Scheme 1.6) (Ortiz de Montellano, 1986). It has been proposed that a two-electron-oxidized intermediate (perhaps analogous to compound I of horse radish peroxidase) containing an unknown high valent iron species, abstracts a hydrogen atom from the substrate. Recombination of the substrate radical with an enzyme bound hydroxyl radical equivalent leads to the hydroxylated product and the resting ferric enzyme. Studies with inorganic model complexes have provided support for such a mechanism (McMurry and Groves, 1986).
Scheme 1.6: Hypothetical catalytic mechanism for cytochrome P-450.

Mechanisms Proposed for R2 based on Heme Peroxidases and Oxygenases

Prior to the work which is described herein, little information had been reported concerning the mechanism of the R2 reaction with O₂. Despite the lack of experimental evidence, several authors had seized upon the evident analogy between the dinuclear iron proteins and the heme iron proteins, and had proposed mechanisms for the R2 reaction.
(Fontecave, et al., 1990a; Ochiai et al., 1990; Sahlin, et al., 1990; Stubbe, 1990). Not surprisingly, the majority of the mechanisms put forth invoke high valent (Fe(IV) or Fe(V)) iron intermediates. A mechanism which is representative of those proposed is shown in Scheme 1.7 (Sahlin, et al., 1990). In this scheme, the diferrous cluster of R2 reduces O2 by two electrons to the peroxide oxidation state (as in hemerythrin). Subsequent to this step, the mechanism is quite reminiscent of that of the peroxidases. The peroxodiferrie intermediate decomposes by cleavage (in this case heterolytic cleavage is implied, but homolytic cleavage has also been proposed) of the O-O bond.

Scheme 1.7: Schematic mechanism for the R2 reaction which is representative of those proposed prior to this work.
The resulting intermediate is proposed to store its two oxidizing equivalents in the form of two Fe(IV) ions. Thus, this hypothetical intermediate is analogous to peroxidase compound I, with the exception that the second oxidizing equivalent is stored as a second Fe(IV) ion rather than as a porphyrin π-cation radical or as an amino acid radical. Successive one electron reductions of this compound I-like intermediate by Y122 of R2 and by an additional, exogenous reductant (no order of these reductions is implied, nor is the identity of the exogenous reductant specified) is proposed to generate the product, native R2.

**Current Working Hypothesis for the R2 Reconstitution Mechanism**

In contrast to mechanisms previously proposed for the R2 reaction, the results of this work indicate that the tyrosyl radical is not generated by a ferryl intermediate (although the participation in prior steps of a fleeting ferryl species is not ruled out). The simplest mechanism for the R2 reaction which is consistent with the data related in Chapters 2-7 is shown in Scheme 1.8. It is proposed that addition of Fe^{2+} at 5 °C in the presence of O_{2} to apo R2 results in the rapid (k_{obs} = 5-8 s^{-1}) formation of an intermediate, (I), which contains a tryptophan radical (perhaps at W48) and an intermediate diiron cluster. The intermediate cluster (X) does not contain Fe(IV), but instead consists of two high-spin ferric ions coupled to a ligand radical. X is therefore designated a "diferric-radical species." (The identity of the ligand radical is not known.) It is further proposed that I can partition between two distinct reaction pathways, and that the partition ratio depends on the availability of exogenous reducing equivalents (from Fe^{2+} or ascorbate). When the reaction is carried out with limiting Fe^{2+} (defined in Chapter 3), I accumulates and decomposes predominantly by intramolecular electron transfer from Y122 of R2 to the tryptophan radical (Pathway A, k_{obs} = 3-7 s^{-1}). This reaction generates II', which contains the tyrosyl radical (•Y122) and the diferric-radical cluster, X. II' reacts by electron transfer from Fe^{2+} to X, to give the product, native R2. When the reaction is
Scheme 1.8: Our current working hypothesis for the mechanism of the R2 reconstitution reaction. The circle representing R2 refers to one monomer of the dimer.

Pathway A
(limiting Fe$^{2+}$)

Pathway B
(excess Fe$^{2+}$ or lim. Fe$^{2+}$ and ascorbate)

\[
\text{Y122} \quad \text{apo R2} \quad \text{W48} \\
+ \quad 2\text{Fe}^{2+} + \text{O}_2 \\
k_{\text{obs}} = 5-10 \text{ s}^{-1}
\]

\[
\text{Y122} \quad (\text{Fe}^{3+})_2\text{L}^\cdot \\
\quad \cdot \text{W48} \\
k_{\text{obs}} = 3.4-5.5 \text{ s}^{-1} \quad k > 20 \text{ s}^{-1}
\]

\[
(\text{Fe}^{3+})_2\text{L}^\cdot = \text{X} \\
\text{L}^\cdot = \text{ligand radical}
\]

\[
\text{Y122} \quad (\text{Fe}^{3+})_2\text{L}^\cdot \\
\quad \text{W48} \\
k_{\text{obs}} = 0.72-1.0 \text{ s}^{-1}
\]

\[
\text{Fe}^{3+} \quad \text{O}^2- \quad \text{Fe}^{3+} \\
\quad \cdot \text{Y122} \\
\quad \quad \cdot \text{W48} \\
\text{native R2}
\]

\[
\text{e}^- \quad (\text{from Fe}^{2+} \text{ or from ascorbate})
\]
carried out with limiting Fe²⁺, a smaller fraction of I reacts by electron transfer from Fe²⁺ to the tryptophan radical (Pathway B). This reaction generates II, which contains only the diferric-radical cluster, X. II reacts by intramolecular electron transfer from Y122 to X (kobs = 0.7-1 s⁻¹), to generate the product, native R2. When the reaction is carried out with excess iron (defined in Chapter 3) or in the presence of ascorbate, I reacts exclusively by Pathway B and is converted to II sufficiently rapidly that I does not accumulate.

In the chapters that follow, the evidence which has led us to the mechanism of Scheme 1.8 is related. The results of Chapter 2 demonstrate that the "extra" electron required for electron balance (for converting II' to native R2 in Pathway A and I to II in Pathway B) is supplied either by Fe²⁺ or by ascorbate, and suggest that this electron may be delivered via intramolecular electron transfer from the opposite monomer of R2, as was proposed by Elgen and coworkers (Elgrecn, et al., 1991). The stopped-flow absorption experiments of Chapter 3 provide the first evidence for accumulation of intermediates in the reconstitution of R2 and suggest that more than one species can generate the tyrosyl radical. In this chapter, the 560 nm absorption transient associated with I is observed (Bollinger, et al., 1991a). This absorption band is ultimately the best direct evidence for the presence of a tryptophan radical. The schematic mechanism of Chapter 3, which contains no proposed structures, nevertheless serves as a basis for interpretation of the experiments of Chapters 4-7.

The rapid freeze-quench EPR experiments of Chapter 4 demonstrate the accumulation of a kinetically competent intermediate iron cluster (X), which is oxidized by one electron relative to the diferric cluster, and which is a component of II and of either I or II' (or both) (Bollinger, et al., 1991a). The experiments also indicate that either I or II' (or both) is associated with a broad g = 2.0 EPR resonance which is not a property of II. This broad signal is ultimately taken as evidence which supports the presence of a tryptophan radical in I.
In Chapter 5, the rapid-freeze quench method is employed to characterize the intermediate iron cluster, X, by Mössbauer spectroscopy. This characterization indicates that X consists of two high-spin ferric ions coupled to a ligand radical (Bollinger et al., 1991b). The identity of the radical component cannot be determined from these experiments, but speculation regarding its identity is presented.

In Chapter 6, the entire time-course of the reaction of apo R2 with excess Fe$^{2+}$ is followed by rapid freeze-quench Mössbauer spectroscopy. These experiments establish the consistency of the rapid freeze-quench results with the stopped-flow results, and demonstrate unambiguously that the intermediate X is a precursor to the diferric cluster. The results of these experiments provide convincing evidence for Pathway B of Scheme 1.8, and suggest, in apparent contradiction with the results of Chapter 2, that a ferric species distinct from the diferric cluster is produced when Fe$^{2+}$ donates an electron to convert I to II$'$.

In Chapter 7, the time-course of the reaction of apo R2 with limiting Fe$^{2+}$ is probed by rapid freeze-quench Mössbauer spectroscopy. The results of this chapter indicate that the diferric-radical cluster X is associated both with I and with II$'$. Thus, I must contain an additional species which stores its second oxidizing equivalent. The experiments also show that the 560 nm transient absorption is not associated with an Fe-containing species. Since previous results indicate that this transient arises from an EPR active, oxidized, the finding that this species does not contain Fe strongly suggests that it is an amino acid radical. The precedent of CCP compound I and studies on amino acid radicals generated by pulse radiolysis both suggest that this amino acid radical is a tryptophan radical, which is most likely protonated. The experiments of Chapter 7 indicate that formation of the diferric cluster lags behind formation of the tyrosyl radical, providing evidence for the intermediate II$'$. Finally, comparative analysis of the Mössbauer spectra of this chapter and the EPR spectra (obtained on samples prepared
identically to the Mössbauer samples) of Chapter 4 provides additional evidence for the occurrence of II'.

New Feature of Current R2 Mechanism

Many of the results described in Chapters 3-5 were previously published in two separate papers, and served as the basis for the R2 mechanism which we proposed therein (Bollinger, et al., 1991a; Bollinger, et al., 1991b). The major difference between our current working hypothesis (Scheme 1.8) and that which we previously put forth concerns the identities of the oxidizing equivalents in the intermediate I. On the basis of the similarity of the 560 nm absorption band which we detected in the limiting Fe$^{2+}$ reaction to that of an inorganic model complex (Menage et al., 1990), we previously proposed that I (then called U) contains a μ-peroxodiferric cluster. Several more recent results have led to the abandonment of this hypothesis in favor of the current one. First, the observation of the broad g = 2.0 EPR signal in R2-Y122F (Chapter 4) demonstrates that I is associated with a broad EPR feature. More importantly, in the rapid freeze-quench Mössbauer experiments of Chapter 7, no spectral feature is observed of the type which would be expected for the postulated μ-peroxodiferric cluster. In fact, no spectral feature whatsoever is observed which develops and decays with time in the manner of I. Moreover, the diferric-radical species (X) accumulates in a fashion which indicate that it is a component of I. Taken together, these results indicate that I contains X and an additional species, which does not contain Fe and which stores its second oxidizing equivalent. A protein radical is the only possible identity of such a species.

Two lines of evidence indicate that a tryptophan radical cation is the best candidate for the protein radical of I. First, the literature on pulse radiolysis of amino acids and proteins indicates that tryptophan radicals exhibit absorption bands at 325 nm and 510 nm in neutral or alkaline solution and at 335 nm and 560 nm in acidic solution (Adams et al., 1972; Posener et al., 1976; Solar et al., 1991). This literature suggests that
the tryptophan radical cation has a pK_a of 4.3 (Posener, et al., 1976; Jovanovic & Simic, 1985), and that deprotonation blue shifts the absorption bands. The bands observed for I (in Chapter 3) match exactly with those of the tryptophan radical cation. Furthermore, the pulse radiolysis literature provides direct precedent for the proposal that a tryptophan radical in I generates •Y122 (Pathway A). Electron transfer from Y to •W has been observed both in proteins (such as chymotrypsinogen A, concanavalin A and α-lactalbumin) (Prütz et al., 1980; Sloper & Land, 1980) and in peptides containing Y and W (Sloper and Land, 1980; Jovanovic et al., 1986). The second line of evidence which suggests that the amino acid of I is derived from a W residue comes from the literature on cytochrome c peroxidase. As mentioned above, evidence indicates that compound I of CCP contains a radical at W191 (Yonetani, et al., 1966; Ho, et al., 1983; Finzel, et al., 1984; Edwards, et al., 1987; Mauro, et al., 1988; Erman, et al., 1989; Scholes, et al., 1989; Miller, et al., 1992). Like I, CCP compound I exhibits a visible absorption band (centered near 570 nm) (Coulson, et al., 1971; Ho, et al., 1983) and a broad g = 2.0 EPR signal (Yonetani, et al., 1966). Furthermore, Nordlund (1990) has pointed out the similarity between the hydrogen bonding network in CCP which connects W191 to the heme site (Finzel, et al., 1984) and the network in R2 which connects W48 to Fe1 (Nordlund, et al., 1990). This insightful observation identifies W48 as the obvious candidate for the radical component of I. Thus, while the data are inconsistent with our original proposal that I contains a μ-peroxodiferrie cluster, they are entirely consistent with the current hypothesis that I contains the diferric-radical cluster (X) and a tryptophan radical cation.

References


Chapter 2: Stoichiometry of Reconstitution of

Native R2 from Apo R2, Fe$^{2+}$ and O$_2$
An essential step in studying any reaction mechanism is determining the stoichiometry of the reactants and the products. When this work was initiated, the available data were consistent with the formulation of the R2 cofactor assembly reaction which is shown in Scheme 2.1 (Atkin et al., 1973; Salowe, 1987). The reduction of $O_2$ to

**Scheme 2.1**: Electron counting formulation of the reconstitution of native R2 from apo R2, Fe$^{2+}$ and $O_2$.

$$\text{Y122} + 2\text{Fe}^{2+} + \text{O}_2 + e^- + 2\text{H}^+ \rightarrow \text{•Y122} + \text{Fe}^{3+}\text{-O}^2\text{-Fe}^{3+} + \text{H}_2\text{O}$$

the oxidation state of $\text{H}_2\text{O}$ requires four electrons. In the reconstitution reaction, two electrons are provided by the conversion of two ferrous ions to the diferric cluster, and a third is provided by the oxidation of Y122 to the tyrosyl radical (•Y122). As indicated, an additional electron is required for electron balance. It is reasonable to expect that Fe$^{2+}$ provides this fourth electron when the reaction is carried out in the absence of other reductants; thus, a stoichiometry of 3 Fe$^{2+}$ oxidized per •Y122 produced is predicted. When the reaction is carried out in the presence of an additional, facile one electron donor (ascorbate, for example), this reductant might provide the fourth electron. In this case, a stoichiometry of 2 Fe$^{2+}$ oxidized per •Y122 generated would be predicted. In the experiments which are described in this chapter, the stoichiometry of Fe$^{2+}$/•Y122 was determined in order to identify the source(s) of the fourth electron required for electron balance. Air-saturated solutions of apo R2 were titrated with known quantities of Fe$^{2+}$, in the presence and in the absence of the reductant ascorbate, and the quantity of tyrosyl radical produced was determined by EPR spectroscopy. These titrations were also monitored by UV-visible absorption spectroscopy and by enzymatic activity assay in order to determine the ratio of Fe$^{2+}$ to apo R2 required for complete reconstitution. In other experiments, $^{57}$Fe$^{2+}$ was added in varying amounts to anaerobic solutions of R2,
and the resulting ferrous-R2 complex was exposed to O2. Mössbauer spectra of these reconstituted samples were acquired in order to quantify the diferric cluster produced and to determine whether any other Fe3+ species is produced in the reaction.

While these experiments were in progress, other groups were also studying the stoichiometry of the R2 reconstitution reaction. Elgren and co-workers carried out a thorough study, measuring the Fe2+/•Y122 and O2/•Y122 ratios for the reaction, as well as the •Y122/R2 ratio after complete reconstitution (Elgren et al., 1991). This group also employed Mössbauer spectroscopy to determine whether any Fe3+ species other than the diferric cluster is produced, and extended their study to include a mutant R2 subunit (R2-Y122F) in which Y122 is replaced by the less easily oxidized residue, F. In reconstituting wild-type R2 (R2-wt) by addition of Fe2+ to aerobic solutions of apo R2 at 25 °C, these authors determined an Fe2+/•Y122 stoichiometry of 3.9 ± 0.5, which led them to conclude that Fe2+ provides the fourth electron. In addition, they measured an Fe2+/O2 ratio of 3.1 ± 0.18, and a final •Y122/R2 ratio of 1.0 ± 0.07. They examined the reconstituted R2 by EPR and Mössbauer spectroscopies and found that >92% of the added Fe had been incorporated into diferric clusters. Drawing on a previous report that added Fe3+ is not incorporated by R2 into dinuclear clusters (Petersson et al., 1980), the authors reasoned that, because virtually all of the Fe2+ added was incorporated, electron transfer among R2 monomers (intramolecular and intermolecular) must occur, allowing Fe2+ to supply the extra reducing equivalent without producing mononuclear Fe3+. They proposed the model for the reconstitution reaction which is shown in Scheme 2.2. Additional support for this model came from the O2 reaction of the R2-Y122F mutant, for which the group measured an Fe2+/O2 ratio of 3.9. As with R2-wt, >92% of the Fe2+ added was incorporated into diferric clusters. These results were taken as an indication that the electron obtained by oxidation of Y122 in R2-wt is supplied in R2-Y122F by Fe2+ bound at the dinuclear cluster binding site. Thus, they propose that, in the mutant protein, electron transfer among R2 monomers allows Fe2+ to provide two "extra" (in
Scheme 2.2: Schematic representation of the mechanistic model proposed by Elgren, et al. (1991) for the reconstitution of R2. The adjacent circles represent the two monomers of R2. The key features of the model are the postulated intra- and intermolecular electron transfer between Fe sites in the R2 monomers.
addition to the two provided by the diferrous cluster at which the O$_2$ reacts) electrons to complete the four electron reduction of O$_2$.

Ochiai and coworkers obtained quite different results in studying the reconstitution of the R2 subunit from mouse (Ochiai et al., 1990). These authors added known quantities of Fe$^{2+}$ to anaerobic solutions of apo R2, exposed the resulting complex to O$_2$, and quantified by EPR spectroscopy the tyrosyl radical produced. They determined an Fe$^{2+}$/tyrosyl radical ratio of 3.4 (averaging 6 of the 7 determinations which they report). They also found by EPR spectroscopy a significant quantity of mononuclear Fe$^{3+}$ ion (0.7 Fe$^{3+}$ per tyrosyl radical produced, averaging the values from the 6 experiments used above) at completion of the reaction. Later, this group found that paramagnetic metal ions (such as Mn$^{2+}$) added in low concentrations (10-100 μM) to native mouse R2 broaden the EPR spectrum of the tyrosyl radical (Ochiai et al., 1991). On the basis of these observations, they proposed that an additional (lower affinity) Fe$^{2+}$ binding site exists in R2 specifically to provide the fourth electron in the reconstitution reaction. Thus, these authors reached a distinctly different conclusion regarding delivery of the extra electron by Fe$^{2+}$ in mouse R2 than was drawn by Elgren et al. (1991) for the E. coli protein. The results of the two groups are discussed further below.

The results of the titration experiments described in this chapter provide support for the conclusions of Elgren et al. (1991) and Ochiai et al. (1991) that the fourth electron, which is required for electron balance, is provided by Fe$^{2+}$ when the reconstitution is carried out in the absence of other reductants. The Mössbauer results, as well as results from other titration experiments, suggest that the Fe$^{2+}$ which provides the extra electron is (in at least a fraction of events) incorporated into a diferric cluster, in agreement with the results of Elgren et al. (1991). As proposed by these authors, this finding raises the possibility of electron transfer among R2 monomers and provides a plausible explanation for the lower than theoretical •Y122 content of R2. Finally, the results of these experiments indicate that ascorbate can also provide the fourth electron,
an observation which provides a useful probe of the reconstitution mechanism, which is exploited in Chapters 3-5.

Materials and Methods

DEAE-Biogel A was purchased from BioRad. Sephadex G-25 and QAE-Sephadex were purchased from Sigma. S-200 was purchased from Pharmacia. $^{57}$Fe (95.1 atom%) metal was purchased from U.S. Services, Inc. All other chemicals were of the highest purity commercially available.

UV-visible absorption spectra were recorded on an HP-8452A Diode Array Spectrometer maintained at constant temperature with a Brinkmann Instruments Lauda K-2/R circulating water bath. X-band EPR spectra were recorded at 100 K on a Brüker Model ESP 300 spectrometer maintained at constant temperature with a Brüker ER4111VT variable temperature controller. Mössbauer spectra were recorded by Prof. B. H. Huynh at Emory University.

Purification of R2

Protein R2 was isolated from the overproducing strain N6405/pSPS2 as previously reported (Salowe & Stubbe, 1986), but with minor modifications. All steps in the purification were performed at 5°C. Frozen wet cell paste (50 g) was suspended in 250 ml of 50 mM Tris buffered at pH 7.6 (at 4 °C), containing 5% glycerol (hereafter referred to as Tris buffer). Phenylmethylsulfonylfluoride (PMSF) was added to 500 μM, (Sjöberg et al., 1986) and the cell suspension was passed through a french pressure cell at 12-14,000 psi to rupture the bacteria. To the cell lysate were added 0.2 g each of Fe(NH$_4$SO$_4$)$_2$ and sodium ascorbate dissolved in a small volume (<10 ml) of Tris buffer. The crude lysate was stirred for several minutes, and then centrifuged at 14,000 x g for 20 min. The supernatant was brought to 1% in streptomycin sulfate by the addition (over 10 min, with stirring) of 0.2 volumes of a 6% (w/v) solution in Tris buffer. This solution
was stirred for an additional 10 min, and then centrifuged at 14,000 x g for 20 min. The supernatant was brought to 60% saturation in (NH₄)₂SO₄ by addition of the solid (390 g/L), over 20 min, with stirring. This solution was stirred for an additional 30 min, and then centrifuged at 14,000 x g for 20 min. The pellet was redissolved in 40 ml of Tris buffer containing 500 μM PMSF. This solution was centrifuged at 14,000 x g for 10 min to pellet any remaining undissolved material, and was desalted through a 5 x 50 cm G-25 column equilibrated in Tris buffer. The fractions containing protein (typically 450 ml) were pooled and diluted with an equal volume of Tris buffer. This solution was brought to 500 μM in PMSF, and then loaded on a 5 x 19 cm DEAE-Biogel A column equilibrated (by washing the free amine form of the resin with ~5 column volumes or until the pH of the eluate reached 7.6) in Tris buffer. After it was loaded, the column was washed with 1.5 column volumes of Tris buffer containing 500 μM PMSF. The column was then washed with 1.6 column volumes of Tris buffer containing 110 mM NaCl and 500 μM PMSF. R2 was then eluted from the column with Tris buffer containing 140 mM NaCl and 500 μM PMSF. Fractions containing R2 were pooled (400-500 mL), and the pool was diluted with an equal volume of Tris buffer. PMSF was added to this solution to 500 μM, and the solution was loaded onto a 5 x 18 cm QAE-Sephadex column equilibrated (by washing with ~5 column volumes) in Tris buffer containing 70 mM NaCl. This column was washed with 2.5 volumes of Tris buffer containing 225 mM NaCl and 500 μM PMSF. R2 was then eluted from the column with Tris buffer containing 300 mM NaCl and 500 μM PMSF. Fractions containing R2 (typically 250-300 mL) were pooled and concentrated to 3-4 mL in an Amicon Diaflow Ultrafilter equipped with a PM-30 or with a YM-30 membrane (they worked equally well). This solution, which generally contained 500-600 mg of R2, was loaded onto a 2.5 x 110 cm S-200 column equilibrated in Tris buffer. (At this point PMSF was no longer required.) The column was eluted at 0.5 ml/min. Fractions which contained R2 were pooled, and the pool was concentrated by ultrafiltration to a final [R2] of >60 mg/ml, as judged by the
absorbance at 280 nm (assuming $\varepsilon_{280} = 131 \text{ mM}^{-1}\text{cm}^{-1}$ (Thelander, 1973)). The protein was frozen in liquid N$_2$ and stored at -80 °C. Isolation of R2 in this manner typically yielded 500 mg of >95% pure (as assessed by SDS-PAGE and coomassie blue staining) protein from 50 g of wet N6405/pSPS2 cell paste. The protein typically had a specific activity of 7500-8500 U/mg, when assayed by a previously described procedure (Thelander, et al., 1978).

**Preparation of Apo R2**

Apo R2 was prepared from the native protein as previously described (Atkin, et al., 1973; Salowe, 1987). The tyrosyl radical was reduced by a 10 min incubation at ambient temperature with 20-30 mM hydroxyurea. The resulting met R2 (30-50 mg/ml) was dialyzed at 5°C for 4-5 hr against the chelator solution described by Atkin, et al. (1973) (without the NH$_2$OH). The resulting dark green-to-black solution was passed through a G-50 column equilibrated either in 50 mM Tris buffered at pH 7.6 or in 100 mM HEPES buffered at pH 7.7 (depending on which buffer was desired for storage). Fractions containing R2 (as judged by $A_{280}$) were pooled, centrifuged to pellet denatured protein, and concentrated by ultrafiltration. Concentrated apo R2 (>60 mg/ml) was frozen in liquid N$_2$ and stored at -80 °C. The concentration of apo R2 was determined by absorbance at 280 nm (assuming $\varepsilon_{280} = 120 \text{ mM}^{-1}\text{cm}^{-1}$ (Thelander, 1973)).

**Titration of Apo R2 with Fe$^{2+}$**

Fe$^{2+}$ stock solutions were prepared by anaerobic dissolution of FeSO$_4$·7H$_2$O in 0.01 M HNO$_3$. The concentration of Fe$^{2+}$ ([Fe$^{2+}$]) was determined by a previously described colorimetric procedure which employs the ferroin chelator, ferrozine (Massey, 1957; Stookey, 1970; Salowe, 1987). Assays were carried out in the absence of ascorbate in order to determine [Fe$^{2+}$], and with ascorbate present in order to determine total [Fe] ([Fe$^{2+}$] + [Fe$^{3+}$]).
In titration 1, 215 µL aliquots of air-saturated 87 µM apo R2 in 100 mM HEPES buffer, pH 7.6 were allowed to reach equilibrium at 5 ºC by incubation for approximately one hour in a 5 ºC constant temperature room. Some of the aliquots were made 4.9 mM in sodium ascorbate immediately prior to addition of Fe²⁺. Varying amounts of the 1.92 mM Fe²⁺ stock were added (such that 0-8 molar equivalents of Fe²⁺ relative to apo R2 were delivered) to the samples, with repeated mixing (by inversion) immediately after addition. The samples were incubated for several minutes, and a 20 µL aliquot of each was diluted (for activity assay) to 400 µL with 50 mM HEPES buffer, pH 7.6, containing 20% glycerol. (Glycerol was used to ensure against protein precipitation which can occur upon freezing dilute solutions of R2. The samples for activity assay were stored frozen at -80 ºC until the next day, when the assays were carried out.) The specific activity of each of these diluted samples was determined at 25 ºC by using a previously reported coupled assay (Thelander et al., 1978). The assay mix contained in a final volume of 400 µL: 50 mM HEPES buffered at pH 7.6, 15 mM MgSO₄, 1 mM EDTA, 1.5 mM ATP, 0.16 mM NADPH, 1.0 mM CDP, 0.5-2.0 µg R2, 30 µg R1, 50 µg thioredoxin, and 3 µg thioredoxin reductase. Each concentrated sample (the entire volume) was diluted with 215 µL of 40% glycerol in H₂O (again, to ensure that the protein would not precipitate when it was frozen for EPR) and a UV-visible absorption spectrum of each was recorded at 5ºC. A 400 µL aliquot of each diluted sample (with glycerol) was transferred to an EPR tube and was frozen in liquid N₂. An EPR spectrum of each was acquired at 100 K with the spectrometer settings listed below. The concentration of tyrosyl radical in each sample was determined by double integration of the first derivative spectrum and comparison of the double integral to those of 3 different concentration standards. The first standard contained 0.973 mM CuSO₄, 2 M NaClO₄, 0.01 M HCl, and 20%(v/v) glycerol (Malmström et al., 1970). The second contained 0.343 mM CuSO₄ and 3.4 mM EDTA (Broman et al., 1962). The third contained 134 µM K₂(SO₃)₂NO in 50 mM HEPES buffer, pH 7.6, with 20% glycerol (Jones, 1963). For the R2 samples and the
Cu\textsuperscript{2+} standards, the microwave power was 1 mW, and for the K\textsubscript{2}(SO\textsubscript{3})\textsubscript{2}NO standard the power was 3.1 \mu W. (It was verified that the signals were not saturated at these powers.) Other EPR parameters were: microwave frequency, 9.38 GHz; modulation amplitude, 4G; modulation frequency, 100 kHz; center field, 3000 G; sweep width, 1000 G; resolution of field axis, 4096 data points per spectrum; scan time, 160 s; receiver gain, 5x10\textsuperscript{4}. Double integrals of samples and standards were corrected for difference in g-value, as previously described (Aasa & V"{a}nng"{a}rd, 1975). (It should be noted that, after correction for g-value, all three of the EPR spin concentration standards agreed with one another to within 3%).

In a titration experiment designed to determine the Fe\textsuperscript{2+}/R2 ratio required for complete reconstitution (titration 2), a 600 \mu L aliquot of 65 \mu M apo R2 in 50 mM HEPES buffered at pH 7.6 was placed in a 1 mL cuvette. The cuvette was allowed to come to equilibrium at ambient temperature (19 °C). For the titration in the presence of ascorbate, a 9 \mu L aliquot of 43 mM sodium ascorbate was added immediately before the titration was performed. For the titration in the absence of ascorbate, a 9 \mu L aliquot of H\textsubscript{2}O was added. Aliquots (5 \mu L) of a 1.95 mM Fe\textsuperscript{2+} stock solution prepared by dissolution of FeSO\textsubscript{4}·7H\textsubscript{2}O in 10 mM HNO\textsubscript{3} were added to the cuvette, with manual stirring immediately after addition. A UV-visible absorption spectrum was recorded after each addition.

Reconstitution Probed by M"{o}ssbauer Spectroscopy

An \textsuperscript{57}Fe\textsuperscript{2+} stock solution was prepared by anaerobic dissolution of 1.3 mg \textsuperscript{57}Fe foil in 50 \mu l 1 N H\textsubscript{2}SO\textsubscript{4}. (During dissolution, which took 4 days, the microcentrifuge tube containing the \textsuperscript{57}Fe was open to N\textsubscript{2}, leading to evaporation. The volume upon complete dissolution was approximately 10 \mu l.) The dissolved \textsuperscript{57}Fe was diluted anaerobically with 650 \mu l H\textsubscript{2}O. The \textsuperscript{57}Fe stock solution was assayed for [Fe\textsuperscript{2+}], and found to be 35.2 mM (theoretical 35.4 mM).
Apo R2 (1.7 mM) was dialyzed anaerobically against 100 mM HEPES buffered at pH 7.6 for 13-38 h. The protein was transferred via cannula to a stopped, argon-purged flask. In an anaerobic box, aliquots (76-94 µl) of the $^{57}$Fe$^{2+}$ stock solution were added to aliquots (1.0-1.1 ml of 0.62-1.4 mM) of apo R2. Samples were prepared containing 2, 3, and 4 equivalents of Fe$^{2+}$ relative to apo R2, with 153-191 µg of $^{57}$Fe per sample. (In the sample with 2 Fe$^{2+}$/R2, cloudiness was observed upon addition of Fe$^{2+}$ stock but disappeared immediately upon mixing.) The ferrous-R2 samples were transferred to Mössbauer sample cups, which were wrapped in parafilm in order to exclude oxygen. The samples were frozen in liquid nitrogen immediately upon removal from the anaerobic box. Mössbauer spectra of the ferrous-R2 samples were acquired at 4.2 K, verifying that anaerobiosis was complete. The samples were then thawed and incubated on ice for ~3 h, with occasional shaking and pipetting to facilitate diffusion of O$_2$ into the samples. Mössbauer spectra of the reconstituted samples were acquired.

Results

Titration of Apo R2 with Fe$^{2+}$

As apo R2 is titrated with Fe$^{2+}$ in the absence of sodium ascorbate (Fig. 2.1A) or in its presence (Fig. 2.1B), the absorption spectrum characteristic of the tyrosyl radical-diferric cluster cofactor develops. In addition, the tyrosyl radical content as determined by EPR (Figs. 2.2A and 2.3A), the absorbance at 410 nm (Figs. 2.2B and 2.3B), and the specific enzyme activity (Figs. 2.2C and 2.3C) all increase proportionally to the quantity of Fe$^{2+}$ added, until a critical ratio of Fe$^{2+}$/R2 is reached, at which point the reconstitution is complete. In the absence of ascorbate, the observed Fe$^{2+}$/R2 ratio at completion is 4.0 ± 0.1 and the •Y122/R2 ratio is 1.2 ± 0.1. From these values, a Fe$^{2+}$/•Y122 stoichiometry of 3.3 ± 0.3 is calculated. This ratio suggests that Fe$^{2+}$ provides the fourth electron under these conditions.
Fig. 2.1: Development of the absorption spectrum of the cofactor in the titration of air-saturated apo R2 with Fe$^{2+}$ (A) in the absence of ascorbate and (B) in the presence of ascorbate. The experimental conditions are given in Materials and Methods (titration 1).
Fig. 2.2: Titration of apo R2 with Fe\textsuperscript{2+} in the absence of ascorbate monitored (A) by absorbance at 410 nm (sharp feature of •Y122), (B) by equiv •Y122 produced (as determined by EPR), and (C) by specific enzyme activity. The values of A_410 are normalized to account for dilution during the titration. The figure refers to titration 1.
Fig. 2.3: Titration of apo R2 with Fe$^{2+}$ in the presence of 4.9 mM ascorbate monitored (A) by absorbance at 410 nm (sharp feature of •Y122), (B) by equiv •Y122 produced (as determined by EPR), and (C) by specific enzyme activity. The values of $A_{410}$ are normalized to account for dilution during the titration. The figure refers to titration 1.
When the titration is carried out in the presence of 4.9 mM sodium ascorbate, the Fe\(^{2+}\) ratio at completion is 3.1 ± 0.1 and the •Y122/R2 ratio is 1.2 ± 0.1. From these values, a Fe\(^{2+}/\cdot\text{Y122}\) stoichiometry of 2.6 ± 0.3 is calculated. Because this ratio is significantly less than that observed in the absence of ascorbate, and significantly less than the theoretical stoichiometry were ascorbate not to participate in the reaction (3 Fe\(^{2+}/\cdot\text{Y122}\)), this result indicates that ascorbate can also provide the fourth electron. The observed stoichiometry is significantly greater, however, than the theoretical limit (2 Fe\(^{2+}/\cdot\text{Y122}\)). Possible reasons for this discrepancy are presented below.

A Spectrophotometric Method for Tyrosyl Radical Quantitation

The numerous samples generated in these titration experiments made it possible to develop a method to quantify •Y122 by using UV-visible absorption spectra. The absorption feature of •Y122 at 410 nm is much sharper than any of the other spectral features of R2. When the absorbance at 410 nm (or 412 nm on our diode-array spectrometer) of a sample of R2 is corrected to the line defined by the absorbance at 406 nm and the absorbance at 416 nm (Fig. 2.4), only the sharp feature of the tyrosyl radical contributes (since any broader feature is well approximated by a straight line over this 10 nm wavelength range). Thus, this "dropline corrected" absorbance at 412 nm (\(A_{412,\text{dropline}}\)) should be directly proportional to [•Y122], irrespective of the conditions under which the R2 is reconstituted. Plotting \(A_{412,\text{dropline}}\) versus [•Y122] for several of the titration samples (Fig. 2.5) demonstrates that this proportionality holds. The slope of this plot gives the proportionality constant (\(\varepsilon_{412,\text{dropline}}\)) as 1.85 ± 0.05 mM\(^{-1}\)cm\(^{-1}\). With \(\varepsilon_{412,\text{dropline}}\) accurately determined, [•Y122] can now readily be calculated from the absorption spectrum of an R2 sample.

In the course of carrying out these experiments, it was discovered that \(\varepsilon_{412,\text{dropline}}\), and indeed the entire UV-visible absorption spectrum of R2, exhibits a significant dependence on temperature (Fig. 2.6). The spectra used to construct Fig. 2.5
Fig. 2.4: Definition of $A_{412, \text{dropline}}$, a spectrophotometric method to determine $\cdot Y_{122}$ concentration.
Fig. 2.5: Illustration of the proportionality between $A_{412,\text{dropline}}$ and the concentration of •Y122 as determined by EPR. The slope of the line is $\varepsilon_{412,\text{dropline}}$, which for this spectrophotometer at 5 °C is $1.85 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$. It should be emphasized that $\varepsilon_{412,\text{dropine}}$ varies significantly with temperature (see Table 2.1 and Fig. 2.6) and varies slightly from one spectrophotometer to another. It should therefore be determined separately for each instrument used.
Fig. 2.6: Temperature dependence of the light absorption spectrum of R2. The spectrum shown results from subtraction of the spectrum of 75 μM R2 at 33 °C from the spectrum at 1 °C. A_{412} of this sample was 0.427 at 5 °C.
Table 2.1: Temperature dependence of $A_{412,dropline}$.

<table>
<thead>
<tr>
<th>Temp. ($^\circ$C)</th>
<th>$A_{412,dropline}$</th>
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<tr>
<td>1</td>
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<tr>
<td>5</td>
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<tr>
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<td>0.1166</td>
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<td>0.1138</td>
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were acquired at 5 $^\circ$C. For experiments carried out at temperatures other than 5 $^\circ$C, $\varepsilon_{412,dropline}$ was corrected appropriately (Table 2.1). It should also be mentioned that $\varepsilon_{412,dropline}$, because of the sharpness of this absorption feature, is dependent on the spectral resolution and on the wavelength calibration of the spectrophotometer used. Therefore, $\varepsilon_{412,dropline}$ (or $\varepsilon_{410,dropline}$) was determined separately for each spectrometer which was used.

Reconstitution Reaction Probed by Mössbauer Spectroscopy

It is reasonable to expect that the Fe$^{3+}$ which is produced in the absence of ascorbate when Fe$^{2+}$ donates the fourth electron might be bound to R2 in mononuclear fashion, or might not be bound at all (in which case it would precipitate). To assess the fate of the Fe$^{2+}$ that provides the fourth electron, ferrous-R2 samples were prepared by anaerobic addition of varying quantities of $^{57}$Fe$^{2+}$ to apo R2. The samples were exposed to oxygen, and Mössbauer spectra were acquired. For both the sample containing 2 Fe$^{2+}$/R2 (Fig. 2.7A) and the sample containing 4 Fe$^{2+}$/R2 (Fig. 2.7B), >80% of the
Fig. 2.7: Mössbauer spectrum of R2 reconstituted (A) with 2.0 equiv $^{57}\text{Fe}^{2+}$ or (B) with 4.0 equiv $^{57}\text{Fe}^{2+}$. The spectra were acquired at 4.2 K with a magnetic field of 50 mT applied parallel to the $\gamma$-beam. The solid line plotted over the data in each is the spectrum of the diferric cluster scaled to 80% of the integrated intensity of the experimental spectrum. The arrows in A denote the features which are generally associated with "adventitiously bound" high-spin ferric ion.
absorption area of the Mössbauer spectra can be accounted for by the spectrum of the diferric cluster (solid line) (Lynch et al., 1989). If the Fe\textsuperscript{2+} which provides the fourth electron were to form some Fe\textsuperscript{3+} species other than the diferric cluster, only \( \sim 67\% \) of the absorption area should correspond to the diferric cluster, and \( \sim 33\% \) of the area should arise from this additional Fe\textsuperscript{3+} species. These observations suggest that the Fe\textsuperscript{2+} that provides the fourth electron is incorporated into a diferric cluster in at least a fraction of events.

*Continuous Titration of Apo R2 with Fe\textsuperscript{2+}*

Further evidence for this conclusion is provided by titration 2. The •Y122/R2 ratio which is observed at completion of a titration in which successive, small additions of Fe\textsuperscript{2+} are made to a single aliquot of apo R2 in the absence of ascorbate (1.06 •Y122/R2, Fig. 2.8A) is significantly less than in an identical titration carried out in the presence of 0.64 mM ascorbate (1.22 •Y122/R2, Fig. 2.8B). This result suggests that the extra Fe\textsuperscript{3+} produced when the reaction is carried out in the absence of ascorbate occupies the Fe-binding sites of R2, thus preventing production of a fraction of the •Y122 which would otherwise form.

*Discussion*

As stated above, the observed Fe\textsuperscript{2+}/•Y122 stoichiometry of 3.3 ± 0.3 in the absence of ascorbate suggests that Fe\textsuperscript{2+} provides the fourth electron under these conditions. In this qualitative aspect, our results are in agreement with those of Elgren, *et al.* (1991). Likewise, our finding from Mössbauer spectroscopy that >80\% of the added Fe\textsuperscript{2+} is incorporated into dinuclear clusters is consistent with the proposal by Elgren and coworkers that the Fe\textsuperscript{2+} which donates the fourth electron is (in at least a fraction of events) incorporated into a diferric cluster. This conclusion is further supported by the finding that, under some conditions, less tyrosyl radical is formed after complete
Fig. 2.8: Continuous titration of air-saturated apo R2 with Fe$^{2+}$ (A) in the absence of ascorbate and (B) in the presence of 0.64 mM ascorbate (titration 2). The Fe$^{2+}$/Y122 ratios calculated from this experiment were (A) 3.0 and (B) 2.5.
reconstitution when the reaction is carried out in the absence of ascorbate than when the reaction is carried out in the presence of ascorbate.

The quantitative agreement of our results with those of Elgren, et al. (1991) is, at best, fair. While the differences between our Fe^{2+}/\cdot \text{Y122} and \cdot \text{Y122}/R2 ratios and their values are less than the sum of the quoted experimental errors, our ratios are not within experimental error of the values which are predicted from their mechanistic model for the reaction (Fe^{2+}/R2 = 4, \cdot \text{Y122}/R2 = 1). Our ratios are more similar to the values of 3 Fe^{2+}/\cdot \text{Y122} and 1.33 \cdot \text{Y122}/R2 which would be predicted from the model shown in Scheme 2.3. Moreover, in other experiments of ours in which Fe^{2+} and R2 were rapidly mixed (see the stopped-flow experiments of Chapter 3), the observed Fe^{2+}/\cdot \text{Y122} ratio has been as low as 3.0 ± 0.2 and the \cdot \text{Y122}/R2 ratio as high as 1.3 ± 0.1. Thus, if the notion of electron transfer among R2 monomers is accepted, our data would seem to support the model of Scheme 2.3 in preference to the model of Elgren, et al. (1991). (The relatively minor difference between the two concerns the fate of the Fe(II), Fe(II) center which, in both models, is produced by intersubunit disproportionation of two Fe(II), Fe(III) centers. In the model of Elgren, et al. (1991), this Fe(II), Fe(II) center reacts with O_2 to generate only an Fe(III), Fe(III) cluster and no \cdot \text{Y122}. In the model of Scheme 2.3, one out of two of the Fe(II), Fe(II) centers reacts with O_2 to produce both an Fe(III), Fe(III) cluster and \cdot \text{Y122}.)

Another example of poor quantitative agreement between our results and those of Elgren, et al. (1991) concerns the Mössbauer spectra following reconstitution of R2. The spectra in our experiments reflect a much higher percentage of species other than the diferric cluster (~18-20%, compared to the 8% which they observed). It is interesting to note that the nature of these other species seems to be sensitive to the Fe^{2+}/R2 ratio in the reaction. The sample reconstituted with an Fe^{2+}/R2 ratio of 2.0 exhibits the magnetic features (denoted by arrows in Fig. 2.7A) generally associated with "adventitiously bound" high-spin ferric ion, whereas the sample with Fe^{2+}/R2 = 4.0 exhibits different,
**Scheme 2.3:** Derivative of the model of Elgren, et al. (1991) (Scheme 2.2) which is more consistent with our stoichiometry data.
unresolved features. The significant quantity of other species which we observe suggests that the Fe$^{3+}$ produced by delivery of the fourth electron is not always incorporated into a diferric cluster, which might indicate that a second mechanism (in addition to electron transfer from the opposite monomer) exists for delivery of the fourth electron by Fe$^{2+}$. Results of Chapter 6 provide support for this suggestion. In these experiments, a significant quantity (0.5 equiv relative to the quantity of diferric cluster produced) of a stable (or very slowly-decaying) ferric species is produced with a rate constant which is (according to our mechanistic hypothesis) identical to that at which Fe$^{2+}$ donates an electron to the assembling cluster. This observation is consistent with the results of Ochiai, et al. (1990) from their stoichiometry study on mouse R2. As stated above, these authors found that significant quantities of mononuclear ferric ion are produced in the reconstitution reaction, and proposed that a third binding site for Fe$^{2+}$ exists in mouse R2 to facilitate delivery of the fourth electron.

Thus, while the results of Elgren, et al. (1991) (and some of the results of this chapter) provide support for the hypothesis that electron transfer between paired R2 monomers allows Fe$^{2+}$ bound in the cluster binding site of one monomer to provide the fourth electron to the assembling cofactor in the other monomer, the results which are described in Chapter 6 and those of Ochiai, et al. (1990) support the notion that an additional Fe$^{2+}$ binding site may be present in R2 to provide the fourth electron. The discrepancies between results on the E. coli protein and those on the mouse protein could be a reflection of divergent evolution. Significant differences (involving aspects such as primary sequence (Thelander & Berg, 1986), susceptibility of the cofactor to reduction (Thelander et al., 1985), and tyrosyl radical content after reconstitution) between the proteins have been reported. For example, Ochiai and coworkers reported that the mouse R2 in their study contained 1.6 tyrosyl radicals per R2 dimer after complete reconstitution (Ochiai, et al., 1990), whereas reconstituted E. coli R2 was found in our study and in that of Elgren, et al. (1991) to contain 1.1–1.2 •Y122/R2. That the tyrosyl radical/R2 ratio more
closely approaches the theoretical value of 2 is consistent with the notion that the Fe$^{2+}$ which donates the fourth electron does not end up in a dinuclear cluster. Alternatively, the apparent discrepancies between results on the *E. coli* protein and those on the mouse protein, as well as the discrepancies between our results on the *E. coli* protein and those of Elgren, *et al.* (1991) (in the •Y122/R2 and Fe$^{2+}$/•Y122 ratios), might reflect differences in experimental protocol. Both in our titration experiments and in those of Elgren, *et al.* (1991), Fe$^{2+}$ was added to aerobic solutions of apo R2. However, the temperature at which the reactions were carried out differed in the two studies (5 °C in our experiments versus 25 °C in their experiments). It is conceivable that temperature might affect the observed Fe$^{2+}$/•Y122 and •Y122/R2 ratios or the fate of the Fe$^{3+}$ which is produced by delivery of the fourth electron. An effect on the Fe$^{2+}$/•Y122 ratio seems unlikely considering that titration 2 of this chapter was carried out at 19 °C, and that the observed Fe$^{2+}$/•Y122 stoichiometry was slightly less than that observed at 5 °C. An effect on delivery of the fourth electron is more plausible. Stopped-flow results indicate that intermediate I (see Scheme 1.8), which accumulates at 5 °C only when potential sources of the fourth electron are scarce (i.e. limiting Fe$^{2+}$ in the absence of ascorbate), also does not accumulate at temperatures of 25 °C or higher (Tong and Stubbe, unpublished results). This observation suggests that the temperature dependencies of the rate of formation of I and the rate at which it accepts the fourth electron are different. An effect of this sort might explain the apparent discrepancies with regard to the fate of the Fe$^{3+}$ which is produced by delivery of the fourth electron.

We have observed that the method of addition of Fe$^{2+}$ to aerobic R2 and rapidity of mixing subsequent to addition seem to have detectable effects on the Fe$^{2+}$/•Y122 stoichiometry, with addition of higher molar ratios of Fe$^{2+}$/R2 and delayed mixing leading to higher observed Fe$^{2+}$/•Y122 ratios. From the experimental procedure of Elgren, *et al.* (1991), it seems likely that their reaction mixtures were continuously stirred (although this is not explicitly stated). Thus, the reaction mixture in their experiments
should have become homogeneous as rapidly or more rapidly than in our experiments, a consideration which makes delayed mixing an unattractive explanation for the higher than optimal Fe^{2+}/Y122 stoichiometry observed in their experiments.

In the study of Ochiai, et al. (1990), Fe^{2+} was added to O_2-free solutions of apo R2. Thus, these authors allowed a ferrous R2 complex to pre-form before exposure to oxygen. This difference could potentially affect the fate of the Fe^{2+} which delivers the fourth electron. Finally, in our experiments of Chapter 6, an O_2-saturated solution of apo R2 was mixed with an excess of O_2-saturated Fe^{2+}. The difference in the O_2/R2 ratio might also affect the fate of the Fe^{2+} which donates the fourth electron.

The speculative nature of the above discussion reveals the current state of knowledge regarding the manner in which Fe^{2+} delivers the fourth electron to the assembling cluster. To date, the data are ambiguous as to whether this Fe^{2+} is bound in the dinuclear site of the opposite monomer (Elgren, et al., 1991) or in an as yet undefined "third" binding site (Ochiai, et al., 1990; Ochiai, et al., 1991). The main points of this chapter which are important in interpretation of later results are that Fe^{2+} donates the fourth electron in the absence of other reductants, and that ascorbate can also serve this function.

References


Chapter 3: The Reaction of Apo R2 with Fe\textsuperscript{2+}

and O\textsubscript{2} Probed by Stopped-Flow Absorption Spectroscopy
The distinctive UV-visible light absorption spectra of the diferric cluster (Fig. 3.1A) and of the tyrosyl radical (Fig. 3.1B) make the assembly of the cofactor of R2 ideally amenable to study by kinetic methods, as the formation of each of the cofactor's components can readily be monitored. In the experiments which are described in this chapter, the reaction of \textit{E. coli} apo R2, Fe$^{2+}$, and O$_2$ was studied by stopped-flow light absorption spectroscopy. Apo R2 was mixed with varying ratios of Fe$^{2+}$, and the development of the absorption spectrum of the cofactor was monitored. The kinetic behavior of the reaction was observed to depend markedly on the ratio of Fe$^{2+}$/R2 present in the reaction mixture. On the basis of the Fe$^{2+}$/R2 ratio employed, the experiments which were carried out can be divided into three categories: Fe$^{2+}$/R2 = 0-1.1 (hereafter referred to as sub-limiting Fe$^{2+}$); Fe$^{2+}$/R2 = 2.1-2.4 (limiting Fe$^{2+}$); and Fe$^{2+}$/R2 = 5-10 (excess Fe$^{2+}$). Experiments were also performed in which apo R2 was mixed with limiting Fe$^{2+}$ in the presence of ascorbate. The results of the above experiments provide evidence for the accumulation of at least two distinct, kinetically competent intermediates in the reaction, and suggest a mechanism involving a partition between two reaction pathways, with a different oxidizing species generating the tyrosyl radical in each pathway, and with the partition ratio between the pathways being controlled by the availability of exogenous reducing equivalents.

In addition to the stopped-flow experiments on wild-type R2 (R2-wt), identical experiments were carried out on a mutant R2 subunit in which the oxidizable Y122 was replaced by site-directed mutagenesis with the less easily oxidized residue, F. Previous work by Sjöberg and coworkers on the R2-Y122F mutant had established that it contains an unaltered diferric cluster but lacks the tyrosyl radical (Larsson & Sjöberg, 1986). (This was the result which established that the radical resides on Y122.) We studied the reaction of apo R2-Y122F with Fe$^{2+}$ and O$_2$ with the expectation that the intermediate(s) responsible for generating the tyrosyl radical in R2-wt would decay more slowly in the mutant subunit. Evidence described in this chapter suggests that both of the intermediates
**Fig. 3.1:** UV-Visible light absorption spectra of the diferric cluster (A) and of the tyrosyl radical (B). Spectra were obtained by subtraction, as described in Materials and Methods. Spectrum C is the sum of A and B.
detected in the reconstitution of R2-wt exhibit increased lifetimes in R2-Y122F, providing additional evidence that each may be responsible for tyrosyl radical production in R2-wt.

The results of this chapter provide insight into the binding of Fe\(^{2+}\) to apo R2, and represent the first evidence for the accumulation of intermediates in the R2 reconstitution reaction. In addition, they allow a schematic mechanism to be proposed for the reaction. This mechanism, though lacking in structural detail, nevertheless serves as the intellectual basis for the experiments which are described in Chapters 4-7.

**Materials and Methods**

**Materials**

β-Cyanoethyl diisopropyl phosphoramidites were purchased from Millipore. M13mp19, the kit for site-directed mutagenesis, and deoxyadenosine 5'-α-[\(^{35}\)S]thiotriphosphate (specific activity 40 Ci/mmol) were obtained from Amersham. Restriction enzymes and DNA modifying enzymes were purchased from Bethesda Research Laboratories, from International Biotechnologies, Inc. and from New England Biolabs. The Sequenase DNA sequencing kit was obtained from U.S. Biochemicals. *E. coli* strain K38 containing the plasmid pGP1-2 and strain HMS1741 containing the plasmid pT7-5 were the generous gifts of Prof. S. Tabor at the Harvard Medical School. Apo R2-wt was isolated as described in Chapter 2. Ferrozine and the Fe atomic absorption standard were purchased from Sigma.

**Preparation of Oligonucleotides for Mutagenesis and Sequencing**

Oligonucleotides for use in mutagenesis and sequencing were synthesized according to the manufacturer's instructions on a Biosearch 8600 DNA Synthesizer. Following detritylation of the final residue, oligomers were removed from the solid support by treatment with 29% ammonium hydroxide, and then were fully deprotected by
heating in this solution at 55 °C for 14 h. Oligomers for sequencing (listed in Table 3.1) were used without purification. The oligomer used in mutagenesis (5'-TCC-CGT-TCC-TTC-ACT-CAT-ATC-3') was purified by preparative gel electrophoresis on a 20% polyacrylamide gel. It was excised from the gel, eluted by soaking (with shaking) in 0.3 M sodium acetate at 37 °C for 12 h, and precipitated with ethanol in order to remove the sodium acetate.

Table 3.1: Oligonucleotide primers used in sequencing nrdB.

<table>
<thead>
<tr>
<th>Seq. Primer</th>
<th>First Base of nrdB added</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-25</td>
<td>5'-GCG-TAA-AAT-GCC-TTA-TCC-GGC-3'</td>
</tr>
<tr>
<td>2</td>
<td>125</td>
<td>5'-ACA-TCT-TCG-AAA-AGC-TGA-TCG-3'</td>
</tr>
<tr>
<td>3</td>
<td>278</td>
<td>5'-TCC-ATT-CAG-GGT-CGT-AGC-CCG-3'</td>
</tr>
<tr>
<td>4</td>
<td>443</td>
<td>5'-TCG-TCA-CCA-ACG-AGC-AGA-TCC-3'</td>
</tr>
<tr>
<td>5</td>
<td>575</td>
<td>5'-GTT-AGC-CTG-CGC-GAG-CTG-AAG-3'</td>
</tr>
<tr>
<td>6</td>
<td>717</td>
<td>5'-TTC-GCC-TGA-TTG-CCC-GCG-ACG-3'</td>
</tr>
<tr>
<td>7</td>
<td>854</td>
<td>5'-GTT-CAG-GCA-GCT-CAA-CAG-GAG-3'</td>
</tr>
<tr>
<td>8</td>
<td>1010</td>
<td>5'-TCC-AAC-CCG-ATC-CCG-TGG-ATC-3'</td>
</tr>
</tbody>
</table>

Testing of Expression System for R2-Y122F

It was anticipated that expression of the inactive R2 mutant, R2-Y122F, might be deleterious to the host cell. Therefore, the system developed by Tabor and Richardson for expression of toxic gene products was used (Tabor & Richardson, 1985). First, a vector (pTB2) was constructed to express R2-wt, in order to assess the efficacy of R2 overproduction with this system. The 2.6 kilobase pair PstI-StuI restriction fragment
(containing the nrdB gene, which encodes R2) from pLC19-24 was ligated into the multicloning region of pT7-5 (encoding ampicillin resistance), to give pTB2. K38 cells containing pGP1-2 (encoding kanamycin resistance and T7 RNA polymerase) were transformed with pTB2. Colonies resistant to both ampicillin and kanamycin were shown by restriction analysis to contain pTB2. These cells (K38-pTB2) were grown in enriched medium (per liter of medium: 20 g tryptone, 10 g yeast extract, 5 g NaCl, 2 g glycerol, 50 mM potassium phosphate buffered at pH 7.2) at 30 °C to an OD_{600} of 1.2, at which time expression of R2 was induced by heating the cells to 42 °C for 30 min. The cells were grown at 37 °C for an additional 3 h, after which they were harvested by centrifugation. The yield in this fermentation was 5 g of wet cell paste/L of culture. Isolation of R2 from these cells as described in Chapter 2 gave 38 mg pure (specific activity 8,000 U/mg) R2 from 10 g of wet cell paste.

Construction and Sequencing of nrdB-Y122F

Site-directed mutagenesis was performed according to the method of Taylor, et al. (1985) according to the instructions included in the Amersham kit. The template was prepared by ligation of the 2.6 kilobase pair EcoRI-PstI restriction fragment (containing nrdB) from pTB2 into the multicloning region of M13mp19, to give M13nrdB. Single stranded M13nrdB was isolated by a standard method (Sambrook et al., 1989) and was subjected to the mutagenesis procedure. Mutants were identified by sequencing of single stranded DNA from plaques which were obtained following mutagenesis. DNA sequencing was performed by the Sanger dideoxy chain termination method (Sanger et al., 1977) according to the instructions in the U.S. Biochemicals Sequenase kit. The sequence of the entire nrdB gene from one plaque (which was shown to contain the desired Y122→F mutation) was determined, in order to verify that no other mutations had been introduced.
Expression and Purification of R2-Y122F

The replicative form of M13nrdB-Y122F was isolated (from the plaque for which the entire nrdB sequence was verified) by a standard procedure (Sambrook, et al., 1989), and the 2.6 kilobase pair EcoRI-PstI restriction fragment (containing nrdB-Y122F) was ligated into pT7-5, to yield pTB2-Y122F. K38 cells containing pGP1-2 were transformed with pTB2-Y122F, and colonies resistant to ampicillin and kanamycin were shown by restriction analysis to contain pTB2-Y122F.

These cells (K38-pTB2-Y122F) were grown as described above for K38-pTB2. A typical yield in this fermentation was 2-3 g of wet cell paste per liter of culture. R2-Y122F was isolated from the cells as described in Chapter 2 for isolation of R2-wt. Apo R2-Y122F was prepared as described in Chapter 2 for preparation of apo R2-wt, except that the chelation procedure had to be performed twice in order to remove all Fe$^{3+}$ from the protein. (Following two chelation cycles, Fe assay of the apo R2-Y122F indicated that the Fe content was less than 0.1 equiv.) A typical yield of pure apo R2-Y122F was 600-800 mg per 100 g of wet cell paste.

Reconstitution of R2 Monitored by Stopped-Flow Absorption Spectroscopy

Stopped-flow experiments were carried out on three different apparatus. Survey experiments designed to detect intermediates with unknown spectra and experiments in which it was desirable to monitor multiple wavelengths in a single shot were carried out with an Applied Photophysics RX.1000 Rapid Kinetics Spectrometer Accessory in conjunction with a Hewlett Packard 8452A Diode Array Spectrometer. With this apparatus, both the rapid mixing accessory and the spectrometer were actuated manually, resulting in a long and somewhat variable deadtime (estimated to be 100-250 ms by comparison of data obtained on this apparatus to those obtained on the other two). Experiments which required increased time resolution or deadtime reproducibility were carried out either in the laboratory of Professor J. R. Norton at Colorado State University
on a Hi-Tech SF-41 Canterbury Stopped-Flow with an SU-40 spectrophotometer or in this laboratory on an Applied Photophysics DX.17MV Sequential Stopped-Flow Spectrofluorimeter.

Stopped-flow experiments were carried out in two different ranges of R2 concentration. In the "dilute protein" experiments, 40-100 μM apo R2 or apo R2-Y122F in 100 mM HEPES buffered at pH 7.7 was mixed with an equal volume of Fe$^{2+}$ solution containing 1-10 molar equivalents of Fe$^{2+}$. The Fe$^{2+}$ stocks for these experiments were prepared by dissolution of FeSO$_4$.7H$_2$O in 5 mM HNO$_3$, HCl, or H$_2$SO$_4$. (The identity of the acid was irrelevant.) The concentration of Fe$^{2+}$ was determined as described in Chapter 2. Typically, both the protein solution and the Fe$^{2+}$ solution were air-saturated in the "dilute protein" experiments. Thus, in these experiments, the concentration of O$_2$ should have been approximately 300 μM (Hitchman, 1978), an excess over apo R2 by at least a factor of 6. In some experiments, the Fe$^{2+}$ was maintained under an atmosphere of argon, but maintaining the Fe$^{2+}$ stock O$_2$-free was found to be unnecessary and was shown to have no effect on the reaction kinetics. The exact conditions employed in each experiment are given in the figure legends.

In the "concentrated protein" experiments, 550-640 μM apo R2 or apo R2-Y122F was mixed with an equal volume of Fe$^{2+}$ stock solution containing 2.2-5.1 molar equivalents of Fe$^{2+}$. The Fe$^{2+}$ stocks for these experiments were prepared as described below (in order to be consistent with the rapid freeze-quench experiments of Chapters 4-7). A 1.5-14 mg piece of Fe metal was dissolved anaerobically in 2N H$_2$SO$_4$. The volume of H$_2$SO$_4$ added was such that 4 molar equivalents of H$^+$, relative to Fe$^{0}$, were initially present. The Fe$^{0}$ and H$_2$SO$_4$ were heated at 60 °C to speed dissolution, which took 12-24 h. Upon complete dissolution, the Fe$^{2+}$ stock was assumed to contain 2 mol H$^+$ per mol Fe$^{2+}$, since dissolution of Fe$^{0}$ under these conditions consumes two mol H$^+$ per mol Fe$^{2+}$ produced (Scheme 3.1). This stock was diluted for storage with H$_2$O to 5 mM Fe$^{2+}$. In preparation of the Fe$^{2+}$ stocks for stopped-flow, an aliquot of the 5 mM

122
Fe\(^{2+}\) solution was diluted either with H\(_2\)O or with dilute H\(_2\)SO\(_4\), in order to give the desired concentration of Fe\(^{2+}\) in 2-3 mM H\(_2\)SC\(_4\). In order to ensure that O\(_2\) was in excess in these experiments, both the protein stock solution and the Fe\(^{2+}\) stock solution were saturated with 1 atm of O\(_2\) prior to their being loaded into the stopped-flow apparatus. Aliquots of the stock solutions were placed in separate 100 ml tonometers. The tonometers were connected to house vacuum and to a source of humidified O\(_2\). The tonometers were gently evacuated by opening to house vacuum. After several seconds, the vessels were refilled with H\(_2\)O-saturated O\(_2\). This vacuum/O\(_2\) cycle was repeated 6-8 times, and then the solutions were incubated on ice for 20-30 min. This routine of 6-8 cycles of vacuum/O\(_2\) followed by incubation on ice for 20-30 min was repeated 3-4 times. The solutions were then loaded into the stopped-flow apparatus. The exact conditions employed in each experiment are given in the figure legends.

**Scheme 3.1:** Reaction stoichiometry for anaerobic dissolution of iron metal in H\(_2\)SO\(_4\).

\[
\text{Fe}^0 + 2\text{H}^+ \rightarrow \text{Fe}^{2+} + \text{H}_2
\]

*Acquisition of Absorption Spectra of •Y122 and Diferric Cluster*

A 570 µL aliquot of 62 µM native R2 in 100 mM HEPES buffered at pH 7.6 was placed in a cuvette and an absorption spectrum was recorded. To this solution was added 30 µL of 1 M hydroxyurea. This solution was incubated at room temperature for 15 min to allow for complete reduction of the tyrosyl radical. An absorption spectrum of the resulting met R2 was acquired. The spectrum of met R2 was subtracted from that of the native protein, and the resulting difference spectrum was divided by the concentration of •Y122 (as determined by A\(_{412,\text{ext}}\), drop line), to give Fig. 3.1B, the spectrum of •Y122. The spectrum of apo R2 was then subtracted from that of met R2. This difference spectrum
was divided by the concentration of diferric cluster (which was taken to be the concentration of Fe\(^{3+}\) in the protein solution divided by 2) to give Fig. 3.1A, the spectrum of the diferric cluster. The Fe\(^{3+}\) concentration was determined by precipitation of the protein and extraction of the Fe\(^{3+}\) with HCl (Lynch et al., 1989), followed by assay of the supernatant with ferrozine (Salowe, 1987). A 350 \(\mu\)L aliquot of the 62 \(\mu\)M native R2 or of the 59 \(\mu\)M met R2 was acidified by addition of 650 \(\mu\)L of 4 N HCl. The resulting solution was incubated in boiling H\(_2\)O for 15 min. The sample was centrifuged to pellet the denatured protein, and the supernatant was transferred to a 2 mL volumetric flask. The pellet was twice washed with 500 \(\mu\)L portions of 2 N HCl, and the washes were added to the 2 mL volumetric flask. The flask was diluted to the mark with 2 N HCl. A 500 \(\mu\)L aliquot of this sample was treated with 20 \(\mu\)L of 75 mM ascorbic acid, 20 \(\mu\)L of 10 mM ferrozine, and 200 \(\mu\)L of saturated ammonium acetate. The absorbance at 562 nm of this sample was compared to those of a set of standards (prepared analogously) to which varying amounts of an Fe atomic absorption standard were added.

**Results**

*Reaction of Apo R2-wt with Excess Fe\(^{2+}\)*

When apo R2-wt is mixed at 5 °C with excess Fe\(^{2+}\) (5-10 molar equivalents), development of the absorption spectrum characteristic of the tyrosyl radical-diferric cluster cofactor is complete within 30 s. The time-dependent spectra of the reaction (Fig. 3.2) indicate that an intermediate rapidly accumulates prior to formation of the product cofactor. In the deadtime (dt) + 0.2 s spectrum of this representative experiment, 47% of the final absorbance at 360 nm has developed, while only 10% of the final •Y122 has formed (as assessed by \(A_{412,\text{dropline}}\)). Although this early spectrum seems to have the ~360 nm broad band associated with the diferric cluster, the 325 nm shoulder of the diferric cluster is absent. Therefore, this spectrum must arise from some species other than the diferric cluster. As the spectrum at completion is that characteristic of the
Fig. 3.2: Development of the absorption spectrum of the tyrosyl radical-diferric cluster cofactor upon mixing of apo R2-wt with excess Fe$^{2+}$ in the presence of O$_2$. The reaction conditions were: 27 µM apo R2-wt, 250 µM Fe$^{2+}$, 50 mM HEPES (air-saturated), pH 7.6, 5 °C. The spectra were acquired on the HP8452A diode array apparatus. The time after mixing at which each spectrum was acquired is indicated, with the deadtime of the apparatus denoted dt. The contribution to each spectrum from the protein has been subtracted away for clarity.
product cofactor, the species which exhibits the ~360 nm band must decay during the reaction, which implies that it is a kinetically competent intermediate.

The $A_{410,\text{dropline}}$-versus-time curve of the reaction (Fig. 3.3 shows a representative trace) illustrates that, after a significant lag phase, formation of $\cdot \cdot \cdot \cdot Y122$ is approximately first order. Non-linear least squares fitting of the equation for a first order growth to the region of this curve between 0.5 and 5 s (Fig. 3.3) gives an observed first order rate constant ($k_{\text{obs}}$) of $0.75 \pm 0.04 \text{ s}^{-1}$ (Table 3.2). (This value represents the average of results from 6 separate experiments, with the results of 3-6 individual trials averaged in each experiment. The quoted uncertainty is the difference between the mean and extreme values.) As illustrated in Table 3.2, in the range of apo R2 concentration ([apo R2]) which was investigated (22-290 μM), the $k_{\text{obs}}$ is independent of [apo R2]. The $\cdot \cdot \cdot \cdot Y122$/R2 ratio at completion (calculated from the magnitude of $A_{410,\text{dropline}}$ in each experiment) is also independent of [apo R2] in the reaction. The mean value of 1.19 $\cdot \cdot \cdot \cdot Y122$/R2 agrees well with the ratio determined in the titration experiments of Chapter 2.

The lag phase in the $A_{410,\text{dropline}}$-versus-time curve is consistent with the inference made from Fig. 3.2 that an intermediate accumulates prior to formation of $\cdot \cdot \cdot \cdot Y122$. In order to calculate a rate constant for formation of this intermediate, the 0-5 s regions of traces from 3 of the experiments of Table 3.2 were analyzed according to Eq. 3.1, which gives $A_{410,\text{dropline}}$ as a function of time ($A_{410,\text{dropline}}(t)$) for the hypothetical reaction sequence of Scheme 3.2, in which an intermediate species (B) intervenes between the reactant (A, corresponding to the Fe$^{2+}$-R2 complex) and the product cofactor (•Y122 and the diferric cluster) (Atkins, 1986). $A_{410,\text{dropline}}(t)$ is related to the concentration of $\cdot \cdot \cdot \cdot Y122$ at completion ([•Y122]$_{\infty}$, also equal to the initial concentration of species A), the dropline-corrected molar absorptivity at 410 nm of $\cdot \cdot \cdot \cdot Y122$ ($\varepsilon_{410,\text{dropline}}$), and the rate constants, $k_1$ and $k_2$. (The assumption is made in Eq. 3.1 that species A and B both have $\varepsilon_{410,\text{dropline}} = 0$.) A representative fit from this analysis is shown in Fig. 3.4, and the results are summarized in Table 3.3. The data are consistent with a rate constant
Fig. 3.3: $A_{410, dropline}$ used to assess the time-course of tyrosyl radical production in the reaction of apo R2-wt with excess Fe$^{2+}$. The reaction conditions were: 0.29(5) mM apo R2-wt, 1.4(5) mM Fe$^{2+}$, 50 mM HEPES (O$_2$-saturated), pH 7.6, 5°C. The data were acquired on the Applied Photophysics DX.17MV apparatus. The experimental trace was constructed as $A_{410}-(A_{404}+A_{416})/2$ by averaging 3 trials for each wavelength. The theoretical curve generated by fitting the equation for a first order growth to the 0.5 s to 5.0 s region of the trace to is also shown.
Table 3.2: Summary of rate constants for \( \cdot Y122 \) formation and of \( \cdot Y122/R2 \) ratios at completion for reaction of apo R2-wt with excess Fe\(^{2+}\).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>[Apo R2] (μM)</th>
<th>Fe(^{2+}/R2)</th>
<th>( k_{obs} ) (s(^{-1}))</th>
<th>( (\cdot Y122/R2)_\infty )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>4.9</td>
<td>0.78</td>
<td>1.20</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>9.5</td>
<td>0.76</td>
<td>1.12</td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>10.0</td>
<td>0.74</td>
<td>1.23</td>
</tr>
<tr>
<td>4</td>
<td>290</td>
<td>5.0</td>
<td>0.78</td>
<td>1.27</td>
</tr>
<tr>
<td>5</td>
<td>295</td>
<td>5.0</td>
<td>0.71</td>
<td>----</td>
</tr>
<tr>
<td>6</td>
<td>295</td>
<td>4.9</td>
<td>0.72</td>
<td>1.15</td>
</tr>
<tr>
<td>Average</td>
<td>----</td>
<td>----</td>
<td>0.75 ± 0.04</td>
<td>1.19 ± 0.08</td>
</tr>
</tbody>
</table>

\(^a\)Fits were made to 0.5-5 s of the experimental traces

Scheme 3.2

\[ A \xrightarrow{k_1} B \xrightarrow{k_2} \cdot Y122 + Fe^{3+}-O^{2-}-Fe^{3+} \]

Eq. 3.1

\[ A_{410,dropline}(t) = [\cdot Y122]_\infty e_{410,dropline} \left( 1 + \frac{k_1 \exp(-k_2 t) - k_2 \exp(-k_1 t)}{k_2 - k_1} \right) \]

Table 3.3: Summary of rate constants calculated from non-linear least-squares fitting of Eq 3.1 to \( A_{410,dropline} \)-versus-time traces from the reaction of apo R2-wt with excess Fe\(^{2+}\).

<table>
<thead>
<tr>
<th>Experiment(^a)</th>
<th>[Apo R2] (μM)</th>
<th>Fe(^{2+}/R2)</th>
<th>( k_1 ) (s(^{-1}))</th>
<th>( k_2 ) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>54</td>
<td>10.0</td>
<td>4.9</td>
<td>0.77</td>
</tr>
<tr>
<td>5</td>
<td>295</td>
<td>5.0</td>
<td>7.5</td>
<td>0.72</td>
</tr>
<tr>
<td>6</td>
<td>295</td>
<td>4.9</td>
<td>7.1, 5.9</td>
<td>0.73, 0.75</td>
</tr>
</tbody>
</table>

\(^a\)experiments are the same as those of Table 3.2
Fig. 3.4: Representative least-squares analysis (according to Eq. 3.1, Scheme 3.2) of $A_{410,\text{dropline}}$-versus-time trace from reaction of apo R2-wt with excess Fe$^{2+}$. The experimental trace is from Fig. 3.3. The theoretical trace corresponds to $k_1 = 6.4$ s$^{-1}$, $k_2 = 0.75$ s$^{-1}$, and $[\text{Y122}]_{\infty} L_{(410,\text{dropline})} = 0.133$. 
(k₁) of 4.9-7.5 s⁻¹ for formation of the inferred intermediate (B), and a rate constant (k₂) of 0.72-0.77 s⁻¹ for formation of •Y122.

In a further attempt to assess the validity of Scheme 3.2 and to determine values for k₁ and k₂, a multicomponent, multivariate analysis (Applied Photophysics Global Analysis) was applied to the time-dependent absorption spectra of the reaction. The spectra, which were constructed by acquisition of absorbance versus time traces at many wavelengths on the Applied Photophysics DX.17MV apparatus, are shown in Fig. 3.5. (These spectra differ somewhat from those of Fig. 3.2 only because the contribution due to the protein has not been subtracted away.) In fitting the kinetic model of Scheme 3.2 to the multivariate data, a k₁ of 3.9 s⁻¹ and a k₂ of 0.49 s⁻¹ are obtained if the values of both rate constants are allowed to vary. In this case, the calculated spectra of the intermediate and reactant are incompletely resolved from the spectrum of the product cofactor (Fig. 3.6A). The spectrum of the intermediate exhibits a sharp positive feature at 410 nm, while that calculated for the reactant (apo R2 + Fe²⁺) exhibits a sharp negative feature at this wavelength. (This incomplete resolution merely suggests that Scheme 3.2 is an oversimplified description of the reaction. As already shown in Fig. 3.4, the assumed model of two consecutive first order reactions results in imperfect fitting to the experimental data.) When k₂ is held fixed at 0.75 s⁻¹ (the mean value calculated from the experiments of Tables 3.1 and 3.2), a value of 5.4 s⁻¹ is calculated for k₁. In this case the spectra calculated for the three components (Fig. 3.6B) are satisfactorily resolved. (In other words, the spectrum of the reactant is featureless and that of the intermediate resembles the early spectra of Figs. 3.2 and 3.5.) Thus, assuming a rate constant of 0.75 s⁻¹ for conversion of the intermediate into the product cofactor, these data are consistent with a rate constant (k₁) of 5.4 s⁻¹ for formation of the intermediate. This value of k₁ agrees well with that obtained by analysis of the A₄₁₀,dropline versus time curves (Table 3.2).
Fig. 3.5: Development of the absorption spectrum of the diferric cluster-tyrosyl radical cofactor upon mixing of apo R2-wt with excess Fe$^{2+}$ in the presence of O$_2$. The spectra were constructed from absorbance-versus-time traces acquired at multiple wavelengths on the Applied Photophysics DX.17MV apparatus. The reaction conditions were the same as for Fig. 3.3.
Fig. 3.6: Spectra calculated for A, B, and the product cofactor (Scheme 3.1) from application of multicomponent, multivariate analysis to the time-dependent absorption spectra of the excess Fe$^{2+}$ reaction. A: spectra calculated from minimization in which both $k_1$ (3.9 s$^{-1}$) and $k_2$ (0.49 s$^{-1}$) were allowed to vary. B: spectra calculated from minimization in which $k_2$ was fixed at 0.75 s$^{-1}$, and $k_1$ (5.4 s$^{-1}$) was allowed to vary.
Reaction of Apo R2-wt with Limiting Fe\textsuperscript{2+}

Development of the absorption spectrum of the cofactor differs markedly in the reaction (at 5 °C) of apo R2 with limiting Fe\textsuperscript{2+} (2.0-2.4 molar equivalents) from that observed in the excess Fe\textsuperscript{2+} reaction. The limiting Fe\textsuperscript{2+} reaction requires 60-120 s to reach completion (depending somewhat on the exact ratio of Fe\textsuperscript{2+}/R2 and on the concentration of apo R2 employed), compared to less than 30 s for the excess Fe\textsuperscript{2+} reaction. In spite of this fact, and in spite of the fact that the final quantity of •Y122 produced is greater in the excess Fe\textsuperscript{2+} reaction, formation of •Y122 (as assessed by A\texttextsubscript{410,dropline}) is initially faster and has a significantly shorter lag phase in the limiting Fe\textsuperscript{2+} reaction (Fig. 3.7, trace A) than in the excess Fe\textsuperscript{2+} reaction (Fig. 3.7, trace B). This unusual, apparently negative kinetic order in Fe\textsuperscript{2+} was observed in each of 5 separate experiments (with multiple shots taken in each experiment), in which the [apo R2] ranged from 22 μM to 290 μM. Fig. 3.7 shows representative traces. At completion of the limiting Fe\textsuperscript{2+} reaction, the magnitude of A\texttextsubscript{410,dropline} indicates a Fe\textsuperscript{2+}/•Y122 stoichiometry of 3.0 ± 0.2 (Table 3.4). This ratio is consistent with the results of Chapter 2 and with the conclusion that Fe\textsuperscript{2+} supplies the required fourth electron.

In an attempt to extract rate constants for the processes leading to •Y122 production in the limiting Fe\textsuperscript{2+} reaction, non-linear least squares fitting to representative A\texttextsubscript{412,dropline}-versus-time traces was carried out. The significant lag phase in the curves suggests that, as in the excess Fe\textsuperscript{2+} reaction, an intermediate accumulates prior to production of •Y122. In fitting Eq. 3.1 to the curves, values calculated for k\textsubscript{1} and k\textsubscript{2} vary according to the fit range used. (This behavior is expected, since the multiphasic nature of the curves indicates that a 3-component model is of insufficient complexity to account for the data.) When the region between 0.01 and 0.80 s is fit (Fig. 3.8A), the values of k\textsubscript{1} and k\textsubscript{2} vary only slightly, with k\textsubscript{1} = 5.0-8.0 s\textsuperscript{-1} and k\textsubscript{2} = 3.6-5.4 s\textsuperscript{-1}. Increasing the fit-range beyond 0.80 s results in increasingly poor fits (Fig. 3.8B), obvious biasing of the rate constant for formation of •Y122 toward lesser values (due to contribution from the
Fig. 3.7: Time-course for formation of •Y122 (as assessed by A410,dropline) in the reaction of apo R2-wt with limiting Fe\textsuperscript{2+} compared to that in the reaction with excess Fe\textsuperscript{2+}. The reaction conditions were: 54 µM apo R2, 50 mM HEPES (air-saturated), pH 7.6, 5 °C, and A: 124 µM Fe\textsuperscript{2+}, or B: 504 µM Fe\textsuperscript{2+}. The data were acquired on the Applied Photophysics DX.17MV apparatus. The traces were constructed as A410-(A404+A416)/2 from the averages of 3 trials at each wavelength.
Fig. 3.8: Least-squares analysis of $A_{410,\text{dropline}}$-versus-time trace from the reaction of apo R2-wt with limiting Fe$^{2+}$. The reaction conditions were: 0.29(5) mM apo R2-wt, 0.67 mM Fe$^{2+}$, 50 mM HEPES (O$_2$-saturated), pH 7.6, 5 °C. The data were acquired on the Applied Photophysics DX.17MV apparatus. The experimental trace was constructed as $A_{410} - (3A_{406} + 2A_{416})/5$, from the averages of four trials at each wavelength. The theoretical curve in A was obtained by fitting Eq. 3.1 to 0.025-0.700 s of the experimental trace. It corresponds to $k_1 = 6.6 \text{ s}^{-1}$, $k_2 = 4.4 \text{ s}^{-1}$, and $[\cdot Y122]_{\infty}e_{410,\text{dropline}} = 0.044$. The theoretical curve in B was obtained by fitting Eq. 3.1 to 0.025-1.40 s of the experimental trace. It corresponds to $k_1 = 12.5 \text{ s}^{-1}$ $k_2 = 2.5 \text{ s}^{-1}$ and $[\cdot Y122]_{\infty}e_{410,\text{dropline}} = 0.050$. 
Table 3.4: Summary of Fe\(^{2+}/•\)Y122 stoichiometries measured in the reaction of apo R2-wt with limiting Fe\(^{2+}\).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>[Apo R2] ((\mu\text{M}))</th>
<th>Fe(^{2+}/\text{R2})</th>
<th>((•\text{Y122}/\text{R2})_\infty)</th>
<th>Fe(^{2+}/•)Y122</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>2.2</td>
<td>0.70</td>
<td>3.1</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>2.1</td>
<td>0.72</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>27</td>
<td>2.2</td>
<td>0.74</td>
<td>3.0</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
<td>1.8, 1.9, 2.1, 2.2, 2.3, 2.5</td>
<td>0.59, 0.65, 0.73, 0.77, 0.85</td>
<td>3.0, 2.9, 2.8, 3.0, 2.9</td>
</tr>
<tr>
<td>5</td>
<td>54</td>
<td>2.3, 2.5</td>
<td>0.79</td>
<td>2.9</td>
</tr>
<tr>
<td>6</td>
<td>295</td>
<td>2.2</td>
<td>0.77</td>
<td>2.9</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>-----</strong></td>
<td><strong>-----</strong></td>
<td><strong>-----</strong></td>
<td><strong>3.0 ± 0.2</strong></td>
</tr>
</tbody>
</table>

Slower phase(s) of •Y122 production), and biasing of the rate constant for formation of the intermediate toward greater values (Table 3.5). Thus, when analyzed according to Eq. 3.1, the A\(_{410}\), dropline traces of the limiting Fe\(^{2+}\) reaction are most consistent with a rate constant \((k_1)\) of 5-8 s\(^{-1}\) for formation of the inferred intermediate and a rate constant \((k_2)\) of 3.6-5.4 s\(^{-1}\) for the fast phase of •Y122 formation. Fig. 3.8A shows a representative experimental trace along with the fit corresponding to \(k_1 = 6.7\) s\(^{-1}\) and \(k_2 = 4.5\) s\(^{-1}\). The amplitude associated with \(k_2\) which is indicated by the least-squares analysis corresponds to 60% of the total amplitude of A\(_{410}\), dropline in this experiment. This result suggests that ~60% of the •Y122 produced in the limiting Fe\(^{2+}\) reaction is generated in the initial fast phase. The results of selected fits are summarized in Table 3.5.
Table 3.5: Summary of least-squares fits of Eq. 3.1 to the early portion of A$_{410,dropline}$-versus-time traces from the reaction of apo R2-wt with limiting Fe$^{2+}$.

<table>
<thead>
<tr>
<th>Experiment$^a$</th>
<th>fit range (s)</th>
<th>$k_1$ (s$^{-1}$)</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$A_{fit}/A_{total}^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5$^c$</td>
<td>0.010-0.700</td>
<td>5.1</td>
<td>5.0</td>
<td>0.66</td>
</tr>
<tr>
<td>6$^d$</td>
<td>0.025-0.400</td>
<td>5.6</td>
<td>5.0</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>0.025-0.600</td>
<td>5.5-6.7$^e$</td>
<td>4.4-5.4$^e$</td>
<td>0.61-0.62$^e$</td>
</tr>
<tr>
<td></td>
<td>0.025-0.800</td>
<td>5.6-8.0$^e$</td>
<td>3.6-5.0$^e$</td>
<td>0.62-0.64$^e$</td>
</tr>
<tr>
<td></td>
<td>0.025-1.00</td>
<td>9.7</td>
<td>3.0</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>0.025-1.20</td>
<td>11.2</td>
<td>2.7</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>0.025-1.60</td>
<td>13.9</td>
<td>2.3</td>
<td>0.72</td>
</tr>
</tbody>
</table>

$^a$experiments are the same as those in Table 3.4
$^b$the amplitude for A$_{410,dropline}$ given in the fit compared to the total amplitude at completion
$^c$4-6 trials averaged at each wavelength
$^d$3-4 trials averaged at each wavelength
$^e$varied within this range depending on the initial estimates for $k_1$ and $k_2$

Consistent with the temporal differences in •Y122 production, the time-dependent absorption spectra of the limiting Fe$^{2+}$ reaction differ significantly from those of the excess Fe$^{2+}$ reaction, especially in the first 1 s of the reaction. (Fig. 3.9 shows spectra which are representative of the 7 sets of trials which were carried out.) For example, at dt + 0.2 s of the excess Fe$^{2+}$ reaction (see Fig. 3.2), the sharp 410 nm feature of •Y122 is barely visible, and the broad ~360 nm feature of the intermediate species dominates the spectrum. In contrast, in the dt + 0.2 s spectrum of the limiting Fe$^{2+}$ reaction, the 410 nm peak is clearly visible, but there is no distinct feature from 320 nm to 400 nm, despite significant absorbance in this region. Nevertheless, as in the excess Fe$^{2+}$ reaction, the spectra of the limiting Fe$^{2+}$ reaction indicate that one or more intermediate accumulates. For example, at dt + 0.2 s, the absorbance at 346 nm (this wavelength is chosen arbitrarily
Fig. 3.9: Development of the absorption spectrum of the tyrosyl radical-diferric cluster cofactor upon mixing of apo R2-wt with limiting Fe$^{2+}$ in the presence of O$_2$. The reaction conditions were: 27 µM apo R2-wt, 59 µM Fe$^{2+}$, 50 mM HEPES (air-saturated), pH 7.6, 5 °C. The spectra were acquired on the HP8452A diode array apparatus. The time after mixing at which each spectrum was acquired is indicated, with the deadtime of the apparatus denoted dt. The contribution to each spectrum from the protein has been subtracted away for clarity.
for purposes of illustration) has reached 70% of its final value, while only 28% of the final quantity of •Y122 is present (as assessed by A412,dropline). This observation indicates that an intermediate contributes to the absorbance in the 346 nm region of the spectrum. The spectra of Fig. 3.9 also seem to indicate that •Y122 initially forms faster than does the diferric cluster. In the dt + 0.4 s spectrum, 48% of the final •Y122 is present, but it appears that much smaller percentages of the 325 nm shoulder and the 365 nm band of the diferric cluster are present. The precedence of •Y122 formation over that of the diferric cluster is also suggested by the 364 nm and 412 nm absorbance-versus-time traces. When the total change in absorbance at each wavelength is normalized to 1 and the curves are plotted together (Fig. 3.10), the increase in absorbance in the region dominated by the diferric cluster (364 nm, A) lags behind that in the region dominated by •Y122 (412 nm, B). If •Y122 does initially form faster than the diferric cluster, it would imply that an intermediate iron species accumulates.

Additional evidence for the accumulation of intermediates in the limiting Fe2+ reaction is provided by spectral changes in the 480-660 nm region. A broad, transient absorption band centered at 560 nm, which is not observed in the excess Fe2+ reaction (Fig. 3.11B, and elaborated below), develops to maximum intensity in the deadtime of the diode array apparatus, and decays on a time-scale comparable with formation of the product cofactor (Fig. 3.11A). In experiments carried out on the Hi-Tech apparatus or on the Applied Photophysics DX.17MV apparatus, the time of maximum absorbance (tmax) for this 560 nm transient was observed to vary between 0.18 and 0.25 s. (More than 7 experiments were carried out, with [apo R2] ranging from 10 μM to 290 μM.) Fig. 3.12 shows a representative trace. In an attempt to estimate rate constants for formation and decay of the species giving rise to the transient absorption and its molar absorptivity at 560 nm (ε560), non-linear least-squares analysis of A560-versus-time traces from three experiments was carried out. The 0-3 s region of the traces were analyzed according to the general equation (Eq. 3.2) for the absorbance as a function of time for a reaction
Fig. 3.10: Comparison of the time-courses of increase in absorbance at 364 nm and at 412 nm upon mixing of apo R2-wt with limiting Fe$^{2+}$ in the presence of O$_2$. The reaction conditions were: 22 μM apo R2-wt, 48 μM Fe$^{2+}$, 50 mM HEPES (air-saturated), pH 7.6, 5 °C. The data were acquired on the HP8452A apparatus. For purposes of comparison, the real absorbance values at each wavelength were multiplied by a constant in order to make the total change in absorbance (from dt to completion) equal to one.
Normalized Absorbance

412 nm

364 nm

Time (s)
Fig. 3.11: Detection of a transient visible absorption band centered at 560 nm in the reaction of apo R2 with limiting Fe$^{2+}$. The reaction conditions were (A): the same as in Fig. 3.9 (limiting Fe$^{2+}$), or (B): the same as in Fig. 3.2 (excess Fe$^{2+}$). The spectra were acquired on the HP8452A diode array apparatus. In A, the time after mixing at which each spectrum was acquired is indicated, with the deadtime of the apparatus denoted dt. In B, the times at which the spectra were acquired are the same as in A. The contribution to each spectrum from the protein has been subtracted away for clarity.
Fig. 3.12: Time-course of the 560 nm transient absorption in the reaction of apo R2-wt with limiting Fe$^{2+}$. The reaction conditions were: 54 μM apo R2-wt, 124 μM Fe$^{2+}$, 50 mM HEPES (air-saturated), pH 7.6, 5 °C. The data were acquired on the Applied Photophysics DX.17MV apparatus. The experimental trace represents the average of 3 trials. The $t_{\text{max}}$ of this trace is 0.22 s. The theoretical trace was obtained by fitting Eq. 3.2 (with $A_0$ fixed) to 0.005-3.00 s of the experimental trace, as described in the text. It corresponds to $k_1 = 6.0$ s$^{-1}$ and $k_2 = 4.0$ s$^{-1}$. 
involving two consecutive, first order processes (Scheme 3.3), where \( A_t \) is the absorbance of the reaction at any time \( t \), \( A_0 \) is the absorbance at \( t = 0 \), \( A_\infty \) is the absorbance at \( t = \infty \), and \( \alpha \) is related to the molar absorptivities of species A, B, and C (\( \varepsilon_A \), \( \varepsilon_B \), and \( \varepsilon_C \), respectively), the rate constants \( k_1 \) and \( k_2 \), and the concentration of species A at \( t = 0 \) ([A]0) by Eq. 3.3 (Espenson, 1981). (Although three components are insufficient to account for the data, fitting according to a model with four components would require use of an excessive number of parameters. The three component model is sufficient to provide estimates for the rate constants of formation and decay of the transient species.)

Fits were generated by two methods. In one method, the value of \( A_0 \) in the fit was fixed to the observed initial absorbance. In the second method, \( A_0 \) was allowed to vary. When the value of \( A_0 \) is fixed, the analysis gives values of 5.3-8.8 s\(^{-1}\) for one rate constant, and values of 3.4-5.5 s\(^{-1}\) for the second rate constant (Fig. 3.12 shows a representative fit).

Due to the existence of dual solutions in consecutive reactions (Espenson, 1981), and because the value of \( \varepsilon_B \) is not known, it is not possible to deduce \textit{a priori} which rate constant corresponds to formation of the 560 nm absorbing intermediate (\( k_1 \)) and which corresponds to its decay (\( k_2 \)). Nevertheless, the values for the larger \( k_{\text{obs}} \) correspond well with the range of 5.1-8.0 s\(^{-1}\) calculated for \( k_1 \) by fitting the \( A_{412, \text{dropline}} \) versus time curves. Likewise, the range for the smaller \( k_{\text{obs}} \) agrees well with the range of 3.6-5.4 s\(^{-1}\)

\[
\text{Scheme 3.3} \quad \begin{array}{ccc}
  & k_1 & \\
 A & \rightarrow & B \\
 \downarrow & & \downarrow \\
 & k_2 & \\
 & B & \rightarrow & C \\
\end{array}
\]

\[
\text{Eq. 3.2} \quad A_t = A_\infty + \alpha \exp(-k_1 t) + (A_0 - A_\infty - \alpha) \exp(-k_2 t)
\]

\[
\text{Eq. 3.3} \quad \alpha = \frac{[(\varepsilon_B - \varepsilon_A)k_1 + (\varepsilon_A - \varepsilon_C)k_2]}{(k_2 - k_1)} \times [A]_0
\]
calculated for $k_2$ by fitting the $A_{412,\text{dropline}}$-versus-time curves. Therefore, an accordant interpretation of the data is that the 560 nm absorbing intermediate generates $\cdot Y_{122}$ in the fast phase of the limiting $Fe^{2+}$ reaction. In this interpretation, the $k_{\text{obs}}$ of 5.3-8.8 s$^{-1}$ corresponds to formation ($k_1$) of the intermediate, and the $k_{\text{obs}}$ of 3.4-5.5 s$^{-1}$ corresponds to its decay ($k_2$) (as well as to the fast phase of $\cdot Y_{122}$ production). This analysis suggests that rate constant for formation of the 560 nm absorbing species in the limiting $Fe^{2+}$ reaction ($k_1 = 5.3-8.8$ s$^{-1}$) is identical (within the experimental variation) to the rate constant for formation of the 360 nm absorbing intermediate ($k_1 = 4.9-7.5$) in the excess $Fe^{2+}$ reaction.

As demonstrated by Fig. 3.12, the data are poorly fit according to the oversimplified kinetic model of Scheme 3.3. In the rise phase of the transient, the experimental trace lags behind the theoretical trace. This observation suggests that there is a lag phase in formation of the 560 nm absorbing intermediate, which in turn suggests that some intermediate accumulates prior to the absorbing species. In the region of $t_{\text{max}}$, the theoretical curve does not rise and fall as sharply as the experimental trace, and fails to reach as great a maximum absorbance as the experimental trace. These observations suggest that the values of $k_1$ and $k_2$ obtained in the fit may be underestimates of the true values. The shape of the experimental trace in the region of $t_{\text{max}}$ can be more accurately reproduced if the lag phase is excluded from the fit-range and if the value of $A_0$ is allowed to vary in the fit (Fig. 3.13). Fitting to the experimental traces in this manner gives maximum values for the two rate constants of 9.7 s$^{-1}$ and 6.6 s$^{-1}$, indicating that fitting with $A_0$ fixed at most only slightly underestimates the rate constants. Results obtained in analysis of the $A_{560}$-versus-time traces are summarized in Table 3.6.

From the parameters obtained in fitting the $A_{560}$ versus time curves, the molar absorptivity of the intermediate ($\varepsilon_B$ in Eq. 3.3) can be estimated. Solving Eq. 3.3 for $\varepsilon_B$ gives Eq. 3.4. In modelling the reaction according to Scheme 3.3 (to which Eqs. 3.2-3.4 apply), species A corresponds either to an $Fe^{2+}$-R2 complex or to $Fe^{2+}$ and apo R2 in
Fig. 3.13: Least-squares fit of Eq. 3.2, with $A_0$ allowed to vary, to the $A_{560}$-versus-time trace from the limiting $\text{Fe}^{2+}$ reaction. The reaction conditions were the same as in Fig. 3.8. The experimental trace was acquired on the Applied Photophysics DX.17MV apparatus and represents the average of 4 trials. The $t_{\text{max}}$ of this trace is 0.185 s. The theoretical curve was obtained by fitting to 0.035-0.695 s of the experimental trace (as described in the text), and corresponds to $k_1 = 9.7 \text{ s}^{-1}$, $k_2 = 6.6 \text{ s}^{-1}$. 
Table 3.6: Summary of selected least-squares fits to Eq. 3.2 of A560-versus-time traces from the reaction of apo R2-wt with limiting Fe²⁺.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>$t_{max}$ (s)</th>
<th>Fit-range (s)</th>
<th>A₀</th>
<th>$k_1$ (s⁻¹)ᵇ</th>
<th>$k_2$ (s⁻¹)ᵇ</th>
<th>$\varepsilon_{560}$ (mM⁻¹cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5ᵈ</td>
<td>0.220</td>
<td>0.005-3.00</td>
<td>fixed</td>
<td>5.5</td>
<td>4.4</td>
<td>1,900</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.005-3.00</td>
<td>fixed</td>
<td>3.8</td>
<td>6.2</td>
<td>2,800</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.005-0.945</td>
<td>fixed</td>
<td>6.6</td>
<td>3.4</td>
<td>1,700</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.055-1.10</td>
<td>varied</td>
<td>7.2</td>
<td>6.7</td>
<td>1,500</td>
</tr>
<tr>
<td>6ᵈ</td>
<td>0.185</td>
<td>0.005-2.50</td>
<td>fixed</td>
<td>6.7</td>
<td>5.5</td>
<td>2,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.005-2.50</td>
<td>fixed</td>
<td>5.4</td>
<td>6.9</td>
<td>2,300</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.035-0.695</td>
<td>varied</td>
<td>9.7</td>
<td>6.6</td>
<td>2,900</td>
</tr>
</tbody>
</table>

ᵃExperimental conditions are identical to those in the corresponding experiments of Table 3.4
ᵇThe assignment of one $k_{obs}$ as $k_1$ and the other as $k_2$ is arbitrary, since the relative magnitudes of $k_1$ and $k_2$ depend on the initial estimate for $\alpha$
ᶜcalculated according to Eq. 3.5, as described in the text
ᵈA560-versus-time trace analyzed represents the average of 3 trials

solution. Neither of these species absorbs at 560 nm, so $\varepsilon_A$ of Eq. 3.4 can be set to zero. (Since species A does not absorb, the non-zero A₀ is merely an offset to account for the small amount of light scattering by the protein.) Eq. 3.4 thus reduces to Eq. 3.5. $[A]_0\varepsilon_C$ is simply the value of $A_\infty$-$A_0$ from the fit. $[A]_0$ can be set to the concentration of •Y122 at completion, which is 1/3 the Fe²⁺ concentration after mixing. With these assumptions, the values calculated for $\varepsilon_B$ at 560 nm from all the fits described above range from 1500 M⁻¹cm⁻¹ to 2900 M⁻¹cm⁻¹ (Table 3.6). (However, the above analysis of the A410,dropline versus time traces suggests that only ~60% of the •Y122 generated in the reaction is produced in the fast phase. This result suggests that the value of $[A]_0$ used above is an...
overestimate, and should be multiplied by 0.6. The range calculated for \( \varepsilon_B \) would then be
2500-4800 M\(^{-1}\)cm\(^{-1}\).)

\[
\text{Eq. 3.4} \quad \varepsilon_B = \frac{\alpha(k_2 - k_1) - [A]_0(\varepsilon_A - \varepsilon_C)k_2}{[A]_0k_1} + \varepsilon_A
\]

\[
\text{Eq. 3.5} \quad \varepsilon_B = \frac{\alpha(k_2 - k_1) + [A]_0\varepsilon_Ck_2}{[A]_0k_1}
\]

*Titration to Determine Fe\(^{2+}/R2\) Required to Suppress 560 nm Transient*

As demonstrated above, the 560 nm transient absorption band is not observed in the reaction of apo R2 with excess Fe\(^{2+}\) (see Fig. 3.11B). A stopped-flow titration was carried out to determine the ratio of Fe\(^{2+}/R2\) required to completely suppress the transient (Fig. 3.14). In the reaction of apo R2 with 4.0 molar equivalents of Fe\(^{2+}\), the transient is still observed, but the amplitudes of the rise and fall are decreased markedly with respect to the reaction with Fe\(^{2+}/R2 = 2.3\), and the \( t_{\text{max}} \) is increased. Increasing the Fe\(^{2+}/R2\) ratio to 5.0 further reduces the amplitudes of the rise and fall and further increases the \( t_{\text{max}} \). Fitting Eq. 3.2 to the trace from this reaction gives a \( k_1 \) of 6.0 s\(^{-1}\) and a \( k_2 \) of 0.48 s\(^{-1}\) (Fig. 3.15). These values agree reasonably well with those obtained from fitting to the \( A_{412,\text{dropline}} \) versus time traces from the excess Fe\(^{2+}\) reaction and from the multicomponent analysis. This agreement suggests that the contribution to this trace from the 560 nm absorbing species (which decays with a rate constant much greater than 0.48 s\(^{-1}\)) is minimal, and that the two phases observed are due to formation and decay of the intermediate which exhibits the broad 360 nm band. (This interpretation would indicate that the intermediate has a slightly greater molar absorptivity at 560 nm than does the product cofactor.) Further increasing the Fe\(^{2+}/R2\) ratio to 10 has negligible
Fig. 3.14: Stopped-flow titration to determine the ratio of Fe$^{2+}$/R2 required to suppress the 560 nm transient. Reaction conditions were: 54 μM apo R2-wt, 50 mM HEPES (air-saturated), pH 7.6, 5 °C, and (A) 124 μM Fe$^{2+}$; (B) 217 μM Fe$^{2+}$; (C) 270 μM Fe$^{2+}$; or (D) 504 μM Fe$^{2+}$. The data were acquired on the Applied Photophysics DX.17MV apparatus, and each trace represents the average of 3-5 trials.
Fig. 3.15: Least-squares fit of Eq. 3.2 to the A₅₆₀-versus-time trace from the reaction with Fe²⁺/R2-wt = 5.0. The experimental trace is C from Fig. 3.14. The theoretical trace corresponds to $k_1 = 6.0 \text{ s}^{-1}$ and $k_2 = 0.48 \text{ s}^{-1}$. 
qualitative effect on the $A_{560}$ versus time trace, a result which also suggests that accumulation of the transient species is completely (or almost completely) suppressed by an Fe$^{2+}$/R2 ratio of 5. In the experiment of Fig. 3.14, [apo R2] in the reaction was 54 $\mu$M, but identical results were obtained in experiments in which [apo R2] ranged from 27 $\mu$M to 290 $\mu$M. Thus, suppression of the 560 nm transient by Fe$^{2+}$ is unrelated to the absolute R2 or Fe$^{2+}$ concentration, and depends only upon the ratio of the two.

*Reaction of Apo R2-wt with Limiting Fe$^{2+}$ and Ascorbate*

The ability of ascorbate to participate in the reconstitution reaction by donating the required fourth electron was established in the experiments of Chapter 2. In the hope of using the reductant as a mechanistic probe, stopped-flow reconstitution experiments were carried in its presence. As illustrated in Fig. 3.16, the 560 nm transient is not observed when apo R2 reacts with limiting Fe$^{2+}$ in the presence of 2.5 mM ascorbate. (Fitting the equation for a first order growth to the 0.04-0.54 s region of this curve gives a rate constant of 7.4 s$^{-1}$, in good agreement with the formation rate constants calculated for both the limiting and the excess Fe$^{2+}$ reactions.) Thus, the presence of either excess Fe$^{2+}$ or ascorbate can suppress the transient. The simplest interpretation of these observations is that either of these reagents can reduce the absorbing intermediate sufficiently rapidly to prevent its accumulation. Associated with the failure of the intermediate to accumulate is a marked slowing of •Y122 production in the presence of ascorbate relative to the limiting Fe$^{2+}$ reaction in its absence (Fig. 3.17). This slowing occurs in spite of the fact that more •Y122 is produced in the presence of ascorbate (the magnitude of $A_{410,\text{dropline}}$ at completion in two sets of trials indicates an Fe$^{2+}$/•Y122 stoichiometry of 2.5 ± 0.1). Thus, an invariant correlation emerges between the accumulation of the 560 nm absorbing intermediate and the presence of a fast ($k_{\text{obs}} = 3.6$-5.4 s$^{-1}$) phase of •Y122 formation. This correlation is consistent with the suggestion
Fig. 3.16: Effect of ascorbate on the 560 nm transient absorption in the reaction of apo R2-wt with limiting Fe$^{2+}$. Reaction conditions were: 54 μM apo R2-wt, 50 mM HEPES (air-saturated), pH 7.6, 5 °C, and (A) 124 μM Fe$^{2+}$; or (B) 126 μM Fe$^{2+}$ and 2.5 mM ascorbate. The data were acquired on the Applied Photophysics DX.17MV apparatus. Each trace represents the average of 3-4 trials.
Fig. 3.17: Effect of ascorbate on the time-course of •Y122 formation (as assessed by \( A_{410,dropline} \)) in the reaction of apo R2-wt with limiting Fe\(^{2+}\). The reaction conditions were the same as for Fig. 3.16. The data were acquired on the Applied Photophysics DX.17MV apparatus, and the traces were constructed as \( A_{410} - (A_{404} + A_{416})/2 \), from the averages of three trials at each wavelength.
made above that the 560 nm absorbing intermediate generates •Y122 in the fast phase of
the limiting Fe$^{2+}$ reaction.

Reaction of Apo R2-wt with Sub-Limiting Fe$^{2+}$

When apo R2 is mixed at 5 °C with sub-limiting Fe$^{2+}$ (Fe$^{2+}$/R2 = 1.1), formation
of •Y122 is much slower overall than in either the limiting Fe$^{2+}$ or the excess Fe$^{2+}$
reactions, and is clearly multiphasic (Fig. 3.18). The reaction requires 120 s to reach
completion, at which time the magnitude of A$_{412}$_dropline indicates an Fe$^{2+}$/•Y122
stoichiometry of 3.0 (average of 3 trials).

Reaction of Apo R2-Y122F with Excess Fe$^{2+}$

When apo R2-Y122F is mixed at 5 °C with excess Fe$^{2+}$, a broad absorption band,
which is centered near 360 nm and which is apparently identical to that observed in the
reaction of apo R2-wt with excess Fe$^{2+}$, rapidly develops (Fig. 3.19, dt + 0.4 s). As in the
R2-wt reaction, the rapid development of this band is not accompanied by development
of the 325 nm shoulder characteristic of the diferric cluster. The band must therefore
arise from an intermediate, which is presumably the same species which accumulates in
the reaction of apo R2-wt with excess Fe$^{2+}$. This result indicates that Y122 is not
required for formation of the intermediate. With time, the spectrum of the diferric cluster
develops, but the reaction appears to proceed more slowly than in R2-wt. Fitting the
general equation for two consecutive, first order reactions (Espenson, 1981) to A$_{364}$
versus time traces from two experiments (Fig. 3.20) gives rate constants of 2.6-2.9 s$^{-1}$ and
0.12-0.19 s$^{-1}$. The most reasonable interpretation of these data is that the larger k$_{obs}$
corresponds to formation of the 360 nm absorbing intermediate, and that the smaller k$_{obs}$
to conversion of the intermediate into the diferric cluster. Thus, formation of the
intermediate is slower in R2-Y122F by a factor of (approximately) 2. More importantly,
conversion of the intermediate into the diferric cluster is slower in R2-Y122F by at least a
Fig. 3.18: Time-course for •Y122 formation in the reaction of apo R2-wt with sub-limiting Fe$^{2+}$. The reaction conditions were: 71 µM apo R2-wt, 77 µM Fe$^{2+}$, 50 mM HEPES (air-saturated), pH 7.6, 5 °C. The data were acquired on the HP8452A apparatus. To illustrate the deviation from first order, the curve generated by fitting a first order growth to the data from dt to dt + 5 s is shown.
Fig. 3.19: Development of the absorption spectrum of the diferric cluster upon mixing of apo R2-Y122F with excess Fe$^{2+}$ in the presence of O$_2$. The reaction conditions were: 24 μM apo R2-Y122F, 124 μM Fe$^{2+}$, 50 mM HEPES (air-saturated), pH 7.6, 5 °C. The spectra were acquired on the HP8452A diode array apparatus. The time after mixing at which each spectrum was acquired is indicated, with the deadtime of the apparatus denoted dt. The contribution to each spectrum from the protein has been subtracted away for clarity.
Fig. 3.20: Least-squares analysis of $A_{364}$-versus-time trace from the reaction of apo R2-Y122F with excess $\text{Fe}^{2+}$ according to the equation for two consecutive, first order reactions. The reaction conditions were: 45 $\mu$M apo R2-Y122F, 225 $\mu$M $\text{Fe}^{2+}$, 50 mM HEPES (air-saturated), pH 7.6, 5 °C. The data were acquired on the HP8452A apparatus. The experimental trace is a representative trial. The theoretical curve was generated by fitting to 0-30 s of the experimental trace (as described in the text), and corresponds to $k_1 = 2.8$ s$^{-1}$ and $k_2 = 0.19$ s$^{-1}$. 
factor of 4. Thus, the 360 nm absorbing species exhibits the increased lifetime in R2-Y122F which is predicted for the •Y122-generating intermediate. This result suggests that the 360 nm absorbing intermediate generates •Y122 in the reaction of apo R2-wt with excess Fe$^{2+}$.

Reaction of Apo R2-Y122F with Limiting Fe$^{2+}$

As with apo R2-wt, when apo R2-Y122F is mixed at 5 °C with limiting Fe$^{2+}$, a broad absorption band centered at 560 nm rapidly develops (Fig. 3.21A). This result indicates that residue Y122 is not required for formation of the 560 nm absorbing species. Also as in R2-wt, the transient is not seen in the reaction of apo R2-Y122F with excess Fe$^{2+}$ (Fig. 3.21B) nor with limiting Fe$^{2+}$ and ascorbate (Fig. 3.21C). In the mutant protein, $t_{\text{max}}$ at 560 nm is 300-400 ms (the range observed in 5 experiments, Fig. 3.22 shows a representative trace), which is significantly greater than in R2-wt. This result suggests that formation or decay (or both) of the 560 nm absorbing species is slower in R2-Y122F than in R2-wt. Fitting Eq. 3.2 (with A$_0$ fixed) to A$_{560}$ versus time traces from three separate experiments gives values of 2.8-3.2 s$^{-1}$ for one rate constant and values of 2.3-2.7 s$^{-1}$ for the second rate constant (Fig. 3.22 shows a representative fit). Calculating $\varepsilon_B$ from these fits according to Eq. 3.5 gives values of 1700-2800 M$^{-1}$cm$^{-1}$, in excellent agreement with the range calculated above from the apo R2-wt limiting Fe$^{2+}$ reaction. Each of the observed rate constants is less than each of those determined by fitting comparable traces from the reaction of apo R2-wt with limiting Fe$^{2+}$. This result suggests that formation and decay of the intermediate are both slower in R2-Y122F than in R2-wt. The increased lifetime of the intermediate in the mutant protein is additional evidence that it generates •Y122 in the reaction of apo R2-wt with limiting Fe$^{2+}$.

Concomitantly with decay of the 560 nm transient, a sharp transient absorption band centered at 410 nm develops (Fig. 3.23). The feature reaches maximum intensity (as assessed by A$_{410, \text{dropline}}$) at 1.1 ± 0.2 s, and decays very slowly thereafter, such that
Fig. 3.21: Detection of the 560 nm absorption transient in the reaction of apo R2-Y122F with limiting Fe$^{2+}$. The reaction conditions were: 25 μM apo R2-Y122F, 50 mM HEPES (air-saturated), pH 7.6, 5 °C, and (A) 53 μM Fe$^{2+}$; (B) 125 μM Fe$^{2+}$; or (C) 54 μM Fe$^{2+}$ and 2.5 mM ascorbate. The spectra were acquired on the HP8452A diode array apparatus. In A, the time after mixing at which each spectrum was acquired is indicated, with the deadtime of the apparatus denoted dt. In B and C, the times at which the spectra were acquired are the same as in A. The contribution to each spectrum from the protein has been subtracted away for clarity.
Fig. 3.22: Time-course of the 560 nm absorption transient in the reaction of apo R2-Y122F with limiting Fe$^{2+}$. The reaction conditions were: 45 μM apo R2-Y122F, 90 μM Fe$^{2+}$, 50 mM HEPES (air-saturated), pH 7.6, 5 °C. The data were acquired on the Applied Photophysics DX.17MV apparatus. The experimental trace represents the average of five trials. The $t_{\text{max}}$ of this trace is 0.39 s. The theoretical trace was obtained by fitting Eq. 3.2 to 0.005-3.00 s of the experimental trace (with $A_0$ fixed), as described in the text. It corresponds to $k_1 = 2.9$ s$^{-1}$ and $k_2 = 2.7$ s$^{-1}$. 
Fig. 3.23: Development of the absorption spectrum of the diferric cluster upon mixing of apo R2-Y122F with limiting Fe^{2+} in the presence of O_2. The reaction conditions were: 24 μM apo R2-Y122F, 53 μM Fe^{2+}, 50 mM HEPES (air-saturated), pH 7.6, 5 °C. The spectra were acquired on the HP8452A diode array apparatus. The time after mixing at which each spectrum was acquired is indicated, with the deadtime of the apparatus denoted dt. The contribution to each spectrum from the protein has been subtracted away for clarity.
nearly 60 s are required for completion (Fig. 3.24). Fitting the equation for a first order reaction to the decay phase of the curve gives a $k_{\text{obs}}$ of 0.07 s$^{-1}$. The narrowness of the feature and its $\lambda_{\text{max}}$ strongly suggest that it arises from a tyrosyl radical. If the $\varepsilon_{410}$,dropline of this putative, transient tyrosyl radical is assumed to be identical to that of •Y122, the magnitude of $A_{410}$,dropline at $t_{\text{max}}$ indicates that 0.06 •Y/R2 is present. Like the 560 nm transient, this transient is not observed in the reaction of apo R2-Y122F with excess Fe$^{2+}$ (see Fig. 3.19) nor with limiting Fe$^{2+}$ and ascorbate. It thus appears that, in the absence of Y122 and under limiting Fe$^{2+}$ conditions, a transient radical is generated from another tyrosine in R2 as the 560 nm absorbing intermediate decays. This observation lends further credence to consideration of the 560 nm absorbing species as the •Y122-generating intermediate in the limiting Fe$^{2+}$ reaction.

The early spectra of the reaction (Fig. 3.23B, C) also appear to exhibit a band near 335 nm. The $A_{334}$ versus time trace provides support for this assertion (Fig. 3.25). The curve exhibits more than three phases, of which the first two are characterized by a rapid increase (from $dt$ to $dt+0.4$ s in experiments carried out with the HP8452A apparatus), followed by a plateau (from $dt+0.4$ s to $dt+1.0$ s) or, in some experiments, a slight decrease. Subsequent to this plateau (or decay) phase, the curve rises monotonically. The complexity of this trace precludes meaningful fitting, but it appears that the rapid rise phase corresponds temporally with the formation of the 560 nm absorbing species, and that the plateau (or decay) phase corresponds with decay of the 560 nm transient. In addition, re-examination of the time-dependent spectra of the reaction of apo R2-wt with limiting Fe$^{2+}$ suggests that the 335 nm band forms in this reaction as well. These results suggest that the band near 335 nm may be associated with the 560 nm absorbing species.
**Fig. 3.24:** Time-course of the sharp 410 nm transient in the reaction of apo R2-Y122F with limiting Fe$^{2+}$. The reaction conditions were the same as for Fig. 3.23. The data were acquired on the HP8452A apparatus. The trace is $A_{410} - (2A_{416} + 3A_{406})/5$, and represents the average of three trials.
Fig. 3.25: $A_{334}$-versus-time trace for reaction of apo R2-Y122F with limiting Fe$^{2+}$. The reaction conditions were the same as for Fig. 3.23. The data were acquired on the HP8452A apparatus. The trace represents the average of three trials.
Discussion

Proposed Mechanism for the Reconstitution of R2-wt

The results presented above provide sufficient evidence to allow a schematic mechanism to be proposed for the reconstitution of R2 (Scheme 3.4). Upon mixing of apo R2 and Fe$^{2+}$ in the presence of O$_2$, an intermediate, I (formerly designated (U) (Bollinger et al., 1991)), rapidly forms ($k_{obs} = 5.3$-$8.8$ s$^{-1}$). I exhibits the 560 nm absorption band (and probably the 335 nm band) seen in the limiting Fe$^{2+}$ reaction and contains two reactive oxidizing equivalents. When reducing equivalents are in excess (excess Fe$^{2+}$ or limiting Fe$^{2+}$ and ascorbate), I is reduced by one electron sufficiently rapidly that it does not accumulate (Pathway B). Iterative simulation of the 560 nm absorbance versus time trace for the excess Fe$^{2+}$ reaction indicates that the reduction of I must occur with an apparent first order rate constant in excess of 20 s$^{-1}$ to give the observed trace. The one-electron reduction of I generates a second intermediate, II, which exhibits the broad 360 nm band seen early in the excess Fe$^{2+}$ reaction (and in the limiting Fe$^{2+}$ and ascorbate reaction, see Fig. 3.26) and which contains one reactive oxidizing equivalent in the form of an intermediate iron cluster. Reaction of II by electron transfer from Y122 to this iron species ($k_{obs} = 0.72$-$0.77$ s$^{-1}$) simultaneously generates •Y122 and the diferric cluster of native R2.

When the reaction conditions are such that exogenous reducing equivalents are limiting (i.e. limiting Fe$^{2+}$, no ascorbate), the intermediate I partitions between Pathways A and B. In Pathway A, I reacts by electron transfer from Y122 to one of its oxidizing equivalents, a reaction which causes the decay of the 560 nm absorption band ($k_{obs} = 3.4$-$5.5$ s$^{-1}$) and the formation of •Y122 (the fast phase of the limiting Fe$^{2+}$ reaction, $k_{obs} = 3.6$-$5.4$ s$^{-1}$), and which generates a third intermediate, II'. II' contains •Y122 and an intermediate iron species which stores its one remaining, reactive oxidizing equivalent. In terms of its formal oxidation state, the iron species of II' is equivalent to that of II.
Scheme 3.4: Schematic hypothesis for the mechanism of assembly of the R2 cofactor. In terms of the reaction stoichiometry, apo R2 in the scheme refers to one monomer of the dimer.
Fig. 3.26: Development of the absorption spectrum of the diferric cluster upon reaction of apo R2-Y122F with limiting Fe$^{2+}$ and ascorbate. The reaction conditions were the same as for Fig. 3.21C. The data were acquired on the HP8452A apparatus. The time after mixing at which each spectrum was acquired is indicated, with the deadtime of the apparatus denoted dt.
(thus, the derivative designation). One electron reduction of \( \text{II}' \) by \( \text{Fe}^{2+} \) converts the intermediate iron species into the diferric cluster, giving native R2.

**Mechanism for Reaction of Apo R2-Y122F with \( \text{Fe}^{2+} \) and \( \text{O}_2 \)**

A similar mechanism is proposed for the reaction of apo R2-Y122F with \( \text{Fe}^{2+} \) and \( \text{O}_2 \). In the mutant subunit, the accumulation both of I in the limiting \( \text{Fe}^{2+} \) reaction (\( k_{\text{obs}} = 2.8-3.2 \text{ s}^{-1} \) or \( 2.3-2.7 \text{ s}^{-1} \)) and of II in the excess \( \text{Fe}^{2+} \) reaction (\( k_{\text{obs}} = 2.6-2.9 \text{ s}^{-1} \)) is slower than in R2-wt by a factor of approximately 2. Likewise, decay of the intermediates, which in R2-wt occurs by electron transfer from Y122, is slower in R2-Y122F. Decay of I (\( k_{\text{obs}} = 2.3-2.7 \text{ s}^{-1} \) or \( k_{\text{obs}} = 2.8-3.2 \text{ s}^{-1} \)) is moderately slower, while decay of II (\( k_{\text{obs}} = 0.12-0.19 \text{ s}^{-1} \)) is slower by at least a factor of 4. The manner in which the intermediate iron cluster of II is reduced to the diferric cluster in R2-Y122F is not apparent, but it is reasonable to expect that \( \text{Fe}^{2+} \) or ascorbate can provide the electron. Reduction of the 560 nm absorbing species of I in the limiting \( \text{Fe}^{2+} \) reaction appears to occur in part by electron transfer from at least one tyrosine residue, as evidence suggests that a tyrosyl radical accumulates to \( \sim 0.06 \) \( \cdot \text{Y/R2-Y122F} \). The formation of intramolecular dityrosine cross-linkages, presumably resulting from the coupling of Y radicals, has been demonstrated in the reaction of metmyoglobin with peroxide (Tew & Ortiz de Montellano, 1988). A similar mechanism involving surface Y radicals has been proposed to account for the cross-linking of CCP which occurs when the ferrous enzyme reacts with \( \text{O}_2 \) in the absence of substrate (Miller et al., 1992). In light of these results, it would be instructive to know if R2-Y122F becomes cross-linked upon decay of the intermediate I, but this question has not yet been addressed experimentally.

**Rationale and Precedent for Two Pathway Mechanism**

Perhaps the two most central features of Scheme 3.4 are the proposal that the reconstitution reaction proceeds by two distinct pathways and the proposal that \( \cdot \text{Y122} \) is
produced by the reaction of a different intermediate in each pathway. Several key observations provide the basis for these proposals. The unexpected finding that •Y122 production is initially faster in the limiting Fe²⁺ reaction than in either the excess Fe²⁺ or the limiting Fe²⁺ and ascorbate reaction, and the fact that the 560 nm transient is only observed in the limiting Fe²⁺ reaction, establish the correlation between the presence of a fast phase of •Y122 production and the accumulation of the 560 nm absorbing intermediate. This correlation, the fact that kₐ₉₅ for formation of the intermediate is equal to the kₐ₉₅ for formation of the •Y122-generating intermediate (indicated by the lag phase of the A₄₁₀,dropline versus time trace) and the fact that kₐ₉₅ for decay of the intermediate is equal to kₐ₉₅ for the fast phase of •Y122 production, strongly suggest that decay of the 560 nm absorbing species is responsible for the fast phase of •Y122 production. Additional evidence for this conclusion are the observations that the species decays more slowly in R2-Y122F and that, even in the mutant protein, a tyrosyl radical forms as the intermediate decays. If the conclusion that decay of the 560 nm absorbing species generates •Y122 is correct, a two pathway model is necessary: since the 560 nm absorbing intermediate does not accumulate in the excess Fe²⁺ reaction nor in the limiting Fe²⁺ and ascorbate reaction, a second intermediate must generate •Y122 under these conditions. The 360 nm absorbing intermediate exhibits all the characteristics expected for this second •Y122-generating intermediate. Its kₐ₉₅ for formation (as assessed by multicomponent analysis) matches the kₐ₉₅ deduced from the lag phase of the A₄₁₀,dropline versus time trace. Furthermore, its lifetime is increased four-fold in R2-Y122F. The results thus provide two excellent candidates for the •Y122 generating intermediate, and the data are most reasonably accounted for by a two pathway mechanism.

Irrespective of the structures of the intermediates I and II, the proposal of two distinct reactive intermediates in the reconstitution reaction is reminiscent of the heme iron peroxidase mechanism (Marnett et al., 1986). This resemblance is emphasized by
designation of the R2 intermediates which generate •Y122 as I and II. Intermediate I, inasmuch as it contains two reactive oxidizing equivalents, is analogous to compound I of the peroxidases, in which one oxidizing equivalent is stored as oxyferryl heme and a second is stored either as a porphyrin π-cation radical (in horse radish peroxidase) or as an amino acid radical (in cytochrome c peroxidase) (Marnett, et al., 1986). Intermediate II is likewise analogous to peroxidase compound II, which contains a single oxidizing equivalent as oxyferryl heme. As demonstrated in Chapters 5-7, the analogy between the R2 intermediates and the peroxidase intermediates does not, however, extend to the nature of the intermediate iron species, as neither I nor II contains Fe(IV).

Another essential feature of Scheme 3.4 is the proposed partition of the intermediate, I, between pathways A and B. The observation that \( k_{\text{obs}} \) for formation of the 560 nm species in the limiting Fe\(^{2+} \) reaction is equal, in both R2-wt and R2-Y122F, to \( k_{\text{obs}} \) for formation of the 360 nm absorbing intermediate in the excess Fe\(^{2+} \) reaction is consistent with a partitioning of I. Equally consistent with the data, however, would be a mechanism in which I does not form at all in the presence of excess exogenous reductant. In such a mechanism, a precursor to I might partition between two pathways. Alternatively, two entirely separate pathways might be operative. Since in any of these mechanisms the intermediate I will not accumulate, distinguishing among them is not possible unless intermediates prior to I can be detected, or unless the reaction mechanism can in some way be perturbed (as by site-directed mutagenesis).

Relative Reactivity of I and II

The greater rate constant with which I generates •Y122 suggests that it is kinetically more reactive with respect to one electron reduction than is II. Consistent with this notion is the proposal in Scheme 3.4 that I rapidly (\( k > 20 \text{ s}^{-1} \)) accepts an electron either from ascorbate or from Fe\(^{2+} \), while reduction of II by either reductant is much slower. (The latter reaction must be slower in order to account for the ability of
Y122 to compete for reduction of the oxidizing species of II.) While the fact that the observed Fe$^{2+}$/Y122 stoichiometry (2.5) is greater than the theoretical value of 2 suggests that ascorbate does reduce II at a significant rate, the observed stoichiometry also implies that the pseudo-first order rate constant for reduction of II by this large excess of ascorbate can be at most one-fourth as large as the rate constant for Y122 production. The relative sluggishness of the quenching of II by ascorbate is sufficient to ensure production of Y122 in the presence of the facile one electron donor.

*Implications for Mechanism of "Fourth Electron" Donation by Fe$^{2+}$*

The manner in which Fe$^{2+}$ donates an electron to reduce I (or the precursor to I) in the excess Fe$^{2+}$ reaction warrants comment. This reduction is sufficiently facile that 5 molar equivalents of Fe$^{2+}$ completely prevents I from accumulating. This slight excess of Fe$^{2+}$ is sufficient both at 27 μM apo R2 and at 295 μM apo R2. The independence of this behavior on absolute concentration of the reactants suggests that the Fe$^{2+}$ which donates the electron might be bound by R2. Elgren and coworkers have suggested that this Fe$^{2+}$ is bound at the cluster binding site of the opposite monomer (Elgren et al., 1991), and evidence presented in Chapter 2 is consistent with this notion. Assuming this proposal to be correct, one would predict that a ratio of Fe$^{2+}$/R2 sufficiently high to occupy all the dinuclear cluster binding sites would be sufficient to prevent I from accumulating. We typically find the iron content of our R2 preparations to be 3.0-3.5 Fe$^{3+}$/R2 (depending on which value of ε$_{280}$ is assumed), whereas Lynch, et al. (1989) and Elgren, et al. (1991) have reported Fe$^{3+}$/R2 ratios as high as 3.9. Thus, assuming that binding of Fe$^{2+}$ by apo R2 is rapid and tight, one would predict that 3-4 molar equivalents of Fe$^{2+}$ per R2 would be sufficient to prevent I from accumulating. Ochiai and co-workers have suggested that, in mouse R2, the Fe$^{2+}$ which donates the electron is bound at an additional (third) binding site (Ochiai et al., 1990). Considering this possibility for *E. coli* R2, one would predict that an Fe$^{2+}$/R2 ratio equal to 3/2 the
Fe\textsuperscript{3+}/R2 ratio of the native protein would be sufficient to prevent I from accumulating, since one extra Fe\textsuperscript{2+} would be required per diferric cluster formed. (Again the assumption of rapid, tight binding between Fe\textsuperscript{2+} and R2 must be made.) Thus, the model of Ochiai, et al. (1990) leads to the prediction that 4.5-6.0 Fe\textsuperscript{2+}/R2 should suppress accumulation of I. As an Fe\textsuperscript{2+}/R2 ratio of 4 clearly is insufficient to completely prevent I from accumulating (see Fig. 3.14), whereas a ratio of 5 is sufficient (or nearly so), the model of Ochiai, et al. (1991) is in better agreement with the experimental observations than is the model of Elgren, et al. (1991). The state of the Fe\textsuperscript{2+} which donates the extra electron is discussed further in Chapter 6.

\textit{Implications for Binding of Fe\textsuperscript{2+} by Apo R2}

The marked slowing and deviation from first order of the reconstitution reaction under conditions of sub-limiting Fe\textsuperscript{2+} may provide insight into the binding of Fe\textsuperscript{2+} by apo R2. This behavior is observed when the Fe\textsuperscript{2+}/R2 ratio is less than 2, irrespective of the absolute concentration of apo R2 and Fe\textsuperscript{2+}. In spite of the fact that the reaction is slower, the final Fe\textsuperscript{2+}/\textgamma 122 stoichiometry is quite close to the theoretical value of 3. The simplest interpretation of these results is that mixing apo R2 with less than 2 molar equivalents of Fe\textsuperscript{2+} results in both mononuclear and dinuclear Fe\textsuperscript{2+} binding. Dinuclear binding is probably required for reaction with O\textsubscript{2}; thus, the slow phase of the reaction is a reflection of the dissociation and reassociation of Fe\textsuperscript{2+} bound in mononuclear fashion, until dinuclear binding is achieved, after which reaction with O\textsubscript{2} is irreversible (Scheme 3.5). If this interpretation is correct, it would indicate that the Fe\textsuperscript{2+} bound in mononuclear fashion is protected from autooxidation, since this side reaction would be expected to compete with the reconstitution reaction on the observed time scale (2 min). It would also provide argument against the idea which has been put forth that binding of Fe\textsuperscript{2+} by apo R2 is "highly cooperative". It has been proposed or implied by several authors that addition of Fe\textsuperscript{2+} to apo R2 results exclusively in dinuclear Fe\textsuperscript{2+} binding.
(Fontecave et al., 1990; Elgren, et al., 1991; Atta et al., 1992). The basis for this proposal has been the observations that the Fe$^{2+}$/\textsuperscript{Y}122 stoichiometry of the reaction is largely independent of the ratio of Fe$^{2+}$/apo R2 and that the majority of Fe$^{2+}$ added is incorporated into diferric clusters. As Scheme 3.5 illustrates, interpretation of these results as an indication of cooperative binding is fallacious, since O$_{2}$ might not react with Fe$^{2+}$ bound in mononuclear fashion. The irreversible reaction of O$_{2}$ only at dinuclear sites would cause the dynamic equilibrium among unbound Fe$^{2+}$, mononuclear Fe$^{2+}$, and dinuclear Fe$^{2+}$ to be driven to the right, allowing all added Fe$^{2+}$ to react productively.

Scheme 3.5: Illustration of the manner in which the irreversible trapping of diferrous-R2 by O$_{2}$ might allow the productive reaction of all added Fe$^{2+}$ in the reaction of apo R2 with sub-limiting Fe$^{2+}$, even if binding of Fe$^{2+}$ by apo R2 is not cooperative. Apo R2 refers to one monomer of the dimer.
Our data indicate that Scheme 3.5 is operative, and suggest that the notion of cooperative binding of Fe$^{2+}$ by apo R2 should be reassessed.

References


Chapter 4: The Reaction of Apo R2 with $\text{Fe}^{2+}$

and $O_2$ Probed by Rapid Freeze-Quench EPR Spectroscopy
Although the mechanism put forth in Chapter 3 (Scheme 3.4) contains no explicit hypotheses as to the identities of the oxidizing components in the intermediates I, II, and II', it does allow several inferences to be drawn which lead logically to the experiments of this chapter. For example, it is proposed in Scheme 3.4 that both II and II' contain a diiron cluster which is oxidized by one electron relative to the diferric cluster. A cluster of this type would necessarily contain an odd number of electrons, and would have a half-integer electron spin quantum number. It is reasonable to expect that such a cluster might be EPR active. Furthermore, in the case of II, the rate constants of Scheme 3.4 indicate that 0.86-0.96 equivalent (per R2) of the odd electron species should accumulate early in the reaction of apo R2 with excess Fe²⁺, since 1.2 equivalents of •Y122 are formed upon completion of the reaction. Thus, it would seem likely that the intermediate cluster could be detected by EPR spectroscopy.

A second potentially testable proposal of Scheme 3.4 is that II' contains •Y122 and an odd electron diiron cluster. As •Y122 and the diferric cluster of native R2 are only 5.3 Å apart (Nordlund et al., 1990), it is logical to expect that •Y122 and the intermediate cluster of II' would be a comparably short distance from one another. It might be anticipated that two paramagnetic species in such close proximity would affect the EPR characteristics of one another (as the diferric cluster does to the tyrosyl radical at temperatures in excess of 75 K (Sahlin et al., 1987; Hirsh et al., 1992)). Thus, in the event that the intermediate cluster of II' were not directly observable by EPR, it might still be possible to use the EPR signal of •Y122 as a probe for the presence of the nearby paramagnetic iron cluster.

One might also expect that EPR spectroscopy would be useful in distinguishing among several possible identities of the two oxidizing equivalents in I. Both equivalents might be retained in the form of an intermediate iron cluster. Such a cluster could be a complex formed between O₂ and the diferrous cluster of R2, such as a peroxodiferric cluster (Menage et al., 1990), or a species farther along the reaction pathway in which the
O-O bond of O$_2$ has been cleaved (yielding formally an Fe(IV), Fe(IV) cluster). Each of these species would contain an even number of electrons, and might be EPR inactive. As a third alternative, I might contain two oxidized components, each storing a single oxidizing equivalent. In analogy to compound I of yeast cytochrome c peroxidase, one equivalent might be stored as an intermediate iron species and a second stored as an amino acid radical (Yonetani et al., 1966; Marnett et al., 1986). In this case, both the intermediate iron cluster and the amino acid radical might be EPR active.

In the experiments which are described in this chapter, EPR spectroscopy was used to obtain additional evidence for and to probe the nature of the intermediates I, II, and II', and to test aspects of Scheme 3.4. The relatively short lifetimes of the intermediates made it necessary to use the rapid freeze-quench method (Bray, 1961; Ballou & Palmer, 1974) to trap them. Application of this method allowed the trapping of a heretofore unprecedented intermediate iron cluster, which exhibits a sharp, isotropic, $g = 2.00$ singlet (Bollinger et al., 1991). Results of isotopic substitution experiments with $^{57}$Fe, $^{17}$O and $^2$H show that the electron spin of this intermediate cluster is coupled to at least two Fe nuclei, to O nuclei from both O$_2$ and H$_2$O, and to at least one H nucleus from H$_2$O. The intermediate species forms both in the excess Fe$^{2+}$ and in the limiting Fe$^{2+}$ reaction, suggesting that it is a component of II, and either of I or of II' (or of both). In the limiting Fe$^{2+}$ reaction, in addition to the EPR signal of the iron species, a broader EPR signal in the $g = 2$ region is observed. This signal does not develop when the reaction is carried out with limiting Fe$^{2+}$ and ascorbate. Thus, this broad signal must be associated with I, with II', or with both.

The results of these experiments confirm the proposal from Chapter 3 that a one-electron-oxidized iron cluster accumulates in the reconstitution of R2, and provide additional evidence for the two pathway mechanism of Scheme 3.4. Furthermore, the demonstration that the R2 intermediates can be trapped by the freeze-quench method provides the technical basis for the experiments of Chapters 5, 6, and 7.
Materials and Methods

Materials

Apo R2-wt and apo R2-Y122F were prepared as described in Chapter 2. $^{57}$Fe (98 atom %) was purchased from Advanced Materials and Technology (of the former USSR). $^{17}$O$_2$ (37 atom % $^{17}$O) was purchased from Icon (Mt. Marion, NY). H$_2^{17}$O was purchased from Monsanto Research Corp., Mound Facility (Miamisburg, OH). It was originally 40% $^{17}$O, but had been used in a prior experiment and recovered by distillation. It was estimated to be 35% $^{17}$O. $^2$H$_2$O (99%) was purchased from Aldrich. Isopentane for the freeze-quenching bath was purchased from Aldrich.

Control for Rapid Freeze-Quenching of R2

Sample preparation by the rapid freeze-quench method was carried out as previously described (Bray, 1961). The method involves mixing two (or more) reactants rapidly and efficiently, allowing the reaction to proceed for a desired length of time (the time required for the mixture to pass through an aging hose of appropriate volume), freeze-quenching the reaction mixture by squirting it into isopentane cooled to -150 °C, and packing the resulting ice crystals into a receptacle appropriate for spectroscopic analysis. In order to ascertain whether R2 is adversely affected by the freeze-quenching procedure, a control was carried out in which native R2 was squirted into liquid isopentane in contact with the frozen solid. The absorption spectrum of a 25 μM solution of native R2 (in 100 mM HEPES buffered at pH 7.7) was acquired. A 500 μL aliquot of this R2 solution was expelled forcefully from a 1 mL gastight syringe into 2 mL of partially frozen isopentane in a 5 mL test tube. The resulting ice crystals were allowed to melt, and the protein solution (the lower phase) was recovered. The absorption spectrum of the recovered protein solution was recorded. The concentration of tyrosyl radical before and after the mock freeze-quenching procedure was determined by the magnitude of $A_{412,dropline}$. This quantity was the same after the freeze-quenching procedure ($A_{412,dropline} = 0.0481$) as
before \( A_{412, \text{dropline}} = 0.0480 \). It was therefore concluded that the procedure is not deleterious to the cofactor of R2. (Some light scattering was observed in the protein sample recovered following the freeze-quenching procedure. This scattering was attributed to a small quantity of isopentane suspended in the aqueous phase.)

*Preparation of EPR Samples by Rapid Freeze-Quenching*

The apparatus which was used in preparation of the rapid freeze-quench samples consists of an Update Instruments (Madison, WI) Ram Unit and Model 705A Computer Controller, and a home-built quenching bath. A schematic diagram of the quenching bath is shown in Fig. 4.1. The bath holds ~8 L isopentane which is cooled with liquid \( \text{N}_2 \) and maintained at -150 °C with a Bayley Instruments Company (Danville, CA) Precision Temperature Controller. The temperature of the reconstitution reaction was maintained at 5 ± 1 °C by filling the Ram Unit with enough cold water to emerse the drive syringes and by periodically adding ice. The aging hose was submerged in the water bath for several minutes before each sample was prepared, in order to allow the hose to reach 5 ± 1 °C. Immediately before the Ram unit was actuated, the aging hose was removed from the cold water and held over the quenching bath.

*Calibration of Rapid Freeze-Quench Apparatus*

In the rapid freeze-quench method, the reaction time at which a given sample is quenched is the sum of two quantities: the time elapsed after the reactants are mixed until the reaction mixture traverses the aging hose, and the time elapsed after the reaction mixture contacts the cold isopentane until it is cooled to the temperature at which no further reaction occurs (Bray, 1964). The latter quantity (quenching time) can vary from one reaction system to another (Ballou and Palmer, 1974), and is therefore difficult to determine. The quenching times of many reactions are short (5-10 ms) (Ballou and Palmer, 1974) compared to the time-scale of the R2 reconstitution reaction \( (t_{1/2} \text{ for } ^\ast \text{Y}122 \)
Fig. 4.1: Rapid freeze-quench apparatus. A shows a schematic diagram of the isopentane freeze-quenching bath with the EPR sampling apparatus inside. B shows a more detailed schematic diagram of the sampling apparatus. In preparation of the samples, immediately before the Ram drive was actuated, the nozzle through which the reaction mixture was sprayed was positioned less than 1 cm above the level of the isopentane inside the funnel.
Fig. 4.1A

stirrer
sampling apparatus
heater
temperature probe
steel dewar
insulation
isopentane
liquid N$_2$
copper pipe (sealed at bottom)
Fig. 4.1B

- wooden handle
- metal brackets
- metal rod
- glass funnel
- latex connector
- quartz EPR tube (4 mM O.D.) blown to glass funnel
formation = 1 s), and quenching time was therefore neglected in this study. The former quantity depends on the volume of the aging hose, the volume expelled by the drive syringes for a given displacement of the Ram drive, and the velocity of the Ram drive. In these experiments, the velocity of the Ram drive was assumed to be that quoted by the manufacturer. The volume expelled by the drive syringes for a given displacement and the volumes of the aging hoses were measured experimentally, as follows. One of the drive syringes was filled with distilled H$_2$O. The Ram unit was programmed for a given displacement and actuated, while the water expelled from the drive syringe was collected in a previously weighed microcentrifuge tube. The weight of the water collected was determined, and this was converted to volume by assuming a density for H$_2$O of 1 g/ml. The volume expelled by the second drive syringe was determined in identical fashion. The volume of each aging hose was determined by connecting the empty hose to one or both of the H$_2$O-filled drive syringes. The Ram drive was actuated for a given displacement, and the water expelled from the aging hose was collected in a previously weighed microcentrifuge tube. The volume of the aging hose was calculated as the difference between the volume expelled by the drive syringe(s) for that displacement and the volume collected in the tube. In some experiments it was necessary to couple two hoses together to obtain a desired volume. In these cases, the volume of the coupled system was determined as above. Table 4.1 summarizes the volume determinations for the aging hoses and the corresponding reaction times for a 1 cm/s Ram velocity.

**Time-Course of the Reconstitution Reaction Monitored by EPR Spectroscopy**

In analogy with the stopped-flow experiments of Chapter 3, the rapid freeze-quench EPR time-course samples were prepared by reaction of apo R2-wt or apo R2-Y122F, in two different ranges of protein concentration, with Fe$^{2+}$ and O$_2$. In the "dilute protein" experiments, 95-105 μM apo R2 in 100 mM HEPES buffered at pH 7.7 was mixed at 5 ± 1 °C with varying concentrations of Fe$^{2+}$. In these experiments, the Fe$^{2+}$ stock solutions
Table 4.1: Measured volumes of the rapid freeze-quench aging hoses and corresponding reaction times for a Ram velocity of 1 cm/s. Each volume quoted represents the average of three or more separate determinations. The combined volume expelled by the two drive syringes was found to be 670 µL for a 1 cm displacement of the Ram drive.

<table>
<thead>
<tr>
<th>Aging Hose No.</th>
<th>Volume (µL)</th>
<th>Reaction Time for 1 cm/s Ram Vel. (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>64</td>
<td>41</td>
<td>0.061</td>
</tr>
<tr>
<td>125</td>
<td>106</td>
<td>0.16</td>
</tr>
<tr>
<td>200</td>
<td>150</td>
<td>0.22</td>
</tr>
<tr>
<td>250</td>
<td>188</td>
<td>0.28</td>
</tr>
<tr>
<td>272</td>
<td>205</td>
<td>0.31</td>
</tr>
<tr>
<td>400</td>
<td>296</td>
<td>0.44</td>
</tr>
<tr>
<td>500</td>
<td>377</td>
<td>0.56</td>
</tr>
<tr>
<td>500 coupled to 125</td>
<td>483</td>
<td>0.72</td>
</tr>
</tbody>
</table>

were prepared by dissolution of FeSO₄·7H₂O in 5 mM HCl or HNO₃, and the Fe²⁺ concentration ([Fe²⁺]) was determined by assay with ferrozine (Stookey, 1970; Salowe, 1987), as described in Chapter 3. Both the protein solution and the Fe²⁺ solution were air-saturated. In the "concentrated protein" experiments, 580-640 µM apo R2 in 100 mM HEPES buffered at pH 7.7 was mixed at 5 ± 1 °C with varying concentrations of Fe²⁺. The Fe²⁺ stock solutions for these experiments were prepared, as described in Chapter 3, by anaerobic dissolution of ⁵⁶Fe or ⁵⁷Fe metal in 1M H₂SO₄. The resulting concentrated Fe²⁺ stock was diluted with H₂O or dilute H₂SO₄ in order to give the desired Fe²⁺ concentration in 2-3 mM H₂SO₄. In the "concentrated protein" experiments, both the protein solution and the Fe²⁺ solution were saturated with 1 atm of O₂, by the procedure
described in Chapter 3. The exact conditions employed in each experiment are listed in the figure legends.

EPR spectra of the time-course samples were recorded on a Bruker ER 200D-SRC spectrometer equipped with an Oxford Instruments ESR 910 continuous flow cryostat. The spectrometer settings which were used are given in the figure legends. Double integration of EPR spectra was carried out with a program written by Dr. K. C. Chan in Prof. Huynh's laboratory at Emory University. Subtraction of EPR spectra was accomplished with a program written by Dr. Yaning Wang in Prof. Huynh's laboratory. Simulation of EPR spectra was carried out with a program written by Prof. Huynh and with the ESR® program of Calleo Scientific Software Publishers (Fort Collins, CO). Analysis of the kinetic data was carried out with the Git and Gear non-linear regression programs of Drs. R. J. McKinney and F. J. Wiegert, Central Research and Development Department, E. I. du Pont de Nemours and Co.

Isotopic Substitution with $^{17}O_2$

An Fe$^{2+}$ stock solution was prepared by dissolution of ~28 mg of FeSO$_4$·7H$_2$O in 100 mL of 5 mM HNO$_3$. The concentration of this stock was determined by ferrozine assay (see Chapter 3) to be 1.06 mM. A 5 mL aliquot of this solution was placed in the specially designed 10 mL flask illustrated in Fig. 4.2. This flask was connected to an opened ended, capillary mercury monometer. The monometer was also connected to a vacuum/argon line, and to a 30 mL cylinder containing 37 atom % $^{17}O_2$ gas at a pressure of 3 atm. The total gas volume of this system was estimated to be 10 mL. The Fe$^{2+}$ solution was frozen, the flask was evacuated, the solution was thawed under static vacuum, and the flask was refilled with argon. The solution was again frozen, the flask was evacuated, the solution was thawed under static vacuum, and the flask was refilled with 1.5 atm of $^{17}O_2$. The stopcocks on both the $^{17}O_2$ cylinder and the flask were closed tightly. The flask containing the 1.06 mM Fe$^{2+}$ under 1.5 atm of 37% $^{17}O_2$ was
Fig. 4.2: Apparatus used in saturating the Fe$^{2+}$ stock with $^{17}$O$_2$ in the isotopic substitution experiment.
ground glass joint (to vacuum line)

ground glass joint (for stopper)

10 mL flask

teflon stopcock
maintained in this condition at ambient temperature for three days until the rapid freeze-quench samples were prepared. (Preparation of the Fe²⁺ stock was carried out at M.I.T., whereas the freeze-quench experiments were performed at Emory University. Three days was the time elapsed for travel to Emory and for preparation to carry out the experiments. A control was previously carried out to show that an identically prepared Fe²⁺ stock did not become oxidized when stored under pure O₂. Incubation of the control Fe²⁺ stock under ~1.5 atm of O₂ at ambient temperature for 44 h resulted in < 2% loss of Fe²⁺.)

A 2 ml aliquot of 144 μM apo R2-Y122F in 100 mM HEPES buffered at pH 7.7 was made anaerobic as follows. The protein solution was placed in a 100 mL tonometer. The vessel was connected to a vacuum line, gently evacuated, and refilled with argon. The vacuum/argon cycle was repeated five times, then the solution was incubated on ice for 30 min. The vacuum/argon cycle was repeated five more times, and the protein was incubated on ice for another 30 min. This process was repeated a third time. The O₂-free apo R2-Y122F was then transferred into one of the drive syringes of the ram unit. The ¹⁷O₂ equilibrated 1.06 mM Fe²⁺ solution was transferred via hypodermic needle (in order to exclude atmospheric O₂) to the second drive syringe. These solutions were allowed to reach equilibrium at 5 ± 1 °C. The solutions were then mixed by actuation of the Ram drive, and the reaction was quenched either at 0.22 s or at 0.34 s.

In order to have a reference spectrum of ¹⁶O₂-labelled intermediate and to verify that the observed effect was due to ¹⁷O₂, a control was performed in which the ¹⁷O₂-saturated Fe²⁺ stock was allowed to reach equilibrium with air. The resulting air-saturated Fe²⁺ was mixed with argon-saturated protein (made O₂-free as described above) at 5 ± 1 °C, and the reaction was quenched at 0.27 s. EPR spectra of the ¹⁷O₂ samples and of the control were acquired on the apparatus described above. The spectrometer settings are given in the figure legends.
Isotopic Substitution with $H_2^{17}O$

To 582 μL of $H_2^{17}O$ (~35% $^{17}O$) were added 15 μL of 42 mM FeSO$_4$ in 10 mM HNO$_3$ and 3 μL of 1 M HNO$_3$, to give 1.05 mM Fe$^{2+}$ in 5 mM HNO$_3$ (~34% $^{17}O$ $H_2^{17}O$). A 600 μL aliquot of 100 mM HEPES buffered at pH = 7.7 was lyophilized, and redissolved in 600 μL of the $H_2^{17}O$. To this buffer was added 71 μL of 1.35 mM apo R2-Y122F. The resulting air-saturated 140 μM apo R2-Y122F solution in (~31% $^{17}O$) $H_2^{17}O$ was loaded into one of the drive syringes, and the air-saturated 1.05 mM Fe$^{2+}$ solution in (~34% $^{17}O$) $H_2^{17}O$ was loaded into the other syringe. These solutions were mixed by actuation of the Ram drive, allowed to react at 5 ± 1 °C for 0.22 s, and then freeze-quenched. A duplicate sample was prepared. EPR spectra of these samples were acquired on the spectrometer described above. The spectrometer settings are given in the figure legends.

Isotopic Substitution with $^2H_2O$

To 1.16 ml $^2H_2O$ (99% $^2H$) were added 30 μL of 42 mM FeSO$_4$ in 10 mM HNO$_3$ and 6 μL of 1 M HNO$_3$. The [Fe$^{2+}$] of this stock solution was determined (as described in Chapter 3) to be 1.04 mM. A 600 μL aliquot of 100 mM HEPES buffered at pH = 7.7 was lyophilized, then redissolved in 600 μL $^2H_2O$. To this buffer was added 71 μL of 1.35 mM apo R2-Y122F. The resulting air-saturated 140 μM apo R2-Y122F solution was loaded into a rapid quench syringe, and the air-saturated 1.04 mM Fe$^{2+}$ stock was loaded into the other syringe. The solutions were mixed and allowed to react at 5 ± 1 °C for 0.22 s before the reaction was freeze-quenched. A duplicate sample was prepared. EPR spectra of these samples were recorded. Spectrometer settings are given in the figure legends.
Results

_EPR Time-course of the Reaction of Apo R2-wt with Excess Fe^{2+}_

When apo R2-wt is mixed at 5 °C in the presence of O_2 with excess Fe^{2+}, an EPR-active intermediate rapidly accumulates. The intermediate exhibits a sharp, isotropic singlet with g-value of 2.00 (Fig. 4.3A). (When the field width of the spectrum was increased, no additional features were observed with the exception of a weak signal at g=4.3, which is generally associated with rhombic, high-spin ferric species.) Comparison of the double integrated intensity of the early spectra of the reaction to the double integral of the spectrum at completion, at which time 1.2 molar equivalents of •Y122 relative to R2 are present (see Chapter 3), allows estimation of the quantity of the transient species which accumulates. Table 4.2 lists the double integrals of the spectra from the two time-course experiments which were carried out. While the values fluctuate a great deal (± ~25%), even among replicate spectra acquired for a single sample (possible reasons for the poor precision of these values are given below), the values obtained for all samples with reaction times greater than 0.27 s are within the limit of variation of the values obtained for the 60 s (completion) samples. This observation indicates that within 0.3 s after mixing, 1.2 ± 0.4 equiv of EPR active species (intermediate + •Y122) accumulates. The early spectra (Fig. 4.3A) are composed predominantly of the isotropic singlet of the intermediate, while with time, the doublet characteristic of •Y122 develops and the singlet of the intermediate decays (Fig. 4.3B, C, D). (A more quantitative analysis, in which the spectrum of each time-point is deconvoluted to give the relative quantities of the intermediate and •Y122 present in each, is presented below.) The spectra of Fig. 4.3 demonstrate that the species which gives rise to the transient EPR singlet (hereafter, designated X) is kinetically competent to be a precursor to •Y122. The observations that X accumulates rapidly (to approximately 1 equiv) in the excess Fe^{2+} reaction and decays as •Y122 forms are consistent with its being the oxidized component of the intermediate II (Scheme 3.4).
Fig. 4.3: Time-course of the reaction of apo R2-wt with excess Fe$^{2+}$ as monitored by EPR spectroscopy. Apo R2-wt (0.59 mM) in O$_2$-saturated 100 mM HEPES buffered at pH 7.7 was mixed at 5 °C with an equal volume of 2.94 mM Fe$^{2+}$ in 2.5 mM H$_2$SO$_4$. The reaction was freeze-quenched (A) at 0.15 s, (B) at 0.63 s, (C) 1.5 s, or (D) at 60 s. The spectra were recorded at 20 K with a microwave power of 2 μW, a frequency of 9.43 GHz, a modulation frequency of 100 kHz, a modulation amplitude of 4 G, a time constant of 200 ms, a scan time of 200 s, and a receiver gain of 4 x 10$^4$. 
Table 4.2: Summary of double-integrated intensities for the spectra from two time-course experiments on the reaction of apo R2-wt with excess Fe$^{2+}$. The units for the double integrals are arbitrary.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>[R2] (μM)</th>
<th>Fe$^{2+}$/R2</th>
<th>time (s)</th>
<th>double-integrated intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>10.</td>
<td>0.078</td>
<td>3.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.27</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.67</td>
<td>12.6, 12.8, 11.9$^a$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.4</td>
<td>8.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.9</td>
<td>8.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60</td>
<td>8.64, 11.1$^b$</td>
</tr>
<tr>
<td>2</td>
<td>295</td>
<td>5.0</td>
<td>0.061</td>
<td>7.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.16</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.22</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.31</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.44</td>
<td>16.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.63</td>
<td>14.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.02</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.52</td>
<td>13.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.22</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.02</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.02</td>
<td>13.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.02</td>
<td>13.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60</td>
<td>13.1</td>
</tr>
</tbody>
</table>

$^a$two samples prepared, and replicate spectra taken for one of the two

$^b$replicate spectra taken for a single sample
Table 4.3: Summary of double-integrated intensities for the spectra from the reaction of apo R2-Y122F with excess Fe$^{2+}$. The units for the double integrals are arbitrary.

<table>
<thead>
<tr>
<th>[R2-Y122F] (µM)</th>
<th>Fe$^{2+}$/R2</th>
<th>time (s)</th>
<th>double-integrated intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>9.8</td>
<td>0.12</td>
<td>7.4, 5.9$^a$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.22</td>
<td>5.5, 4.3$^a$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.44</td>
<td>5.5, 4.3, 3.9$^a$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.94</td>
<td>5.6$^b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.4</td>
<td>3.3$^b$</td>
</tr>
</tbody>
</table>

$^a$ replicate spectra taken for a single sample
$^b$ samples were heterogeneous with respect to reaction time (as explained in text)

Reaction of Apo R2-Y122F with Excess Fe$^{2+}$

As with the wild-type protein, when apo R2-Y122F is mixed at 5 °C in the presence of O$_2$ with excess Fe$^{2+}$, the sharp isotropic $g = 2.00$ singlet characteristic of X rapidly develops. In contrast to the reaction of apo R2-wt, the lineshape of the spectrum does not change with time (Fig. 4.4), suggesting that in the absence of the oxidizable Y122, the intermediate decays to give an EPR inactive product. The double integrals of the time-dependent spectra suggest that decay of the intermediate is slower in R2-Y122F than in R2-wt (Table 4.3). For example, the value obtained for the 5.4 s sample is only ~50% less than the maximum integral. This result suggests that 50% of the quantity of X which accumulates at $t_{\text{max}}$ is still present after 5.4 s. This contrasts with R2-wt, in which X decays almost completely within 5 s (see Table 4.4 below). Unfortunately, the 0.94 s and 5.4 s samples of Table 4.3 were heterogeneous with respect to reaction time. Approximately 20% of each was made up of reaction mixture which was quenched after only 0.35 s. (This error was a result of erroneous volumes for the aging hoses.) If the
**Fig. 4.4:** Time-course of the reaction of apo R2-Y122F with excess Fe$^{2+}$. Apo R2-Y122F (0.10 mM) in air-saturated 100 mM HEPES buffered at pH 7.7 was mixed with an equal volume of 0.99 mM FeSO$_4$ in air-saturated 5 mM HNO$_3$. The reaction was freeze-quenched (A) at 0.15 s, (B) at 0.44 s or (C) at 5.4 s. (As stated in the text, sample C was heterogeneous with respect to reaction time. Approximately 20% of the sample reacted for only 0.35 s.) The spectra were acquired at 20 K with a power of 6.7 μW, and a receiver gain of $1.6 \times 10^5$. All other spectrometer settings were as in Fig. 4.3.
\( t_{\text{max}} \) of X in R2-Y122F is close to 0.35 s, the heterogeneity of the 0.94 s and 5.4 s samples renders this already rough spin quantitation even more dubious. Thus, while the data suggest that decay of X is slower in R2-Y122F than in R2-wt, this conclusion must be considered tentative.

**Quantitative Analysis of the EPR Spectra from the Reaction of Apo R2-wt Excess \( \text{Fe}^{2+} \)**

The observation that the lineshape of the EPR spectrum does not change with time in the reaction of apo R2-Y122F with excess \( \text{Fe}^{2+} \) suggests that X is the only EPR active component which accumulates in this reaction. Therefore, the spectrum of this reaction serves as a reference spectrum for X which can be used to estimate the relative amounts of X and •Y122 present in the time-course samples from the R2-wt reaction. The relative quantities of the two species were estimated for each sample from Exp. 2 of Table 4.2 by iterative addition of the spectra of X and •Y122 in varying ratios, until the experimental spectrum was satisfactorily reproduced. At all times during the reaction the experimental spectrum can be accounted for as the sum of the spectra of X and •Y122 (Fig. 4.5 shows a representative example of this analysis). The relative quantities of X and •Y122 in each sample are listed in Table 4.4, along with the double integral for each (from Table 4.2B). By using the values from Table 4.4, and by using the 60 s sample as a radical concentration standard, the absolute amounts of X and of •Y122 in each sample (in terms of molar equiv relative to R2) can be estimated according to Eqs. 4.1 and 4.2, where \( I(t) \) is the double integral for a given sample, \( F_X(t) \) and \( F_{\cdot Y122}(t) \) are the fractions of X and •Y122 present in that sample, and \( I(60) \) is the double integral for the 60 s sample. Table 4.4 lists the values of X/R2 and •Y122/R2 calculated for each sample. These data were analyzed according to the kinetic model of Scheme 4.1. Non-linear least-squares fitting of the equations appropriate for this model to the data gives a rate constant \( (k_1) \) of 10 s\(^{-1}\) for formation of X and a rate constant \( (k_2) \) of 0.95 s\(^{-1}\) for its decay and the concomitant formation of •Y122.

In Fig. 4.6 the data are plotted along with the theoretical curves generated in this analysis.
Fig. 4.5: Example of deconvolution of an EPR spectrum from the reaction of apo R2-wt with excess Fe$^{2+}$ to determine the relative quantities of X and •Y122 present. Spectrum A was acquired on the 1.0 s sample from the experiment depicted in Fig. 4.3. Spectrum B was constructed by summation of the spectra of X and •Y122 in a ratio of 0.45:0.55 (X:•Y122). The reference spectrum for •Y122 was acquired on the 60 s sample from the same experiment. The reference spectrum for X was acquired on the control sample of Fig. 4.9 below. The spectrometer settings for the experimental and reference spectra were as in Fig. 4.3.
\[
\text{Eq. 4.1} \quad \frac{X}{R^2} = 1.2 \frac{I(t)}{I(60)} F_X(t)
\]

\[
\text{Eq. 4.2} \quad \frac{\cdot Y_{122}}{R^2} = 1.2 \frac{I(t)}{I(60)} F_{\cdot Y_{122}(t)}
\]

\[
\text{Scheme 4.1} \quad \text{apo R2} + \text{Fe}^{2+} + \text{O}_2 \xrightarrow{k_1} X \xrightarrow{k_2} \cdot Y_{122}
\]

Table 4.4: Summary of relative and absolute quantities of X and \(\cdot Y_{122}\) as determined by EPR in the time-course samples for the reaction of apo R2-wt with excess Fe\(^{2+}\).

<table>
<thead>
<tr>
<th>time (s)</th>
<th>integral</th>
<th>% X</th>
<th>% (\cdot Y_{122})</th>
<th>X/R2</th>
<th>(\cdot Y_{122}/R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.061</td>
<td>7.0</td>
<td>85</td>
<td>15</td>
<td>0.54</td>
<td>0.095</td>
</tr>
<tr>
<td>0.16</td>
<td>11.6</td>
<td>82</td>
<td>18</td>
<td>0.87</td>
<td>0.19</td>
</tr>
<tr>
<td>0.22</td>
<td>12.9</td>
<td>80</td>
<td>20</td>
<td>0.95</td>
<td>0.23</td>
</tr>
<tr>
<td>0.31</td>
<td>12.4</td>
<td>77</td>
<td>23</td>
<td>0.87</td>
<td>0.26</td>
</tr>
<tr>
<td>0.44</td>
<td>16.4</td>
<td>67</td>
<td>33</td>
<td>1.01</td>
<td>0.50</td>
</tr>
<tr>
<td>0.63</td>
<td>14.7</td>
<td>61</td>
<td>39</td>
<td>0.82</td>
<td>0.53</td>
</tr>
<tr>
<td>1.02</td>
<td>11.9</td>
<td>45</td>
<td>55</td>
<td>0.49</td>
<td>0.60</td>
</tr>
<tr>
<td>1.52</td>
<td>13.7</td>
<td>28</td>
<td>72</td>
<td>0.35</td>
<td>0.90</td>
</tr>
<tr>
<td>2.22</td>
<td>8.8</td>
<td>19</td>
<td>81</td>
<td>0.15</td>
<td>0.65</td>
</tr>
<tr>
<td>3.02</td>
<td>13.3</td>
<td>10</td>
<td>90</td>
<td>0.12</td>
<td>1.10</td>
</tr>
<tr>
<td>4.02</td>
<td>13.6</td>
<td>5</td>
<td>95</td>
<td>0.06</td>
<td>1.18</td>
</tr>
<tr>
<td>5.02</td>
<td>13.1</td>
<td>2</td>
<td>98</td>
<td>0.02</td>
<td>1.18</td>
</tr>
<tr>
<td>60</td>
<td>13.1</td>
<td>--</td>
<td>100</td>
<td>--</td>
<td>(1.20)</td>
</tr>
</tbody>
</table>
Fig. 4.6: Non-linear least-squares analysis of quantities of X and •Y122 as functions of time in the reaction of apo R2-wt with excess Fe^{2+}. The equations appropriate for the kinetic model of Scheme 4.1 (Atkins, 1986) were fit to the data from Table 4.4. The curves generated in the analysis are shown. They correspond to $k_1 = 10 \text{ s}^{-1}$, $k_2 = 0.95 \text{ s}^{-1}$, and $(•Y122)_{\text{final}} = 1.2$. 
The concentrations of \( \mathbf{X} \) and \( \mathbf{\cdot Y122} \) as functions of time suggest that decay of \( \mathbf{X} \) and formation of \( \mathbf{\cdot Y122} \) are concomitant processes. This result is consistent with a mechanism in which \( \mathbf{X} \) generates \( \mathbf{\cdot Y122} \).

*Isotopic Labelling of \( \mathbf{X} \) with \( ^{57}\text{Fe}^{2+} \)*

In Chapter 3, it is proposed that the component of \( \mathbf{II} \) which generates \( \mathbf{\cdot Y122} \) is an intermediate iron cluster. To assess whether \( \mathbf{X} \) contains iron (by looking for hyperfine coupling in its EPR spectrum to the \( I = 1/2 \) \( ^{57}\text{Fe} \) nucleus), the intermediate species was generated both in \( \mathbf{R2-wt} \) and in \( \mathbf{R2-Y122F} \) with \( ^{57}\text{Fe}^{2+} \). The spectra resulting from the reaction of apo \( \mathbf{R2-Y122F} \) (A) with excess \( ^{56}\text{Fe}^{2+} \) or (B) with excess \( ^{57}\text{Fe}^{2+} \) are shown in Fig. 4.7. Pronounced hyperfine broadening of the \( g = 2.00 \) singlet of \( \mathbf{X} \) is observed when the species is generated with \( ^{57}\text{Fe}^{2+} \), indicating that the electron spin of this species is coupled to at least one \( ^{57}\text{Fe} \) nucleus. This hyperfine coupling is also observed when \( \mathbf{X} \) is generated in \( \mathbf{R2-wt} \) with excess \( ^{57}\text{Fe}^{2+} \) (data not shown). The coupling to \( ^{57}\text{Fe} \) exhibited by \( \mathbf{X} \) demonstrates that it is an Fe species.

As stated above, the time-course data suggest that the EPR spectrum which develops upon reaction of apo \( \mathbf{R2-Y122F} \) with excess \( \text{Fe}^{2+} \) is characteristic of pure \( \mathbf{X} \). Therefore, the spectrum of Fig. 4.7B is characteristic of \( \mathbf{X} \) coupled to \( ^{57}\text{Fe} \), and it contains information regarding the number and magnitude of \( ^{57}\text{Fe} \) hyperfine interactions. Simulation of this spectrum by imposition of hyperfine coupling on the spectrum of \( \mathbf{X} \) generated with \( ^{56}\text{Fe}^{2+} \) (the coupling is actually imposed upon a simulated \( ^{56}\text{Fe} \) spectrum) indicates that the species contains at least two Fe nuclei. Imposition of coupling to a single \( ^{57}\text{Fe} \) nucleus produces unsatisfactory agreement with the experimental spectrum. If the coupling constant used is sufficiently large to reproduce the peak-to-trough separation of the \( ^{57}\text{Fe} \) spectrum, a doublet type spectrum is obtained (Fig. 4.8C) which does not approximate the experimental lineshape (Fig. 4.8A). In contrast, satisfactory agreement
Fig. 4.7: Hyperfine coupling of X to $^{57}$Fe. (A) Apo R2-Y122F (0.14 mM) in air-saturated 100 mM HEPES buffered at pH 7.7 was mixed at 5 ± 1 °C with an equal volume of 1.0 mM $^{56}$Fe$^{2+}$ in air-saturated 5 mM HNO$_3$. The reaction was quenched after 0.31 s. (B) Apo R2-Y122F (0.29 mM) in air-saturated 100 mM HEPES buffered at pH 7.7 was mixed at 5 ± 1 °C with an equal volume of 2.9 mM $^{57}$Fe$^{2+}$ in air-saturated 2.5 mM H$_2$SO$_4$, and the reaction was quenched after 0.45 s. The spectra were acquired at 20 K with all spectrometer settings except the microwave power (2 μW) and the receiver gain (2 x 10$^5$) the same as in Fig. 4.2.
can be achieved if hyperfine coupling to two $^{57}$Fe nuclei is considered (Fig. 4.8B). These simulations indicate that at least two Fe nuclei are present in X.

*Isotopic Labelling of X with $^{17}$O$_2$, H$_2^{17}$O, and $^2$H$_2$O*

To further characterize the intermediate Fe cluster, X, isotopic labelling experiments were carried out in which the species was generated in the presence of $^{17}$O$_2$, H$_2^{17}$O or $^2$H$_2$O. The EPR spectrum of X generated in the presence of $^{17}$O$_2$ (37 atom %) by the reaction of apo R2-Y122F with excess Fe$^{2+}$ clearly demonstrates coupling between $^{17}$O and the electron spin of X (Fig. 4.9B). In an attempt to analyze this coupling, it was assumed that the observed coupling arises from a single $^{17}$O nucleus, and the portion of the spectrum (63%) which would correspond to X containing $^{16}$O was subtracted away. The resulting spectrum (Fig. 4.9C) is physically unreasonable in that it cannot be simulated by imposing hyperfine coupling to one (or more than one) I=5/2 nucleus on the EPR spectrum of X. It appears that in order to generate a physically reasonable subtraction spectrum for the $^{17}$O-coupled X, less than 63% of the $^{16}$O spectrum should be subtracted. The implications of this result are discussed below.

X is also coupled to O nuclei from solvent H$_2$O. The spectrum of Fig. 4.10B results from production of X in ~32% H$_2^{17}$O, and clearly shows hyperfine broadening due to $^{17}$O. Likewise, X must be coupled to at least one solvent-exchangeable H nucleus, as the EPR spectrum of the cluster generated in 91% $^2$H$_2$O shows significant narrowing (Fig. 4.11B).

*Microwave Power and Temperature Dependence of the g = 2.0 Signal of X*

The power saturation behavior (at 20 K) of the EPR signal of X formed in R2-wt was also briefly investigated. The signal intensity of the 0.078 s sample from the experiment of Table 4.2A was determined as a function of the applied microwave power. (It was estimated that this sample contained >85% X and <15% *Y122.*) For comparison
Fig. 4.8: Simulation of the EPR spectrum of X coupled to \(^{57}\text{Fe}\). Spectrum A is B from Fig. 4.7. Spectra B and C are simulations obtained by applying the ESR\(^3\) program as follows. First, the spectrum of X generated with \(^{56}\text{Fe}^{2+}\) was simulated. A rhombic, diagonal g-tensor with values of 2.0043, 2.0073 and 2.0177 was assumed. (These values are higher than the actual g of X. This point is insignificant for simulating the line-shape of the spectrum.) A Gaussian lineshape with linewidth 7.2 G was assumed. The spectrum of X coupled to \(^{57}\text{Fe}\) was then simulated by imposing hyperfine coupling on the simulated \(^{56}\text{Fe}\) spectrum. For spectrum B, coupling to two \(^{57}\text{Fe}\) nuclei was assumed. Isotropic A-values of 12 G and 25.5 G were used. For spectrum C, coupling to a single \(^{57}\text{Fe}\) nucleus was assumed. An isotropic A-value of 32 G was used.
Fig. 4.9: Hyperfine coupling of X to $^{17}$O from O$_2$. X was generated by reaction of O$_2$-free apo R2-Y122F with Fe$^{2+}$ stock equilibrated (A) in air or (B) in 1 atm. of (37 atom % $^{17}$O) $^{17}$O$_2$. Preparation of these samples is described in the Materials and Methods section. The spectra were acquired at 20 K, with a microwave power of 6.7 $\mu$W and a receiver gain of $10^5$. All other spectrometer settings were as in Fig. 4.3. C results from subtraction of B (corresponding to 63% of the double integrated intensity of A) from A.
Fig. 4.10: Hyperfine coupling of X to $^{17}\text{O}$ from H$_2$O. X was generated in R2-Y122F (A) in H$_2^{16}$O or (B) in ($\sim32\%\;^{17}\text{O}$) H$_2^{17}$O. A is the same spectrum as Fig. 4.9A and is shown for comparison. Preparation of the sample of B is described in Materials and Methods. The spectra were acquired as in Fig. 4.9.
Fig. 4.11: Hyperfine coupling of X to H from H$_2$O. X was generated in R2-Y122F (A) in H$_2$O or (B) in 91% $^2$H$_2$O. A is the same spectrum as Fig. 4.9A. Preparation of the sample of B is described in Materials and Methods. The spectra were acquired as in Fig. 4.9.
to the saturation behavior of •Y122, the 60 s sample from the same experiment was also investigated. Fig. 4.12 shows the saturation profiles which were obtained. While the data are too poor to allow quantitative conclusions to be drawn, it appears that the power saturation of X at 20 K is not significantly different from that of •Y122, with each signal beginning to saturate at ~10 µW.

At temperatures above 20 K, the EPR singlet of X becomes increasingly broad. Fig. 4.13 illustrates this effect from 20 K to 80 K. Even up to 100 K, the signal of X is still observable.

**EPR Time-Course of the Reaction of Apo R2-wt with Limiting Fe²⁺**

As stated above, the time-dependent EPR spectrum of the reaction of apo R2-wt with excess Fe²⁺ is consistent with the simple 3 component mechanism of Scheme 4.1, in which the intermediate X rapidly \( k_{\text{obs}} = 10 \text{ s}^{-1} \) forms and then decays more slowly \( k_{\text{obs}} = 0.95 \text{ s}^{-1} \) to give •Y122. Development of the EPR spectrum in the reaction of apo R2-wt with limiting Fe²⁺ is more complex (Fig. 4.14). As in the excess Fe²⁺ reaction, the intermediate cluster X forms rapidly. The spectrum at short times (Fig. 4.14A) resembles the isotropic singlet characteristic of X, and demonstrates hyperfine coupling to \(^{57}\text{Fe} \) of the same magnitude as that observed for X (Fig. 4.15). Also as in the excess Fe²⁺ reaction, the spectra at increasingly longer reaction times show increasing contribution from •Y122 and decreasing contribution from X (Fig. 4.14B, C, D). In contrast to the excess Fe²⁺ reaction, however, additional EPR features, which are broader than those of X and •Y122, are observed in the \( g = 2.0 \) region of the limiting Fe²⁺ spectra. These features are present at all times from 0.10 s to beyond 3 s. In the early spectra (0-1 s), the broad features contribute as much as 50 ± 10 % of the double integrated intensity (as estimated by subtraction of the spectra due to •Y122 and X, see Fig. 4.16), indicating that the species giving rise to these features accumulate(s) to concentrations comparable to those of X and
Fig. 4.12: Microwave power saturation profiles for a sample containing predominantly X and for a sample containing exclusively •Y122. The samples were prepared by mixing 99 μM apo R2-wt in air-saturated 100 mM HEPES buffered at pH 7.7 with an equal volume of 1.0 mM Fe²⁺ in air-saturated 5 mM HNO₃. For the sample containing predominantly X (>85%), the reaction was quenched at 0.971 s. For the sample containing exclusively •Y122, the reaction was quenched at 60 s. The solid lines through the data are hand-drawn fits.
Fig. 4.13: Effect of temperature on the EPR spectrum of X. The spectra were acquired at the indicated temperature on the sample of Fig. 4.4B. For the 20 K spectrum, the spectrometer setting were as in Fig. 4.4. For the 80 K spectrum, the settings were as in Fig. 4.4, except that the power was 0.10 mW.
Fig. 4.14: Time-course of the reaction of apo R2-wt with limiting Fe$^{2+}$ as monitored by EPR spectroscopy. Apo R2-wt (0.59 mM) in O$_2$-saturated 100 mM HEPES buffered at pH 7.7 was mixed at 5 ± 1 °C with an equal volume of 1.35 mM Fe$^{2+}$ in O$_2$-saturated 2.5 mM H$_2$SO$_4$. The reaction was quenched (A) at 0.15 s, (B) at 0.44 s, (C) at 1.0 s, or (D) at 60 s. The spectra were acquired at 20 K with a microwave power of 2 μW and a receiver gain of 2.5 x 10$^4$. All other spectrometer settings were as in Fig. 4.3.
**Fig. 4.15:** Formation of X in the limiting Fe$^{2+}$ reaction as demonstrated by hyperfine coupling to $^{57}$Fe. Apo R2-wt (99 μM) in air-saturated 100 mM HEPES buffered at pH 7.7 was mixed at 5 ± 1 °C with an equal volume of 220 μM (A) $^{56}$Fe$^{2+}$ or (B) $^{57}$Fe$^{2+}$ in air-saturated 5 mM HCl. The reactions were quenched at 0.11 s. The spectra were recorded at 40 K with a power of 200 μW, and a receiver gain of $1.25 \times 10^5$. All other spectrometer settings were as in Fig. 4.3.
•Y122. The broad features eventually decay to leave only the spectrum of •Y122 (Fig. 4.14D).

X also forms in the reaction of apo R2-Y122F with limiting Fe$^{2+}$. As with R2-wt, additional broad features are observed in the $g = 2.0$ region. The temporal behavior of the broad features is different, however, in R2-Y122F from that seen in R2-wt. An attempt to illustrate this difference is shown in Figs. 4.16 and 4.17 and in Table 4.5. To create the inner spectra of Fig. 4.16, the spectra of X and of •Y122 were summed in ratios appropriate to approximate the lineshape of the three (outer) experimental spectra, which are from the reaction of apo R2-wt with limiting Fe$^{2+}$. Each summation spectrum was then used to estimate (by using the EPR subtraction program of Prof. Huynh) the percentage of the double integrated intensity of the corresponding experimental spectrum which, in the derivative mode, falls outside the summed spectra of X and •Y122. Table 4.5 summarizes the percentages of X and of •Y122 added to get the inner spectra of Fig. 4.16, and the percentage of the double integrated intensity of each spectrum which is contributed by the broad features. In Fig. 4.17, the spectrum of X formed in the reaction of apo R2-Y122F with excess Fe$^{2+}$ (the inner spectrum of A, B, and C) was used to estimate the percentage of the double integrated intensity of each limiting Fe$^{2+}$ spectrum which, in the derivative mode, falls outside the spectrum of X. Table 4.5 summarizes these estimates. In the reaction of either apo R2-wt or apo R2-Y122F with limiting Fe$^{2+}$, the broad features contribute approximately 50% of the double integrated intensity of the spectrum at early times (Figs. 4.16A and 4.17A). At 1 s this fraction has decreased to ~20% in R2-Y122F (Fig. 4.17C). In contrast, the broad signal still makes up 35% of the intensity in R2-wt at this time (Fig. 4.16C). This analysis, which should be regarded merely as qualitative, suggests that the species associated with the broad features decay(s) more rapidly in R2-Y122F than in R2-wt.

It should be possible to deduce the lineshape of the broad EPR features formed in the reaction of apo R2-Y122F with limiting Fe$^{2+}$ by subtracting away the spectrum of X.

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Fig. 4.16: Qualitative analysis of the temporal behavior of the transient, broad EPR features which develop in the reaction of apo R2-wt with limiting Fe$^{2+}$. The outer (experimental) spectra of A, B, and C are the corresponding spectra from Fig. 4.14. The inner spectra were constructed by summation of the spectra of X and of •Y122 in relative amounts appropriate to reproduce the line-shape of the inner part of each experimental spectrum. The reference spectra were the same as those used in Fig. 4.5.
Fig. 4.17: Qualitative analysis of the temporal behavior of the transient, broad EPR features which develop in the reaction of apo R2-Y122F with limiting Fe$^{2+}$. The outer spectra were acquired on samples prepared as follows. Apo R2 Y122F (0.60 mM) in O$_2$-saturated 100 mM HEPES buffered at pH 7.7 was mixed at 5 ± 1 °C with an equal volume of 1.3 mM Fe$^{2+}$ in O$_2$-saturated 2.5 mM H$_2$SO$_4$. The reaction was quenched (A) at 0.15 s, (B) at 0.44 s, or (C) at 1.0 s. The inner spectrum in A-C is the reference spectrum for X from Fig. 4.5. The spectrometer settings for the experimental spectra were the same as for Fig. 4.14.
Table 4.5: Summary of the analysis of the EPR spectra from the reaction of apo R2-wt or apo R2-Y122F with limiting Fe$^{2+}$.

<table>
<thead>
<tr>
<th>Protein with Lim. Fe$^{2+}$</th>
<th>Time (s)</th>
<th>% X in Inner Spectrum$^a$</th>
<th>% • Y122 in Inner Spectrum$^a$</th>
<th>% Broad Features in Experimental Spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>apo R2-wt</td>
<td>0.15</td>
<td>83</td>
<td>17</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>0.44</td>
<td>56</td>
<td>44</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>22</td>
<td>78</td>
<td>35</td>
</tr>
<tr>
<td>apo R2-Y122F</td>
<td>0.15</td>
<td>100</td>
<td>--</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>0.44</td>
<td>100</td>
<td>--</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>100</td>
<td>--</td>
<td>20</td>
</tr>
</tbody>
</table>

$^a$ refers to Figs. 4.16 and 4.17

Fig. 4.18 shows the spectra which result from subtracting the inner spectra of Fig. 4.17 from the outer spectra. The lineshape of the subtraction spectrum changes somewhat with time. This observation may indicate that the broad features arise from multiple species.

It should likewise be possible to subtract the spectra of X and of • Y122 from the experimental spectra of Fig. 4.16. Fig. 4.19 shows two subtraction spectra generated in this manner. (The corresponding spectra from Fig. 4.18 are shown for comparison.) The spectra are somewhat different from those of Fig. 4.18. The analysis is complicated in this case by the presence of at least 3 EPR active species (X, • Y122, and at least one species giving rise to the broad features). It is not possible, therefore, to say with certainty whether the observed differences in the subtraction spectra reflect real differences between R2-wt and R2-Y122F in the nature of the broad features or merely reflect errors in subtracting the spectra of X and • Y122.
Fig. 4.18: Line-shape of the transient, broad EPR features which develop in the reaction of apo R2-Y122F with limiting Fe$^{2+}$. The inner spectra of Fig. 4.17A-C were subtracted from the outer spectra to give A-C. The percentage of the double integrated intensity of each experimental spectrum which was subtracted is indicated in Table 4.5.
Fig. 4.19: Line-shape of the transient, broad EPR features in the reaction of apo R2-wt with limiting Fe$^{2+}$. The inner spectra of Fig. 4.16A and C were subtracted from the outer spectra to give A and B, respectively. C and D are the subtraction spectra of Fig. 4.18A and C, respectively, and are shown for comparison.
Reaction of Apo R2-wt or Apo R2-Y122F with Limiting Fe$^{2+}$ and Ascorbate

In both R2-wt and R2-Y122F, the broad features observed in the limiting Fe$^{2+}$ reaction do not develop when the reaction is carried out in the presence of 2.5 mM ascorbate (Fig. 4.20). (The spectra shown are of samples prepared with $^{57}$Fe$^{2+}$, but identical behavior was observed with $^{56}$Fe$^{2+}$.) Thus, the broad features are associated only with components of Pathway A of Scheme 3.4 (I and II').

Discussion

The EPR spectrum as a function of time for the reaction of apo R2 with excess Fe$^{2+}$ can readily be interpreted within the schematic mechanism of Scheme 3.4. The rapid accumulation of the intermediate iron cluster (X), which is characterized by the g = 2.00 isotropic singlet, correlates in time with the development of the 360 nm broad absorption band of intermediate II. The rate constant for formation of X as determined by EPR ($k_{\text{obs}} = 10$ s$^{-1}$) is in fair agreement with the rate constant determined in Chapter 3 for formation of II ($k_{\text{obs}} = 5.3-8.8$ s$^{-1}$). The decay of the EPR singlet due to X and the development of the doublet characteristic of •Y122 correspond temporally with the development of the light absorption spectrum characteristic of the product cofactor. The rate constant for decay of X and for formation of •Y122 as determined by EPR ($k_{\text{obs}} = 0.95$ s$^{-1}$) is in fair agreement with the rate constant for formation of •Y122 ($k_{\text{obs}} = 0.72-0.77$ s$^{-1}$) determined in Chapter 3. Furthermore, the results of Chapter 3 indicate, as might be expected from Scheme 3.4, that formation of the diferric cluster from the intermediate Fe species is slower in R2-Y122F than in R2-wt. This observation seems to be reflected in the EPR data, which suggest that X persists longer in R2-Y122F than in R2-wt. Thus, the stopped-flow absorption and rapid freeze-quench EPR data together provide convincing evidence for Pathway B of Scheme 3.4, in which the intermediate II rapidly accumulates, and then decays to give native R2. II contains a single reactive oxidizing equivalent in the form of
Fig. 4.20: Suppression of the broad EPR features by ascorbate. Apo R2-Y122F (0.63 mM) in O$_2$-saturated 100 mM HEPES buffered at pH 7.7 was mixed at 5 ± 1 °C with an equal volume of 1.5 mM $^{57}$Fe$^{2+}$ in O$_2$-saturated (A) 2.5 mM H$_2$SO$_4$ or (B) 5 mM ascorbic acid. Both reactions were quenched at 0.34 s. The spectra were acquired at 20 K with a power of 6.7 μW and a receiver gain of 5 x 10$^4$. All other settings were as in Fig. 4.3.
the iron cluster, $X$, which exhibits the transient 360 nm absorption band and the EPR singlet, and which generates $\cdot Y122$ by one electron oxidation of Y122.

The time-dependent EPR spectrum of the limiting $Fe^{2+}$ reaction is less easily interpreted in terms of Scheme 3.4 than that of the excess $Fe^{2+}$ reaction, both because the reaction is kinetically more complex and because the broad features of unknown lineshape preclude accurate estimation of the amounts of $X$ and $\cdot Y122$ present at each time-point. Although the development of the broad $g = 2.0$ features upon reaction of either apo R2-wt or apo R2-Y122F with limiting $Fe^{2+}$, but not upon reaction of either protein with excess $Fe^{2+}$ or with limiting $Fe^{2+}$ and ascorbate, is consistent with a two pathway mechanism, it is not immediately obvious that the EPR data require the occurrence of two distinct intermediates ($I$ and $I'$) in Pathway A. Nevertheless, the results can be reconciled with the proposed mechanism. The development of the broad features in R2-Y122F suggests that the features are associated with $I$, since $I'$ cannot form in the mutant protein. The unexpected observation that the broad features decay more slowly in R2-wt than in R2-Y122F can be rationalized if the features are also associated with $I'$. In this interpretation, decay of the broad features in R2-Y122F would occur concomitantly with decay of $I$ (again, since $I'$ cannot form in the mutant protein), whereas decay of the broad features in R2-wt would occur only upon conversion of $I'$ into native R2. Thus, the broad features might persist longer in R2-wt. This interpretation leads naturally to the hypotheses that the broad features in $I'$ arise from interaction of its one-electron oxidized intermediate iron cluster (possibly $X$) with $\cdot Y122$ (an interaction which can occur only in R2-wt), whereas the broad features in $I$ arise from an additional EPR active species (which can form in both R2-wt and R2-Y122F). Additional evidence in support of these hypotheses is presented in Chapter 7.

We previously reported that broad features did not develop in the reaction of apo R2-Y122F with limiting $Fe^{2+}$, and the lack of these features in the mutant protein was cited as evidence that the broad features in R2-wt arise from interaction of $X$ and $\cdot Y122$.
Two factors account for our previous failure to recognize the broad features in the reaction of apo R2-Y122F with limiting Fe$^{2+}$. First, lower reactant concentrations were employed in those experiments, leading to an inferior signal-to-noise ratio. Second, the difference in the temporal behavior of the features between R2-wt and R2-Y122F was not anticipated. As noted above, the features seem to decay more rapidly in R2-Y122F than in R2-wt. This trend contrasts with the trends observed both for X and for the 560 nm absorbing species, which exhibit increased lifetimes in the mutant protein.

The $^{57}$Fe hyperfine coupling seen in the early spectra of the limiting Fe$^{2+}$ reaction (see Fig. 4.15) indicates that the intermediate cluster X accumulates in this reaction as well as in the excess Fe$^{2+}$ reaction. Since it is proposed in Scheme 3.4 that two distinct intermediates (I and II') accumulate in the limiting Fe$^{2+}$ reaction, the question arises as to whether X is a component only of I, only of II', or of both (assuming that Scheme 3.4 is correct). The question can be stated more generally as follows: 1) Does the intermediate which is associated with the 560 nm transient absorption (I) contain X? 2) If so, are decay of the 560 nm absorbing intermediate and decay of X concomitant processes, or does the 560 nm absorbing intermediate decay to give a second intermediate (II') which also contains X? Quantitation of X as a function of time (data which is reported in Chapter 7) is required to allow definitive answers to these questions, but the data of this chapter do suggest answers. If X were a component only of II', •Y122 and X should form simultaneously, and the quantity of X present in the reaction should at all times be less than (or equal to) the quantity of •Y122 present. This condition does not appear to be met early in the reaction (see Figs. 4.14A and 4.15). It appears that X initially forms faster than •Y122, which would suggest that the cluster is a component of I. This conclusion must be considered tentative, however, since a partition between Pathway A and Pathway B (in which X is clearly a precursor to •Y122) in the limiting Fe$^{2+}$ reaction would cause X to form faster than •Y122, even if the cluster were not a component of I. Such a partition is expected on the basis of analysis of the A$^{410}$,dpline-versus-time traces (Chapter 3).
The data of this chapter are likewise ambiguous as to whether \( \text{X} \) is a component of \( \text{II}' \). As stated above, the behavior of the broad features in R2-wt and R2-Y122F can be rationalized by proposing that \( \text{II}' \) exhibits broad features which result from interaction of •Y122 and a one-electron-oxidized intermediate iron cluster. In the simplest case, this cluster would be \( \text{X} \). Again, the quantitation of \( \text{X} \) as a function of time which is presented in Chapter 7 provides support for this speculation.

The hyperfine broadening of the EPR spectrum of \( \text{X} \) observed when the reaction is carried out in the presence of 37% \( ^{17}\text{O}_2 \) indicates that the intermediate cluster retains one or both of the O nuclei from \( \text{O}_2 \). The observation that an unreasonable spectrum is obtained when 63% of the spectrum due to unlabelled \( \text{X} \) is subtracted away suggests that both O nuclei from \( \text{O}_2 \) may be retained. In this scenario, 60% of the \( \text{X} \) formed would contain one or two \( ^{17}\text{O} \) nuclei (since 37 atom % \( ^{17}\text{O}_2 \) should have the following composition: 13.6% \( ^{17}\text{O}^{17}\text{O} \), 46.6% \( ^{17}\text{O}^{16}\text{O} \), and 39.7% \( ^{16}\text{O}^{16}\text{O} \), and the observed hyperfine coupling would arise from \( ^{17}\text{O} \) nuclei bound in one or both of two different positions (with each position potentially having a different coupling constant.) Thus, simulation of the spectrum of \( ^{17}\text{O} \)-coupled \( \text{X} \), without additional information concerning these coupling constants, would be uninformative due to the requirement for an excessive number of parameters. Further discussion regarding the structure of \( \text{X} \) is reserved for Chapter 5, in which characterization of the species by Mössbauer spectroscopy is described.

The results of this chapter allow the mechanism of Scheme 3.4 to be elaborated (Scheme 4.2). The presence of an oxidized iron cluster in \( \text{II} \) is confirmed by the accumulation of nearly 1 equivalent of \( \text{X} \) in the excess \( \text{Fe}^{2+} \) reaction. In addition, the presence of \( \text{X} \) either in \( \text{I} \), in \( \text{II}' \), or in both is indicated by the accumulation of this species in the limiting \( \text{Fe}^{2+} \) reaction. The presence of an additional EPR active intermediate in \( \text{I} \) is supported by the development of a relatively broad \( g = 2.0 \) resonance in the reaction of apo R2-Y122F with limiting \( \text{Fe}^{2+} \). Less definitively, the simultaneous presence of \( \text{X} \)
Scheme 4.2: Schematic mechanism for assembly of the R2 cofactor from Chapter 3 (Scheme 3.4) elaborated to reflect the results of the rapid freeze-quench EPR experiments of this chapter. The features of the scheme which are from this chapter are in bold print. The abbreviations SF and RFQ EPR indicate whether the rate constant was determined by stopped-flow or by rapid freeze-quench EPR, respectively.
and •Y122 in II' could explain the slower decayi.g broad features observed in the reaction of apo R2-wt with limiting Fe^{2+}.

More definitive interpretation of the results of this chapter would be facilitated by more precise double integrals for both the limiting Fe^{2+} reaction and the excess Fe^{2+} reaction. The values obtained in these experiments show unacceptably large scatter. The reason for this scatter is not obvious, but may be related to the noticeable heterogeneity in the EPR tubes used (in terms of length and diameter), or to poor temperature control in the EPR cryostat. Accurate spin quantitation as a function of time for both reactions would allow certain predictions based on Scheme 4.2 to be tested experimentally. For example, in Pathway A, the rapidly-accumulating di-radical intermediate (II') is postulated to decay more slowly to native R2, which has a single radical. Thus, the total spin concentration as a function of time for the limiting Fe^{2+} reaction might be expected to exhibit a rise-fall behavior. Likewise, spin quantitation for the reaction of apo R2-Y122F with excess Fe^{2+} might provide more definitive evidence for the tentative conclusion that X decays more slowly in apo R2-Y122F than in apo R2-wt. A primary objective of future efforts should be to solve the unidentified experimental problems which have thus far precluded reliable spin quantitation.

References


Chapter 5: Mössbauer Characterization of a Novel Diferric-Radical

Intermediate which Generates the Tyrosyl Radical in R2
The results of Chapter 4 demonstrate that an intermediate iron cluster (X) accumulates to near stoichiometric quantities in the reaction of apo R2 with excess Fe$^{2+}$ and O$_2$, and that the cluster decays concomitantly with •Y122 formation (Scheme 5.1).

**Scheme 5.1:** Kinetic scheme deduced in Chapter 4 for the reaction of apo R2-wt with excess Fe$^{2+}$ (or with limiting Fe$^{2+}$ and ascorbate) and O$_2$.

\[
\text{apo R2} + \text{Fe}^{2+} + \text{O}_2 \rightarrow_{10 \text{ s}^{-1}} \text{X} \rightarrow_{0.95 \text{ s}^{-1}} \cdot \text{Y122}
\]

These kinetic data, as well as the data of Chapter 3, strongly suggest that X generates •Y122 in the excess Fe$^{2+}$ reaction (and in the limiting Fe$^{2+}$ and ascorbate reaction). In the experiments which are described in this chapter, Mössbauer spectroscopy was used to further characterize this key intermediate in the assembly of the R2 cofactor. This characterization was carried out both in the R2-Y122F mutant protein, in which one equivalent (relative to R2) of X can be trapped which is free from both •Y122 and the diferric cluster, and in R2-wt. The results demonstrate explicitly that X contains two tightly-coupled, high-spin ferric ions, and imply that the two ferric ions are also coupled to a free radical, which most likely derives either from O$_2$ or from an iron ligand. Identification of this heretofore unprecedented "diferric-radical species" is perhaps the most significant result of this work, as it indicates, in contrast to virtually all of the mechanistic proposals which have been put forth in the literature (Fontecave et al., 1990; Sahlin et al., 1990; Stubbe, 1990; Que, 1991), that the species which generates •Y122 does not contain Fe(IV).

A second significant result presented in this chapter is the successful adaptation of the rapid freeze-quenching apparatus for preparation of Mössbauer samples. (Application of the freeze-quench method to Mössbauer spectroscopy was previously reported, but the
sampling apparatus which these investigators used was somewhat cumbersome (Kent et al., 1985). The apparatus described herein allows for efficient, reproducible preparation of freeze-quenched samples in receptacles suitable for Mössbauer spectroscopy. This capability provides the technical basis for the complete kinetic characterization of the R2 reconstitution reaction by the rapid freeze-quench Mössbauer method. The results of this characterization, which are reported in Chapters 6 and 7, provide additional evidence for the schematic mechanism proposed in Chapters 3 and 4, and allow significant chemical details of the mechanism to be filled in.

Materials and Methods

$^{57}$Fe metal (98% isotopic purity) was purchased from Advanced Materials and Technology of the former U.S.S.R. Apo R2-wt and apo R2-Y122F were isolated as described in Chapter 2.

Preparation of $^{57}$Fe$^{2+}$ Stock

The $^{57}$Fe$^{2+}$ stock solution used in preparation of the rapid freeze-quench samples was prepared by anaerobic dissolution of the $^{57}$Fe metal in dilute H$_2$SO$_4$. A 150 μL aliquot of 1 M H$_2$SO$_4$ was added to a 5.3 mg nugget of $^{57}$Fe$^0$. The mixture was incubated at ambient temperature in an anaerobic chamber for 4 days. After this time, it was estimated that one-half of the $^{57}$Fe metal had dissolved. The liquid was taken off and diluted to 7.5 ml with O$_2$-free H$_2$O. This stock was assayed for [Fe$^{2+}$] (as described in Chapter 3) and found to be 6.2 mM, confirming that one-half of the $^{57}$Fe$^0$ had dissolved. A 2 mL aliquot of this 6.2 mM $^{57}$Fe$^{2+}$ stock was diluted with 0.76 mL H$_2$O and 28 μL of 0.5 M sodium ascorbate in H$_2$O. This final stock solution was filtered through a 0.22 μM filter. It was assayed for [Fe$^{2+}$] and found to be 4.5 mM. It was calculated to contain 20 mM H$^+$ (from the H$_2$SO$_4$) and 5 mM ascorbate.
Preparation of Samples for Mössbauer Spectroscopy

Two samples were prepared for Mössbauer characterization of X. For sample 1, 1.5 mM apo R2-Y122F in O₂-saturated 100 mM HEPES buffered at pH 7.7 was mixed at 5 ± 1°C with an equal volume of O₂-saturated 4.5 mM $^{57}$Fe²⁺ stock. The reaction was quenched at 0.31 s. For sample 2, 1.5 mM apo R2-wt was mixed under identical conditions with an equal volume of the 4.5 mM $^{57}$Fe²⁺ stock, and the reaction was quenched at 0.31 s.

The apparatus used to prepare the Mössbauer samples (shown schematically in Fig. 5.1) is a derivative of the EPR sampling apparatus (see Fig. 4.1). It was designed and fabricated by Mr. Bud Puckett in the Department of Physics at Emory University. Sample preparation was carried out as follows. The apparatus was assembled, placed in its holder, and filled with isopentane. It was inserted into the cold quenching bath (containing isopentane at ~ -150 °C) and allowed to reach thermal equilibrium. The spray nozzle at the end of the aging hose was held less than 1 cm above the level of the isopentane in the glass funnel, and the reactants were mixed and expelled into the cold isopentane by actuation of the ram drive. Approximately 500 µL of reaction mixture was required to generate a satisfactory sample. In preparation of all the freeze-quenched Mössbauer samples (including those of Chapters 6 and 7), the velocity of the ram drive was 1 cm/s. This relatively low velocity seemed to be required to obtain ice crystals which were coarse enough to settle properly. The ice crystals were allowed to settle for approximately one min, an then were packed into the Mössbauer cell. The crystals were packed gently at first, so as to avoid breaking them into finer particles, and then were packed forcefully, in order to obtain as dense a sample as possible. As rapidly as possible (total time elapsed of 1-4 s), the sampling apparatus was removed from the quenching bath and from its holder, and the Mössbauer cell was submerged in a slush of partially frozen isopentane. The slush was prepared (prior to removal of the sampling apparatus from the bath) in a styrofoam cup which had been cut to approximately 4 cm in height.
Fig. 5.1: Apparatus used to prepare rapid freeze-quenched samples for Mössbauer spectroscopy. It was designed and fabricated by Mr. Bud Puckett in the Department of Physics at Emory University.
(This styrofoam cup was replaced in the experiments of Chapters 6 and 7 with a broad (~13 cm in diameter), flat (~8 cm in height), stainless-steel Dewar.) With the packer still in the packing column, the latex connector and glass funnel were removed. Again with the packer still pressing on the top of the sample, the apparatus was lifted from the isopentane slush just long enough for the threaded Mössbauer cell to be manually loosened from the threaded coupler which connects it to the packing column. The sample cell was quickly reinserted into the isopentane slush bath. With the packer providing friction against the top of the sample, the packing column and threaded coupler were unscrewed and removed from the sample cell. A hex-nut style, delrin sample cap (not depicted in Fig. 5.1) was inserted into the isopentane slush bath on the end of a hex-key wrench. The cooling of the cap on the wrench had the effect of temporarily fixing the two together. Next, the sample cell in the slush bath was grasped with pre-cooled tweezers, and the threaded sample cap was inserted into the threaded sample cell. With both still emersed in the cold isopentane, the cap was threaded into the sample cell by turning the hex-key wrench while holding the sample cell with the tweezers. Once the threads had grabbed, the two were repeatedly removed from the slush bath, for as short a time as possible (~1 s per removal), in order to manually tighten the cap inside the sample cell. Between tightenings, the two were reinserted into the slush bath and allowed to cool. (The slush bath was kept cold throughout this process by periodic addition of liquid N₂ to the isopentane.) In this manner, the cap was made as tight as possible, in order to compress the sample to the minimum thickness and highest density achievable. When the cap was tight, the sample was removed from the end of the hex-key wrench, very briefly wiped free of isopentane, and then stored in liquid N₂. Acquisition of replicate spectra over a period of months has shown that the samples are indefinitely stable in liquid N₂.
Results

The Mössbauer spectrum at 4.2 K of sample 1 (R2-Y122F) is shown in Fig. 5.2A. The spectrum consists of a central quadrupole doublet (indicated by the bracket) and a magnetic component with resolved absorption peaks at -3.9, -1.8, and 5.0 mm/s. The spectrum of sample 2 (R2-wt, Fig. 5.2B) has identical features. The minor differences between spectra A and B can be explained by the presence in sample 2 of 0.1 equiv of the diferred cluster (features indicated by arrow) and of slightly less (~0.9 times) of the species responsible for the magnetic component. The quadrupole doublet in A and B has Mössbauer parameters ($\Delta E_Q = 3.20$ mm/s and $\delta = 1.30$ mm/s) which are characteristic either of the diferrous cluster of R2 (Lynch et al., 1989) or of high-spin ferrous ions in solution. The magnetic component must be associated with X, for two reasons. First, as stated in Chapter 4, no EPR signal attributable to iron is observed in the freeze-quenched samples other than the $g = 2.0$ signal which is associated with X. Second, experiments described in Chapter 4 indicate that between 0.5 and 1 equiv of X accumulates under these reaction conditions. The three resolved magnetic peaks in Fig. 5.2A contribute greater than 33% of the integrated intensity of the spectrum. Since this area corresponds to 1 equiv of Fe, a lower limit of 0.5 equiv can be set (if one assumes for the sake of argument that the species is dinuclear) for the quantity of the paramagnetic species which is present in sample 1. The association of the $g = 2.0$ EPR signal of X (which implies a system electron spin quantum number of 1/2) with the magnetic Mössbauer component of Fig. 5.2A indicates that the component can be analyzed according to an $S = 1/2$ spin Hamiltonian (Eq. 5.1). The component can be interpreted as the superposition of two

$$\hat{H} = \beta \vec{S} \cdot \vec{\tilde{g}} \cdot \vec{H} + \vec{S} \cdot \vec{\tilde{A}} \cdot \vec{I} + \frac{eQV_{zz}}{4} \left[ I_z^2 - I(I+1)/3 + \frac{n}{3} (I_x^2 - I_y^2) \right] - g_n \beta_n \vec{H} \cdot \vec{I}$$

Eq. 5.1
Fig. 5.2: Mössbauer spectra of freeze-quenched samples from the reaction of (A) apo R2-Y122F or (B) apo R2-wt with Fe$^{2+}$ and O$_2$ (in the presence of ascorbate). The reaction conditions are described in Materials and Methods. The spectra were acquired at 4.2 K with a magnetic field of 50 mT applied parallel to the γ-beam. The solid line plotted over the data in A is the theoretical spectrum of X, which was generated by using Eq. 5.1 and the parameters of Table 5.1. The solid and dashed lines above the data in A are the theoretical spectra for the individual Fe sites of X.
sub-spectral components of equal intensity, with each component originating from an iron site. It can be simulated by using the Mössbauer parameters given in Table 5.1. The solid and dashed lines plotted above the data in Fig. 5.2A are the theoretical sub-spectra for sites 1 and 2 which were generated by using these parameters. The solid line plotted over the data is the superposition of the sub-spectra. Excellent agreement between theory and experiment is achieved with these parameters. By using the theoretical spectrum of the magnetic component as a reference, it can be estimated that 70% of the integrated intensity of the spectrum of sample 1 (Fig. 5.2A) and 63% of the spectrum of sample 2 is contributed by this species. This corresponds to 1 and 0.9 equiv of the species in samples 1 and 2, respectively.

**Table 5.1: Parameters used to simulate the magnetic Mössbauer component of Fig. 5.2A.**

<table>
<thead>
<tr>
<th>parameter</th>
<th>Site 1</th>
<th>Site 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta E_Q$</td>
<td>-1.0 mm/s</td>
<td>-1.1 mm/s</td>
</tr>
<tr>
<td>$\delta$</td>
<td>0.55 mm/s</td>
<td>0.45 mm/s</td>
</tr>
<tr>
<td>$\eta$</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>$A/g_n\beta_n$</td>
<td>-52 T$^a$</td>
<td>25 T$^a$</td>
</tr>
</tbody>
</table>

*The signs of the $A$-values are deduced from Fig. 5.5.*

It should be noted that the values of $\Delta E_Q$ quoted in Table 5.1 were obtained through comparison of the experimental spectrum to a set of simulations in which this parameter was varied. (This analysis suggests an upper limit of 1.5 mm/s for both values of $\Delta E_Q$). The usual practice for obtaining $\Delta E_Q$ for a paramagnetic species is to increase the temperature at which the spectrum is acquired until the electronic relaxation of the
species becomes rapid with respect to the nuclear precession frequency. The magnetic spectrum then collapses into a quadrupole doublet. The presence of frozen isopentane (which has a melting temperature of 112.7 K) in these samples imposes an upper limit of 112 K on the temperature at which spectra can be acquired. (Allowing the frozen isopentane to melt would likely cause the samples to explode.) At temperatures just below 112 K, the Mössbauer spectrum of sample 1 is broad and featureless (Fig. 5.3). Therefore, $\Delta E_Q$ for the iron sites of X cannot be directly measured from these samples.

The absolute values of $A/g_n\beta_n$ from the simulation of Fig. 5.2A (Table 5.1) represent the magnitudes of the internal magnetic field created at each $^{57}$Fe nucleus by the electron spin of the cluster. Likewise, the electron experiences a magnetic field created by the $^{57}$Fe nuclei, and this field accounts for the observed hyperfine coupling in the EPR spectrum of X generated with $^{57}$Fe$^{2+}$. The EPR spectrum of X coupled to $^{57}$Fe can be simulated by using the hyperfine $A$-values obtained from the Mössbauer spectrum (Fig. 5.4). Excellent agreement between the experimental spectrum (C) and the theoretical spectrum (D) is achieved. Thus, the hyperfine coupling constants determined from the Mössbauer spectrum accurately account for the hyperfine coupling observed in the EPR spectrum. This agreement unambiguously establishes the association of the $g = 2.0$ EPR signal of X with the magnetic Mössbauer component of Figs. 5.2A and B.

The Mössbauer spectrum of sample 1 as a function of the magnitude of the applied magnetic field (Fig. 5.5) indicates that the two iron sites of X are tightly coupled. In an applied field (parallel to the $\gamma$-beam) of 1.0 T (the lower spectrum of Fig. 5.5), the magnetic component due to X is nearly indistinguishable (by eye) from the spectrum of Fig. 5.2A (50 mT applied field). When the magnitude of the field is increased to 3.0 T (middle spectrum of Fig. 5.5), the energy difference between the highest and lowest energy lines of site 1 decreases perceptibly, while the separation between the outer lines of site 2 increases. In an applied field of 8.0 T (upper spectrum of Fig. 5.5), the relative positions of the outer lines of sites 1 and 2 are actually reversed, so that the observed
Fig. 5.3: Mössbauer spectrum of sample 1 (from Fig. 5.2A) at 100 K. The spectrum was acquired with a magnetic field of 50 mT applied parallel to the γ-beam.
Fig. 5.4: Simulation of the EPR spectrum of X coupled to $^{57}$Fe by using the $A$-values determined from the Mössbauer spectrum. A is the experimental spectrum of X generated by reaction of apo R2-Y122F with excess $^{56}$Fe$^{2+}$. B is a simulation of this spectrum. C is the experimental spectrum of X generated by reaction of apo R2-Y122F with excess $^{57}$Fe$^{2+}$. D is the simulation of this spectrum. Both the experimental spectra and the simulated spectra are from Chapter 4, and details of both are presented therein (see Fig. 4.8).
Fig. 5.5: Dependence of the Mössbauer spectrum of sample 1 on the magnitude of the applied magnetic field (H). The spectra were acquired at 4.2 K with the indicated magnetic field applied parallel to the \( \gamma \)-beam. The solid line plotted over the data in each spectrum is the theoretical spectrum of X at that applied field. The solid and dashed lines plotted over the upper spectrum are the theoretical spectra of sites 1 and 2 (respectively) at 8 T. Notice that the outer lines of sites 1 and 2 have crossed, so that those of site 1 are now inside those of site 2.
hyperfine coupling of site 2 is larger than that of site 1. The data of Fig. 5.5 indicate that
the internal field at site 1 of X opposes the laboratory field (negative A-value), whereas
the field at site 2 is aligned with the applied field (positive A-value). This observation is
proof that the Fe ions are coupled.

Discussion

The successful simulation both of the magnetic component of Figs. 5.2A and B
and of the EPR spectrum of X coupled to $^{57}$Fe by using the same A-values proves that the
Mössbauer spectrum is associated with X. The spectra of Fig. 5.2 thus confirm the
observation made by EPR that approximately one equiv of X accumulates under these
reaction conditions. The isomer shifts and isotropic A-tensors used in simulation of the
Mössbauer spectrum of X indicate that the two iron ions in the cluster are high-spin ferric
ions. Two high-spin ferric ions ($S = 5/2$) can couple to give only an integer electron spin
quantum number, whereas the EPR spectrum of X indicates that its system spin is 1/2.
Therefore, X must contain an additional component, with half-integer spin, which is
coupled to the two high-spin ferric ions. Since this additional component cannot be Fe$^{3+}$
(a third $^{57}$Fe nucleus is not observed in the Mössbauer spectrum), the only possible
identity of the component is a free radical. We have therefore designated the
intermediate cluster, X, a "diferric-radical species." To our knowledge, this species has
no precedent in the chemical or biochemical literature.

The possible identities of the radical component are limited to 1) an oxygen
radical derived from H$_2$O or from O$_2$ or 2) a radical derived from an amino acid of R2.
The tight coupling of the components of X, even in an applied field of 8 T, suggests that
the free radical component is directly ligated to one or both of the ferric ions. This
consideration further limits the amino acids of R2 from which the radical might be
derived to the aspartate, glutamate and histidine ligands of the iron cluster (Nordlund et
al., 1990) (assuming that no large scale conformational change occurs in R2 during conversion of X to the diferric cluster).

The identity of X is perhaps surprising in light of the numerous proposals in the R2 literature that a ferryl species generates •Y122 (Fontecave, et al., 1990; Sahlin, et al., 1990; Stubbe, 1990; Que, 1991). Its Mössbauer spectrum clearly indicates that X does not contain Fe(IV), as ferryl ions typically exhibit isomer shifts of 0-0.1 mm/s (Schulz et al., 1984; Collins et al., 1990; Leising et al., 1991). Thus, it appears that if the +4 oxidation state of Fe is accessed during the reconstitution reaction, a ligand to the Fe(IV) is of appropriate redox potential to rapidly reduce the high-valent species. Alternatively, an Fe(IV) intermediate may not form at all: an amino acid ligand may be preferentially oxidized, or the radical component of X may be a 3-electron reduced product of O₂ (a peroxyl radical). Thus, our characterization of X adds fuel to the debate as to whether Fe(IV) species can be generated with biologically relevant ligands other than porphyrins. The work of Que and coworkers (Leising, et al., 1991) and of Uffelman and coworkers (Collins, et al., 1990) has elegantly shown that Fe(IV) species can, in fact, be generated in non-heme environments, but the extent to which the ligands of these model complexes are mimetic of biological iron ligands is questionable. Irrespective of this point, our results suggest that species other than ferryl intermediates should be considered as candidates for the reactive intermediates in O₂ activation at dinuclear iron clusters and (perhaps) at other non-heme iron centers.

In addition to the insight which they provide into the structure of X, the spectra of Fig. 5.2 hint at the vast quantity of kinetic information about the reconstitution reaction which is potentially accessible by the rapid freeze-quench Mössbauer technique. The resolved features of X make quantitation of the intermediate cluster from the Mössbauer spectra straight forward. With the known quantity of Fe in the reaction serving as an internal standard, the proportionality between absorption area and quantity of Fe allows the quantity of X to be calculated in terms of molar equivalents per R2 subunit. Varying
the time at which the reaction is quenched should allow for time-resolved quantitation of the species. In addition, Fig. 5.2B reveals that the presence of even a small quantity of the diferric cluster (~ 0.1 equiv) can readily be detected from the Mössbauer spectrum of a freeze-quenched sample. Thus, it appears that the diferric cluster might also be quantified as a function of reaction time by using this method. The potential mechanistic insight to be gained from time-resolved quantitation of the major iron-containing intermediates and products is self evident. In the two chapters which follow, the vast potential of the method is realized in the kinetic characterization of the R2 reconstitution reaction by rapid freeze-quench Mössbauer spectroscopy. Within the context of the results of the preceding chapters, the information gathered in these time-resolved Mössbauer experiments allows a coherent mechanism to be proposed for the reaction.

References


*Biochemistry* 23, 4743-4754.

Chapter 6: Time-Course of the Reaction of Apo R2 with Excess $\text{Fe}^{2+}$ and $\text{O}_2$ Monitored by Rapid Freeze-Quench Mössbauer Spectroscopy
The successful adaptation of the rapid freeze-quench methodology for Mössbauer spectroscopic characterization of the diferric-radical intermediate ($X$, see Chapter 5) provides an excellent method by which to monitor the entire time-course of the reconstitution reaction. It is evident that the time-resolved Mössbauer spectra of the reaction might provide a wealth of mechanistic information, as the concentration at any time of each iron-containing reactant, intermediate, and product might be determined, provided that the spectrum at a given time could be deconvoluted into its components.

In the experiments which are described in this chapter, the time-course of the reaction at 5 °C of apo R2-wt with excess Fe$^{2+}$ and O$_2$ was probed by rapid freeze-quench Mössbauer spectroscopy. Analysis of the spectrum of each time-point allowed for accurate quantitation as a function of time of the diferric-radical intermediate and of the diferric cluster. This quantitation allowed rate constants for formation and decay of the diferric-radical and for formation of the diferric cluster to be determined. Analysis of the time-dependent Mössbauer spectra also allowed for identification and quantitation of additional components of the reaction.

The results of this chapter are quantitatively consistent with the stopped-flow absorption data of Chapter 3 and with the rapid freeze-quench EPR data of Chapter 4, and provide additional evidence for the mechanism proposed in these chapters for the excess Fe$^{2+}$ reaction (see Schemes 6.2 and 6.3). Specifically, these results provide convincing evidence that the diferric-radical species ($X$) is a precursor to the diferric cluster. The experiments further show that an Fe$^{3+}$-containing species, which is distinct both from $X$ and from the diferric cluster, is produced with a rate constant identical to that for formation of $X$. This species may be the Fe$^{3+}$ product which is formed when Fe$^{2+}$ donates the electron to convert $I$ to $II$, although the analysis suggests that it accumulates only to 50% of the quantity expected. Finally, the consistency of the data of this chapter with the stopped-flow absorption data of Chapter 3 demonstrates that the results of the two very different experiments (the continuous method of stopped-flow and the
discontinuous method of rapid freeze-quench) can be directly compared for the reconstitution reaction. In Chapter 7, comparison of stopped-flow absorption data with rapid-freeze quench Mössbauer and EPR results is exploited to gain significant insight into the kinetically and mechanistically more complex reaction of apo R2-wt with limiting Fe$^{2+}$.

Materials and Methods

$^{57}$Fe$^{0}$ (98% $^{57}$Fe) was purchased from Advanced Materials and Technology of the former U.S.S.R. The $^{57}$Fe$^{2+}$ stock solution was prepared by dissolution of the metal in O$_{2}$-free 1 M H$_{2}$SO$_{4}$ (as described in Chapter 3). Apo R2-wt was isolated as described in Chapter 2. 2-Methylbutane was purchased from Aldrich.

Preparation of Diferric Cluster Reference Sample

Although Mössbauer spectra and parameters have been reported for the diferric cluster (in native and met R2) (Atkin et al., 1973; Lynch et al., 1989) and for the ferrous form of R2 (Lynch, et al., 1989), it was deemed desirable to have reference spectra for these species for use in analysis of the time-dependent spectra of the reconstitution reaction. In preparation of the reference sample for the diferric cluster (native R2), 140 µL of 4.38 mM $^{57}$Fe$^{2+}$ in 4.5 mM H$_{2}$SO$_{4}$ containing 14 mM ascorbic acid was added at 2 ± 2 °C to 250 µL of 0.98 mM apo R2-wt in air-saturated 100 mM HEPES buffered at pH 7.7. The resulting solution, which contained 0.64 mM apo R2-wt, 1.6 mM $^{57}$Fe (Fe/R2 = 2.5), and 4.9 mM ascorbate, was incubated (open to air) on ice for 45 min. It was then transferred to a Mössbauer cell and frozen in liquid N$_{2}$. The Mössbauer spectrum of this sample was acquired at 4.2 K with a magnetic field of 50 mT applied parallel to the γ-beam.
Preparation of Ferrous-R2 Reference Sample

A 1 mL aliquot of 0.98 mM apo R2-wt in 100 mM HEPES buffered at pH 7.7 and a 1 mL aliquot of 5.1 mM $^{57}$Fe$^{2+}$ in 5 mM H$_2$SO$_4$ were made O$_2$-free by several cycles of gentle evacuation-refilling with argon-incubation on ice (as described in Chapter 4). A 207 µL aliquot of the apo R2 was mixed in an anaerobic chamber with 200 µL of the 3.1 mM $^{57}$Fe$^{2+}$ solution. A second sample was prepared by addition of 130 µL of the $^{57}$Fe$^{2+}$ stock to 270 µL of the apo R2 solution. The resulting Fe$^{2+}$-R2 solutions, which contained 5.0 Fe$^{2+}$/R2 and 2.5 Fe$^{2+}$/R2 respectively, were transferred to Mössbauer cells, which were placed in septum-stopped flasks. The flasks were removed from the anaerobic chamber and submerged in liquid N$_2$ until the samples in the Mössbauer cells were frozen. Mössbauer spectra of these samples were acquired at 4.2 K with a magnetic field of 50 mT applied parallel to the γ-beam.

Preparation of the Rapid Freeze-Quench Mössbauer Time-Course Samples

The rapid freeze-quench samples for Mössbauer characterization were prepared as described in Chapter 5 (Fig. 5.1 shows the apparatus which was used). In Experiment 1, an O$_2$-saturated solution of 0.59 mM apo R2-wt in 100 mM HEPES buffered at pH 7.7 was mixed at 5 ± 1 °C with an equal volume of O$_2$-saturated 2.97 mM $^{57}$Fe$^{2+}$ in 3 mM H$_2$SO$_4$. (The reactant solutions were saturated with O$_2$ as described in Chapter 3.) Samples were quenched at reaction times which varied between 0.061 and 60 s. In Experiment 2, 0.39 mM apo R2 was mixed with 3.02 mM $^{57}$Fe$^{2+}$, and samples were quenched at reaction times which varied from 0.061 s to 0.44 s. Mössbauer spectra of these samples were acquired at 4.2 K with a magnetic field of 50 mT applied parallel to the γ-beam.
Preparation of "Ferrous Ion in HEPES" Reference Sample

A reference sample for $^{57}$Fe$^{2+}$ in HEPES buffer was prepared by mixing (at 5 °C) equal volumes of O$_2$-saturated 100 mM HEPES buffered at pH 7.7 with O$_2$-saturated 1.5 mM $^{57}$Fe$^{2+}$ stock and freeze-quenching the mixture at 0.44 s. The Mössbauer spectrum of this sample was acquired at 4.2 K with a magnetic field of 50 mT applied parallel to the γ-beam.

Quantitative Analysis of the Rapid Freeze-Quench Time-Course Samples

Analysis of the Mössbauer spectra of the freeze-quenched samples to determine the quantities of diferric-radical intermediate (X) and diferric cluster present in each was carried out by using a subtraction program written by Prof. Huynh. The program computes the total area under the absorption spectrum (which is proportional to the quantity of $^{57}$Fe present in the sample) and allows a theoretical or experimental reference spectrum to be superimposed upon and then subtracted from the original spectrum. The superimposed spectrum can be scaled to represent any percentage of the integrated intensity of the original spectrum. By iteratively superimposing varying percentages of the reference spectrum for a given component of the reaction (ferrous R2, X or the diferric cluster) on the spectrum of a time-course sample, it was possible to determine the fraction of the $^{57}$Fe in the sample which was present as that component and to assign error limits to this value. The known, total quantity of $^{57}$Fe present in the sample served as an internal standard, which allowed the absolute quantity of each component to be calculated from the fraction of $^{57}$Fe in that component.

For each of the three components, ferrous-R2, X, and the diferric cluster, both experimental and theoretical spectra were used as references for analysis of the time-course samples. Experimental spectra for ferrous-R2 and for the diferric cluster were acquired on the samples of which preparation is described above. Theoretical spectra for these components were generated either by non-linear least-squares fitting of two
quadrupole doublets to the data or by simulation (using parameters from the least-squares fit or from Lynch, et al. (1989)). For X, the theoretical spectrum from Chapter 5 was used as a reference for quantitation of the intermediate. Because this spectrum of X shows imperfect agreement with the experimental spectrum in the central region, when an objective of the analysis was to subtract away the spectrum of X in order to identify other components of the reaction, it was necessary to use an experimental spectrum as the reference so as not to distort the central region of the subtraction spectrum. The spectrum of Fig. 5.2 was used as the experimental reference for X. (As reported in Chapter 5, ~70% of the integrated intensity of this spectrum is contributed by X, with the remainder being contributed predominantly by ferrous-R2 or by ferrous ion in solution. Therefore, before this spectrum was used as a reference, the contribution from Fe$^{2+}$ was first subtracted away.) An example of the quantitative analysis applied to the time-course spectra is presented in detail in the Results section. Non-linear regression analysis on the kinetic data was carried out with the Git and Gear programs of Drs. R. J. McKinney and F. J. Wiegert, Central Research and Development Department, E. I. du Pont de Nemours and Co.

Control to Show Equivalence of Mössbauer Samples and EPR Samples

As described in Chapter 5, the apparatus and procedure developed for preparation of rapid freeze-quench Mössbauer samples require somewhat more manipulation after quenching than those used in preparation of EPR samples. In order to verify that quantitative comparison of data for the two types of samples would be meaningful, it was deemed essential to ensure that the slightly different methods of preparation generate identical samples. Therefore, a control was performed in which a Mössbauer sample was prepared in the usual manner and was subsequently transferred and repacked into an EPR tube. (Both transfer and repacking were carried out under cold isopentane.) A second sample was prepared under identical reaction conditions but was quenched and packed in

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the usual manner for preparation of EPR samples. The EPR spectra at 20 K of the two samples were acquired and compared.

Results

Reference Spectra of Ferrous-R2, X, and the Diferric Cluster

The Mössbauer spectra at 4.2 K of the ferrous-R2 samples containing 2.5 Fe$^{2+}$/R2 and 5.0 Fe$^{2+}$/R2 are shown in Figs. 6.1A and B along with the results of least-squares fits of two quadrupole doublets to the data (solid line). The parameters from the fit (Table 6.1) are in good agreement with those reported by Lynch, et. al. (1989). The spectrum of

Table 6.1: Parameters for reference spectra of ferrous-R2, ferrous ion in HEPES buffer, the diferric cluster, and the diferric-radical species.

| Species                  | Site | $\Delta E_Q$ (mm/s) | $\delta$ (mm/s) | $\eta$ | $|A/g_\beta\beta|$ (T) |
|--------------------------|------|---------------------|-----------------|--------|---------------------|
| ferrous-R2               | 1    | 3.24 ± 0.06$^a$    | 1.31 ± 0.03$^a$ | --     | --                  |
|                          | 2    | 2.92 ± 0.06$^a$    | 1.20 ± 0.03$^a$ | (3.13)$^b$ | (1.26)$^b$ | -- | --                  |
| ferrous ion in HEPES buffer | 1    | 3.39 ± 0.06$^a$    | 1.40 ± 0.03$^a$ | --     | --                  |
|                          | 2    | 3.03 ± 0.06$^a$    | 1.36 ± 0.03$^a$ | --     | --                  |
| diferric                 | 1    | 1.64 ± 0.06$^a$    | 0.54 ± 0.03$^a$ | (1.62 ± 0.02)$^c$ | (0.55 ± 0.01)$^c$ | -- | --                  |
|                          | 2    | 2.41 ± 0.06$^a$    | 0.45 ± 0.03$^a$ | (2.44 ± 0.03)$^c$ | (0.45 ± 0.01)$^c$ | -- | --                  |
| diferric-radical (X)$^d$ | 1    | -1.0                | 0.55            | 0.5    | 52                  |
|                          | 2    | -1.1                | 0.45            | 0.7    | 24.5                |

$^a$obtained by a least-squares fit of two quadrupole doublets to the data

$^b$average parameters for the two sites quoted by Lynch, et al. (1989)

$^c$parameters from Lynch, et al. (1989)

$^d$simulation from Chapter 5

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Fig. 6.1: Reference spectra for ferrous-R2 (A and B), ferrous ion in HEPES buffer (C), the diferric cluster (D), and the diferric-radical intermediate (E). A and B were acquired on samples of ferrous-R2 containing 2.5 and 5.0 Fe$^{2+}$/R, respectively. The preparation of these samples and of those used to acquire C and D, is described in Materials and Methods. Preparation of the sample for E is described in Chapter 5. The solid lines in A-D were obtained by least squares fitting of two quadrupole doublets to the data. The parameters obtained in these fits are listed in Table 6.1. The solid line in E is the simulation from Chapter 5. The parameters for this spectrum are also shown in Table 6.1.
the reference sample containing Fe$^{2+}$ in HEPES buffer is shown in Fig. 6.1C, along with the result of a least-squares fit of two quadrupole doublets to the data (solid line).

The spectrum at 4.2 K of the sample of reconstituted R2 is shown in Fig. 6.1D. Plotted over the experimental data is the result of a least-squares fit of two quadrupole doublets to the data (solid line). The parameters from this fit (Table 6.1) also agree with those reported by Lynch, et. al. (1989).

Fig. 6.1E shows the spectrum at 4.2 K of the sample from Chapter 5 which contains 70% X. Plotted over the experimental data is the theoretical spectrum obtained by using the parameters reported in Chapter 5 (see Table 6.1). As mentioned above, the inner region of the theoretical spectrum shows imperfect agreement with the experimental data. The theoretical spectrum is adequate to quantify X, but the experimental spectrum is preferable for subtraction.

**Time-Course of the Excess Fe$^{2+}$ Reaction Monitored by Mössbauer Spectroscopy**

The time-dependent Mössbauer spectrum of the reaction of apo R2-wt with excess Fe$^{2+}$ and O$_2$ (Fig. 6.2) clearly reflects the progress of the reaction. In the first time-point which was taken (0.061 s, Fig. 6.2A), the spectrum is dominated by unreacted ferrous ion (solid line plotted over the experimental data). Although Fe$^{2+}$ is the predominant species at this first time-point, a significant quantity of X has accumulated (solid line plotted just above the experimental spectrum), while the features of the diferric cluster are not yet detectable. At somewhat longer reaction times (0.31 s, Fig. 6.2B), the relative quantity of Fe$^{2+}$ present has decreased significantly, while the contribution due to X has increased (solid line above data). In addition, the spectrum of the diferric cluster is now detectable (solid line over data). With increasing reaction time (1.0 s, Fig. 6.2C), the relative contributions from Fe$^{2+}$ and from X (solid line above data) decrease, while that from the diferric cluster (solid line over data) increases. Finally, at completion of the reaction
Fig. 6.2: Time-course of the reaction of apo R2-wt with excess Fe\textsuperscript{2+} and O\textsubscript{2} as monitored by Mössbauer spectroscopy. The spectra are of samples from Experiment 1 (details are given in Materials and Methods). The reaction was quenched (A) at 0.061 s, (B) at 0.31 s, (C) at 1.0 s, or (D) at 60 s. The solid line plotted over the data in A is a simulation of the ferrous-R2 spectrum which was obtained by using the parameters reported by Lynch, et al. (1989). It is scaled to 45% of the integrated intensity of the experimental spectrum. The solid line plotted just above the data in A is the theoretical spectrum of X scaled to 20% of the intensity of the experimental spectrum. In B and C, the solid line above the data is the theoretical spectrum of X (36% in B and 23% in C), and the line plotted over the data is the theoretical spectrum of the diferric cluster (12% in B and 32% in C). The solid line in D is the theoretical spectrum of the diferric cluster (55%). The diferric cluster reference spectrum is a simulation based on the values of ΔE\textsubscript{Q} and δ from the least squares fit of Fig. 6.1D and on the values of η reported by Lynch et al. (1989). (The signs of the values of ΔE\textsubscript{Q} (-) were taken from Lynch et. al. (1989)).
(60 s, Fig. 6.2D), the spectrum is dominated by the features of the diferric cluster (solid line). Fig. 6.2 clearly demonstrates the utility of the rapid freeze-quench Mössbauer method for monitoring the reconstitution reaction, as the progression from reactant (Fe\(^{2+}\)), to intermediate (X), to product (diferric cluster) can be seen upon inspection.

**Analysis of the Time-Course Spectra to Quantify X and the Diferric Cluster**

In addition to illustrating the progress of the reaction, Fig. 6.2 also shows that both X and the diferric cluster can be quantified as functions of time from the Mössbauer spectra. For X, the highest and lowest energy lines of site 1 (see Table 6.1) and the lowest energy line of site 2 are well resolved from other spectral features (see Figs. 6.2A-C). Likewise, for the diferric cluster, both lines of the outer quadrupole doublet (site 2 of Table 6.1) are sufficiently resolved from other spectral features to allow for reliable quantitation (see Figs. 6.2B-D). To estimate the quantity of X or of the diferric cluster present in a given time-course sample, the reference spectrum of the species was superimposed on the experimental spectrum of that sample. The percentage of the reference spectrum (relative to the integrated intensity of the experimental spectrum) which was plotted was varied until agreement was achieved. The proper percentage of this species was subtracted away, and the correct quantity of the next species was determined by analysis of the subtraction spectrum. In many cases, the analysis was repeated in opposite order to provide a check. Limits of error for the measured quantities of the species were also estimated. The quantitation and error estimates are summarized in Table 6.2. The quantity of X as a function of time exhibits the rise-fall behavior expected of an intermediate, while the quantity of the diferric cluster rises smoothly. The ratio of diferric cluster/R2 at completion (1.38 ± 0.08) is in reasonable agreement with the Fe\(^{3+}\) content of native R2 as determined in our laboratory (2.8-3.2 Fe\(^{3+}\)/R2 assuming \(\varepsilon_{280} = 131 \text{ mM}^{-1}\text{cm}^{-1}\), see Chapter 1). The ratio also is only slightly different from the •Y122/R2 ratio (1.2 ± 0.1, see Chapter 3) observed upon completion of the reaction.
Scheme 6.1: Kinetic model fit to quantities of X and diferric cluster as functions of time.

\[ \text{apo R2} + \text{Fe}^{2+} + \text{O}_2 \xrightarrow{k_1} \text{X} \xrightarrow{k_2} \text{Fe}^{3+} \cdot \text{O}_2^{-} \cdot \text{Fe}^{3+} \]

Table 6.2: Summary of quantitation of X and the diferric cluster as functions of reaction time. The experimental conditions are described in Materials and Methods.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Reaction Time (s)</th>
<th>% X</th>
<th>Equiv X</th>
<th>% Diferric Cluster</th>
<th>Equiv Diferric Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.061</td>
<td>21 ± 4</td>
<td>0.53 ± 0.11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.16</td>
<td>33.5 ± 3.5</td>
<td>0.84 ± 0.10</td>
<td>3 ± 1</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>0.22</td>
<td>35 ± 2</td>
<td>0.88 ± 0.07</td>
<td>6 ± 1</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>0.31</td>
<td>39 ± 3</td>
<td>0.98 ± 0.09</td>
<td>9.5 ± 1.5</td>
<td>0.24 ± 0.04</td>
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<td></td>
<td>0.38</td>
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<td>0.83 ± 0.07</td>
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</tr>
<tr>
<td></td>
<td>0.63</td>
<td>27 ± 3</td>
<td>0.68 ± 0.09</td>
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<td>1.02 ± 0.12</td>
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<td>0.26 ± 0.08</td>
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<td>38 ± 3</td>
<td>0.97 ± 0.12</td>
<td>15 ± 3</td>
<td>0.39 ± 0.08</td>
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</table>
The general equations for two consecutive, first-order reactions (Atkins, 1986) were fit to the measured quantities of X and •Y122 as functions of time. The data are quite consistent with this simple kinetic model, and the analysis gives a rate constant ($k_1$) of $7.3 \pm 1$ s$^{-1}$ for formation of X and a rate constant ($k_2$) of $1.03 \pm 0.1$ s$^{-1}$ for decay of X and concomitant formation of the diferric cluster. In Fig. 6.3, the theoretical curves which are based on these rate constants and on a final diferric cluster/R2 ratio of 1.34 are plotted along with the experimental data. As would be predicted from Scheme 6.1, there is a noticeable lag phase in formation of the diferric cluster. This observation contrasts with the apparent lack of a lag phase in •Y122 formation observed in the rapid freeze-quench EPR experiments of Chapter 4. This discrepancy is discussed below.

**Analysis of the Spectra for Additional Components**

In addition to the obvious features of ferrous-R2, the diferric-radical intermediate, and the diferric cluster, several other features can be discerned in the Mössbauer spectra of the time-course samples. Perhaps the most potentially informative of these is a resolved peak centered at approximately 1 mm/s, just slightly lower in energy than the high energy line of site 1 of the diferric cluster spectrum (Fig. 6.4A). This peak develops rapidly, and is therefore prominent in the early time-points of the reaction (0.061-0.63 s), before it becomes obscured by the features of the diferric cluster. The peak is even more prominent early in the reaction of apo R2-Y122F with excess Fe$^{2+}$ (Fig. 6.4B), because formation of the diferric cluster is slower in the mutant protein, and the peak develops before the features of the diferric cluster begin to appear. The peak does not develop when the reaction is carried out with limiting Fe$^{2+}$ (Fig. 6.4C) nor when the reaction is carried out in the presence of ascorbate (see Fig. 6.1E).

Because the species associated with this peak is apparently not a major component of the reaction (quantitation of the species is discussed below), and because only one of an unknown number of spectral lines associated with the species is resolved,
Fig. 6.3: Non-linear least-squares fitting of the kinetic model of Scheme 6.1 to the measured quantities of X and diferric cluster as functions of time. The fit lines correspond to $k_1 = 7.3 \text{ s}^{-1}$, $k_2 = 1.03 \text{ s}^{-1}$, and a final diferric cluster/R2 ratio of 1.34.
Fig. 6.4: Mössbauer spectra showing that a resolved peak at approximately 1 mm/s develops rapidly only in the excess Fe$^{2+}$ reaction. Spectrum A is of the 0.31 s sample of Experiment 1. B is of a sample which was prepared by mixing (at 5 °C) 1.5 mM apo R2-Y122F in O$_2$-saturated 200 mM HEPES buffered at pH 7.7 with an equal volume of 8.8 mM $^{57}$Fe$^{2+}$ in O$_2$-saturated 10 mM H$_2$SO$_4$, and quenching the reaction at 0.31 s. C is of a sample which was prepared by mixing (at 5 °C) 0.59 mM apo R2-wt in O$_2$-saturated 100 mM HEPES buffered at pH 7.7 with an equal volume of 1.34 mM $^{57}$Fe$^{2+}$ in O$_2$-saturated 2.5 mM H$_2$SO$_4$, and quenching the reaction at 0.28 s. The spectra were acquired at 4.2 K with a magnetic field of 50 mT applied parallel to the γ-beam.
definitive characterization of its oxidation and spin state has not yet been achieved. Nevertheless, analysis of the spectra of all the time-points is consistent with the interpretation that the resolved peak is the high energy line of a quadrupole doublet. The low energy line is obscured in the spectra of early time-points by the low energy line of the ferrous ion spectrum (Fig. 6.5A). In spectra of later time-points, both lines of the doublet can be discerned as apparent shoulders inside the two inner lines of the diferric cluster spectrum (Fig. 6.5B). The simulated spectrum (solid line) in Figs. 6.5A and B corresponds to a quadrupole doublet with \( \delta = 0.56 \) and \( \Delta E_Q = 0.95 \). If this spectrum is representative of the unknown species, these parameters would identify it as containing high-spin ferric ion. The lack of magnetic hyperfine splitting in the spectrum of the species would indicate either that the electronic relaxation of the ferric ion is rapid compared to the nuclear precession frequency or that the ferric ion is in a diamagnetic cluster. High magnetic field spectra indicate that the former explanation is correct, and we have therefore tentatively assigned the resolved peak to a fast-relaxing ferric species.

If the solid line of Figs. 6.5A and B is taken as a reference spectrum for this putative fast-relaxing ferric species, the species can be quantified as a function of time in the same manner as were \( \text{X} \) and the diferric cluster. Table 6.3 summarizes this quantitation. The quantities of \( \text{X} \) and diferric cluster in each sample (from Table 6.2) are included for comparison. It can be seen by inspection of Table 6.3 that the putative fast-relaxing ferric species accumulates rapidly and then remains (nearly) constant throughout the reaction. Again assuming that the theoretical spectrum of Fig. 6.5 is accurate, the species accumulates to 0.6-0.7 equiv Fe\(^{3+} \) per R2 subunit, or to approximately one-half the quantity of diferric cluster present upon completion of the reaction. Fitting the equation for a first order growth to the data for this species gives a formation rate constant of \( 7.3 \pm 1.5 \text{ s}^{-1} \). Fig. 6.6 shows the fit line and experimental data plotted along with the fits for \( \text{X} \) and the diferric cluster (from Fig. 6.3). The putative fast-relaxing ferric species accumulates with a rate constant identical to that for formation of \( \text{X} \). The
Fig. 6.5: Quantitation of the putative fast-relaxing ferric species. Spectrum A is of the 0.31 s time-point of Experiment 1. The solid line plotted over the data is the hypothetical reference spectrum for the fast-relaxing Fe$^{3+}$ species scaled to 10% of the integrated intensity of the experimental spectrum. B is of the 1.5 s time-point of Experiment 1, and the solid line is the reference spectrum of the fast-relaxing Fe$^{3+}$ species scaled to 11% of the intensity of the experimental spectrum. The parameters used to generate the reference spectrum are given in the text.
Table 6.3: Summary of quantitation of putative fast-relaxing ferric species.

<table>
<thead>
<tr>
<th>Reaction Time (s)</th>
<th>% &quot;Fast-Relaxing Fe$^{3+}$&quot;</th>
<th>Equiv &quot;Fast-Relaxing Fe$^{3+}$&quot;</th>
<th>Equiv X</th>
<th>Equiv Diferric Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.061</td>
<td>5 ± 1</td>
<td>0.25 ± 0.06</td>
<td>0.53 ± 0.11</td>
<td>0</td>
</tr>
<tr>
<td>0.16</td>
<td>9 ± 1</td>
<td>0.45 ± 0.06</td>
<td>0.84 ± 0.10</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>0.22</td>
<td>10 ± 1</td>
<td>0.50 ± 0.06</td>
<td>0.88 ± 0.07</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>0.31</td>
<td>12 ± 2</td>
<td>0.60 ± 0.11</td>
<td>0.98 ± 0.09</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td>0.38</td>
<td>12 ± 2</td>
<td>0.60 ± 0.11</td>
<td>0.75 ± 0.09</td>
<td>0.43 ± 0.05</td>
</tr>
<tr>
<td>0.44</td>
<td>12 ± 1</td>
<td>0.60 ± 0.06</td>
<td>0.83 ± 0.07</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td>0.63</td>
<td>13 ± 1</td>
<td>0.65 ± 0.06</td>
<td>0.68 ± 0.09</td>
<td>0.50 ± 0.06</td>
</tr>
<tr>
<td>1.0</td>
<td>14 ± 1</td>
<td>0.70 ± 0.06</td>
<td>0.55 ± 0.09</td>
<td>0.80 ± 0.05</td>
</tr>
<tr>
<td>1.5</td>
<td>13 ± 1</td>
<td>0.65 ± 0.06</td>
<td>0.38 ± 0.06</td>
<td>0.95 ± 0.10</td>
</tr>
<tr>
<td>2.2</td>
<td>13 ± 1</td>
<td>0.65 ± 0.06</td>
<td>0.16 ± 0.04</td>
<td>1.25 ± 0.08</td>
</tr>
<tr>
<td>3.0</td>
<td>12 ± 1</td>
<td>0.60 ± 0.06</td>
<td>0.08 ± 0.03</td>
<td>1.23 ± 0.07</td>
</tr>
<tr>
<td>5.0</td>
<td>12 ± 1</td>
<td>0.60 ± 0.06</td>
<td>--</td>
<td>1.33 ± 0.08</td>
</tr>
<tr>
<td>60</td>
<td>10 ± 2</td>
<td>0.50 ± 0.11</td>
<td>--</td>
<td>1.38 ± 0.08</td>
</tr>
</tbody>
</table>

possible mechanistic significance of these observations is discussed below.

Several additional spectral features, which are not associated with any of the previously defined components, can be discerned in the time-resolved spectra of the reaction. Because these features contribute a small fraction of the integrated intensity of any given spectrum, it is not possible to unambiguously define the spectra of the additional components which give rise to the features. Nevertheless, iterative subtraction of the spectra of known components from the experimental spectra of several time-points has allowed Prof. Huynh to deduce hypothetical spectra for two additional components. By using these hypothetical spectra as references along with those of ferrous-R2, ferrous
Fig. 6.6: Non-linear least-squares fitting of the equation for a first order growth to the quantity of the fast-relaxing Fe$^{3+}$ species as a function of time. The fit line corresponds to $k = 7.3$ s$^{-1}$ and to a final fast-relaxing ferric species/R2 ratio of 0.65. The fit lines for X and the diferric cluster (from Fig. 6.3) are shown for comparison.
ion in solution, the diferric-radical intermediate, the diferric cluster, and the putative fast-relaxing ferric species, the entire spectrum of each time-point can be accounted for. An example of this analysis is depicted in Fig. 6.7, which shows the decomposition of the spectrum of the 2.2 s time-point. It should be emphasized that the reference spectra of Figs. 6.7A and D may not accurately represent the spectra of real components. Nevertheless, by using these spectra along with the other reference spectra, the time-dependent Mössbauer spectrum at all times during the reaction can satisfactorily be accounted for in terms of its components. Table 6.4 summarizes this analysis.

Control to Show Equivalence of Mössbauer Samples and EPR Samples

To ensure that the slight differences in the procedures for preparation of EPR and Mössbauer samples do not cause differences in the samples, a control was performed in which a Mössbauer sample was prepared and then was transferred under cold isopentane into an EPR tube. In terms of the relative contributions from X and •Y122, the EPR spectrum of this sample (Fig. 6.8A) is identical (within experimental variation) to the spectrum of a sample packed into an EPR tube in the usual manner (Fig. 6.8B). The result of this control establishes that the EPR data of Chapter 4 and the Mössbauer data of this chapter can be directly compared.

Discussion

The mechanism proposed in Chapter 3 (Scheme 3.4) and elaborated in Chapter 4 (Scheme 4.3) for the reaction of apo R2-wt with Fe²⁺ and O₂ reduces to Scheme 6.2 for the excess Fe²⁺ reaction. Because conversion of I to II by Fe²⁺ is proposed to be much faster than formation of I, Scheme 6.2 reduces further to Scheme 6.3. (It should be emphasized that the broken circles which represent R2 in these schemes refer only to one monomer of the dimeric subunit, and that no implication is intended regarding either the environment of the Fe²⁺ which converts I to II or the fate of the resulting Fe³⁺.) The
Fig. 6.7: An example of deconvolution of the Mössbauer spectrum of a time-course sample into its known and hypothetical components. A) The spectrum of the 2.2 s time-point. The solid line plotted over the data is the hypothetical reference spectrum of an "unknown paramagnetic species," scaled to 15% of the integrated intensity of the experimental spectrum. This reference spectrum was generated from the spectrum of the 60 s time-point by subtraction of the spectra of known components (as indicated in Table 6.4). Speculation concerning the possible identity of the "unknown paramagnetic species" is presented in the text. B) The result of subtraction of the reference spectrum in A from the data. The solid line in B is the reference spectrum of X scaled to 6% of the intensity of the original spectrum. C) The subtraction result from B. The solid line is the reference spectrum of Fe\(^{2+}\) in HEPES buffer, scaled to 7% of the intensity of the original spectrum. D) The subtraction result from C. The solid line is the hypothetical reference spectrum of an "unknown ferrous species" scaled to 10% of the intensity of the original spectrum. E) The subtraction result from D. The solid line is the hypothetical reference spectrum of the "fast-relaxing ferric species", scaled to 13% of the intensity of the original spectrum. F) The subtraction result from E. The solid line is the experimental reference spectrum of the diferric cluster, scaled to 50% of the intensity of the original spectrum.
Table 6.4: Summary of decomposition of time-dependent Mössbauer spectra into components.

<table>
<thead>
<tr>
<th>Reaction Time (s)</th>
<th>% X</th>
<th>% diferric cluster</th>
<th>% &quot;Fast-Relaxing Fe\textsuperscript{3+}&quot;</th>
<th>% diferrous cluster</th>
<th>% Fe\textsuperscript{2+} in Hepes</th>
<th>% Unidentified Fe\textsuperscript{2+} Species</th>
<th>% Unidentified Paramagnetic Species</th>
<th>Total % Accounted For</th>
</tr>
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<td>21 ± 4</td>
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<td>5 ± 1</td>
<td>45</td>
<td>25</td>
<td>3</td>
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<tr>
<td>0.16</td>
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<td>9 ± 1</td>
<td>18</td>
<td>20</td>
<td>15</td>
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<td>8</td>
<td>22</td>
<td>100</td>
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Fig. 6.8: EPR spectra from a control experiment to show equivalence of the rapid freeze-quench Mössbauer and EPR samples. The sample of A was quenched and packed as a Mössbauer sample and then transferred under cold isopentane into an EPR tube. B was quenched and packed into an EPR tube in the usual manner. The spectra were acquired at 20 K with a microwave power of 2 μW, a microwave frequency of 9.43 GHz, a modulation amplitude of 4 G, a modulation frequency of 100 kHz, a scan time of 200 s, a time constant of 200 ms, and a receiver gain of 4 x 10^4.
results of Chapters 3 and 4 considered together with the results of this chapter provide convincing evidence for this mechanism. The stopped-flow results of Chapter 3 indicate that an intermediate (II) accumulates prior to formation of native R2. The EPR data of Chapter 4 indicate that the oxidized iron cluster, X, is a component of II and is a precursor to •Y122. The Mössbauer data of this chapter establish that X is a precursor to the diferric cluster. Finally, the rate constants for the two steps of Scheme 6.3 measured in the stopped-flow experiments agree reasonably well with those determined in the rapid 

Scheme 6.2: Postulated schematic mechanism for the reaction of apo R2-wt with excess Fe$^{2+}$.

$$\text{Y122} + 2\text{Fe}^{2+} + \text{O}_2 \rightarrow \text{II} \quad k_{\text{obs}} = 5-10 \text{ s}^{-1}$$

$$\text{•Y122} \quad k > 20 \text{ s}^{-1}$$

$$\text{native R2} \quad k_{\text{obs}} = 0.7-1.0 \text{ s}^{-1}$$

$$\text{Y122} + \text{(Fe}^{3+})_2\text{L•} \rightarrow \text{III} \quad \text{k} \approx 20 \text{ s}^{-1}$$

$$\text{(Fe}^{3+})_2\text{L•} = X$$

•L = ligand radical
Scheme 6.3: Minimal mechanism for reaction of apo R2-wt with excess Fe$^{2+}$.

\[ \text{Y122 apo R2} + 3\text{Fe}^{2+} + \text{O}_2 \xrightarrow{k_{\text{obs}} = 5-10 \text{ s}^{-1}} \text{Y122} \]

\[ \xrightarrow{k_{\text{obs}} = 0.7-1.0 \text{ s}^{-1}} \text{(Fe}^{3+}\text{)}_2\text{L}^\cdot \]

\[ \xrightarrow{\text{native R2}} \text{Y122 O}^2- \text{Fe}^{3+} \text{Fe}^{3+} \]

\[ (\text{Fe}^{3+})_2\text{L}^\cdot = X \]
\[ \cdot\text{L} = \text{ligand radical} \]

freeze-quench experiments, although the latter values are marginally greater. (As the method used to ensure constant temperature in the rapid freeze-quench experiments was quite crude, the slight discrepancy in measured rate constants is most likely a trivial result of a slightly higher reaction temperature in these experiments.) Thus, the data provide strong support for the major features of this mechanism.

The nature of the conversion of intermediate II into native R2 warrants discussion. It is implied in Chapters 3 and 4 that intramolecular electron transfer from Y122 to X is the sole step required for this conversion. It is certainly possible (indeed, likely), however, that nuclear reorganization also occurs in this process. Such reorganization might involve subtle changes in the coordination geometry about the Fe$^{3+}$ ions (justifying description of the reaction as electron transfer) or more extensive rearrangements which result in formation of new bonds or cleavage of existing bonds. For example, if the O-O bond of O$_2$ is intact in X, it must cleave upon conversion to native R2. Alternatively, as x-ray crystallographic (and other) evidence suggests that the diferrrous cluster of R2 does not have an oxo- bridge (B. M. Sjöberg, personal
communication), one or both of the short Fe-O bonds of the diferric cluster may form during conversion of II to native R2. As a third possibility, a "carboxylate shift" might occur in this conversion. Evidence for such a shift in the transition from met R2 to the diferrous form of the protein has been obtained by x-ray crystallography. E238, which in met R2 is ligated in monodentate fashion to Fe2 of the diferric cluster, bridges the two Fe$^{2+}$ ions in the diferrous form of the protein (B. M. Sjöberg, personal communication). The shift of this ligand from bridging to monodentate might occur in conversion of II to native R2. Finally, nuclear reorganization might also involve the tyrosyl radical. ENDOR (Bender et al., 1989) and resonance Raman (Backes et al., 1989) studies on R2 indicate that •Y122 is a neutral radical. Therefore, if electron transfer to X occurs from neutral Y122, deprotonation of the resulting cation radical must follow. In Scheme 6.4, the mechanism proposed for the reconstitution reaction is expanded to reflect the possibility that extensive, irreversible nuclear reorganization occurs subsequent to an electron transfer pre-equilibrium. It should be emphasized that none of the results of this work provide direct evidence that this additional complexity is required. Nevertheless, the scheme would allow an apparently anomalous observation to be rationalized. Whereas formation of the diferric cluster as monitored by rapid freeze-quench Mössbauer spectroscopy exhibits the initial lag phase which is required by the mechanism of Scheme 6.3, formation of •Y122 as monitored by rapid freeze-quench EPR spectroscopy (see Fig. 4.6) appears not to exhibit a lag phase. This observation is illustrated in Fig. 6.9. (The EPR and Mössbauer samples were prepared under identical reaction conditions.) While the difference between the quantity of •Y122 and the quantity of diferric cluster measured for any one time-point is less than the sum of the errors associated with these values, the fact that the quantity of •Y122 exceeds the quantity of diferric cluster for each of the first five time-points suggests that the illustrated difference may be real. This apparent difference in lag phases, in identical experiments, is inconsistent with the simple mechanism of Scheme 6.3. It could, however, be rationalized according to Scheme 6.4.
Fig. 6.9: Apparent difference in lag phases for formation of $\cdot$Y122 (monitored by rapid freeze-quench EPR) and the diferric cluster (monitored by rapid freeze-quench Mössbauer). The differences between the experimentally determined quantities of $\cdot$Y122 and diferric cluster are less than the sum of the experimental errors associated with the values. Therefore, additional experiments are required to ascertain whether the apparent difference is real.
Scheme 6.4: Mechanism for excess Fe$^{2+}$ reaction expanded to reflect the proposal that rate-limiting nuclear reorganization ($k_{\text{reorg}}$) may occur subsequent to reversible electron transfer between $X$ and $\cdot Y122$ ($k_{\text{et}}$ and $k_{-\text{et}}$).

\[
\begin{align*}
\text{apo R2} + 3\text{Fe}^{2+} + \text{O}_2 & \quad \xrightarrow{k_{\text{obs}} = 5-10 \text{ s}^{-1}} & \text{(Fe}^{3+})_2\text{L}\cdot + \cdot \text{Fe}^{3+} \\
\cdot Y122 & \quad \xrightarrow{k_{\text{reorg}}} & \cdot Y122 \\
\text{O}_2 & \quad \xrightarrow{?} & \text{native R2}
\end{align*}
\]

$\text{(Fe}^{3+})_2\text{L}\cdot = X$

$\cdot L = \text{ligand radical}$

Molecules trapped on the lower half of the electron transfer pre-equilibrium but prior to the irreversible nuclear rearrangement (the intermediate denoted ?) would contain $\cdot Y122$ but would lack the product diferric cluster. (They would also contain some unknown precursor to the diferric cluster, but the quantity of this precursor might be insufficient to
allow its detection). The presence of the intermediate •Y122 in the freeze-quenched samples could account for the observation that the quantity of •Y122 formed at short reaction times is apparently greater than the quantity of diferric cluster formed. To speculate further, the fact that a lag phase in •Y122 formation is observed when the reaction is monitored continuously at 5 °C (as in the stopped-flow experiments of Chapter 3) but is not observed when the reaction is monitored (discontinuously) by freeze-quench EPR might also be rationalized according to Scheme 6.4, if the position of the electron transfer pre-equilibrium were temperature dependent (in other words if k_{et} < k_{-et} at 5 °C, but Δk_{et}/ΔT > Δk_{-et}/ΔT). In this scenario, formation of •Y122 might still exhibit a lag phase when monitored at 5 °C. Rapid cooling of the reaction mixture (as in the rapid-freeze quench experiments) might allow the electron transfer pre-equilibrium to shift before complete quenching occurs. The shifting of the pre-equilibrium toward •Y122 upon cooling might eliminate the lag phase seen at 5 °C. This rationale is extremely speculative, and insufficient analysis has been carried out to know if it is plausible. Furthermore, additional experiments are required to assess whether the difference in lag phases illustrated in Fig. 6.9 is real. If confirmed, the observation might provide the only evidence to date that conversion of II to native R2 involves a nuclear reorganization step subsequent to electron transfer from Y122 to X.

Other aspects of the reconstitution mechanism on which the data of this chapter may shed light are the site from which Fe^{2+} delivers the electron to convert I into II and the fate of the resulting Fe^{3+}. As discussed in Chapter 2, Elgren and coworkers have suggested (and some of our data of Chapter 2 support) that this Fe^{2+} is bound at the diiron cluster binding site of the opposite monomer, and that the resulting Fe^{3+} is incorporated into a diferric cluster (Elgren et al., 1991). They have proposed a mechanism in which a transient Fe(II), Fe(III) cluster forms (as a result of delivery of the fourth electron by Fe^{2+}) and then decays by disproportionation. In contrast, Ochiai and coworkers have proposed for mouse R2 that the Fe^{2+} which delivers the fourth electron is
bound at an additional site, and that the Fe$^{3+}$ which is produced is weakly bound in mononuclear fashion (Ochiai et al., 1990). The putative fast-relaxing Fe$^{3+}$ species detected in the experiments of this chapter forms with a rate constant identical to that for formation of X (or of II), an observation which suggests that it results from conversion of I to II. Credence is given to this interpretation by the fact that the Fe$^{3+}$ species accumulates only when the reaction is carried out with excess Fe$^{2+}$. In the proposed mechanism, it is solely under these reaction conditions that Fe$^{2+}$ provides an electron to convert I to II. However, although the species forms in a kinetically competent fashion, it accumulates only to one-half the quantity (0.6-0.7 equiv compared to 1.38 equiv diferric cluster at completion) which would be expected if it arises from conversion of I to II. Thus, the data are ambiguous as to whether the putative fast-relaxing Fe$^{3+}$ has mechanistic significance. We are currently considering three possibilities. First, the Fe$^{3+}$ species may be unrelated to delivery of the fourth electron, in which case the correspondence of its rate of formation with that of X would be coincidental. Second, the theoretical spectrum used to quantify the putative fast-relaxing Fe$^{3+}$ might not accurately represent the real spectrum of the species. The real spectrum may have additional features which are unresolved. In this case, the reported quantity of the species would be an underestimate of the true amount (and the identification of the species as fast-relaxing Fe$^{3+}$ would most likely be incorrect). Finally, there may be a partition in delivery of the fourth electron. The putative fast-relaxing ferric species may be produced in only a fraction of the events in which X is produced. In this case, an additional ferric species accounting for the missing ~0.7 equiv of electrons must also be produced. The data of Elgen, et al. (1991) would provide argument in favor of the first possibility. It is interesting to note, however, that the Mössbauer spectra of reconstituted R2 which are presented by these authors also exhibit features like those of the putative fast-relaxing Fe$^{3+}$ species. Because the features make up less than 8% of the integrated intensity of the spectra, the authors disregard these features as arising from a minor Fe$^{3+}$ contaminant. In
contrast, in the work carried out by Ochiai, et al. (1990) on mouse R2, although the quantity of Fe\textsuperscript{3+} which they detect (an average of 0.7 equiv relative to tyrosyl radical produced) by EPR is also less than the predicted amount (1 equiv relative to tyrosyl radical), these authors conclude that the Fe\textsuperscript{3+} produced is mechanistically significant and arises from the Fe\textsuperscript{2+} that delivers the fourth electron. Our results fail to resolve this point, but do demonstrate that a significant quantity of a (putative) Fe\textsuperscript{3+} species accumulates in kinetically competent fashion. Thus, the data may indicate that, in at least a fraction of events, the Fe\textsuperscript{2+} which donates the fourth electron is not incorporated into a diferric cluster. This conclusion would suggest, as Ochiai, et al. (1990) have suggested for mouse R2, that an additional site may be present in R2 to bind the Fe\textsuperscript{2+} which provides this electron.

Irrespective of the site from which Fe\textsuperscript{2+} delivers the fourth electron, the data of Table 6.4 indicate that insufficient Fe\textsuperscript{3+} (in any form other than X) is produced early in the reaction for quantitative consistency with Scheme 6.3. For example, at 0.061 s, 21% of the added Fe has been incorporated into X. Scheme 6.3 requires that at least 10.5% of the added Fe be present as some other Fe\textsuperscript{3+} species. Only 5% of the intensity of the spectrum can be attributed to the putative fast-relaxing Fe\textsuperscript{3+} species, and no other Fe\textsuperscript{3+} species can be identified. Thus, the data fail to account for one-half of the electrons required for conversion of I to II. It seems unlikely that the additional electrons come from a source other than Fe\textsuperscript{2+}, as this occurrence would generate an additional radical which should be detected by EPR (unless it decays very rapidly). Recall from Chapter 4 that the EPR spectrum of the excess Fe\textsuperscript{2+} reaction can at all times be accounted for by summation of the spectra of X and •Y122. In light of the extensive evidence which supports the mechanism of Scheme 6.3, this inconsistency between the data and the model suggests that we have failed to identify an Fe\textsuperscript{3+} containing species (or have failed to accurately quantify the recognized one).
A final aspect of the Mössbauer time-course which warrants comment is the "unknown paramagnetic species" associated with the reference spectrum of Fig. 6.7A. This spectrum, which was deduced by analysis of the spectrum of the 60 s time-point, appears to exhibit large hyperfine splitting, such that its outer spectral lines are resolved outside the spectrum of X. In speculating as to the identity of the "unknown paramagnetic species", we have considered that it might be an Fe(II), Fe(III) cluster. This was deemed a possibility in light of the proposal of Elgen, et al. (1991) that an Fe(II), Fe(III) cluster is an intermediate in the reconstitution reaction. Although the semi-met form of R2 has not been thoroughly characterized due to its instability, there have been three recent reports of the preparation (in low yield) and detection of this form of R2 (Gerez et al., 1991; Hendrich et al., 1991; Gerez & Fontecave, 1992). In the work of Hendrich, et al. (1991), x-irradiation at 77 K of native R2 followed by annealing at 200 K led to the development of an EPR spectrum (accounting for 0.005 spins per R2 monomer) with g-values of 5.4 and 14. The spectrum was interpreted as arising from an Fe(II), Fe(III) cluster in which the Fe ions are ferromagnetically coupled to give a ground state electron spin quantum number of 9/2. (It should be noted that in the two other reports of the preparation of semi-met R2, chemical reduction of the native protein was employed. The EPR spectrum of the species which was detected indicates that its system spin is 1/2, which implies antiferromagnetic coupling between the ferrous and ferric ion.) So that we might compare it to the spectrum of our unknown paramagnetic species, we attempted to simulate the Mössbauer spectrum of the ferromagnetically coupled Fe(II), Fe(III) cluster. In order to obtain guesses for the δ (0.55 for the Fe³⁺ ion and 1.12 for the Fe²⁺ ion), ΔEQ (-0.92 for the Fe³⁺ ion and 2.35 for the Fe²⁺ ion), and a-values (21.2 T for the Fe³⁺ ion and 13.2 T for the Fe²⁺ ion) of the two Fe ions, we drew from the published Mössbauer characterization of an Fe(II), Fe(III) model complex which exhibits ferromagnetic coupling (Surerus et al., 1989). We also assumed the E/D of 0.03 reported by Hendrich, et al. (1991). In the spectrum generated by using these parameters (Fig. 6.10, solid line),
Fig. 6.10: Possible evidence for the accumulation of an $S = 9/2$ Fe(II), Fe(III) cluster in the excess Fe$^{2+}$ reaction. The experimental spectrum was generated by Prof. Huynh by analysis of the spectrum of the 60 s sample from Experiment 1. The other spectral components were subtracted away as indicated in Table 6.4. The solid line is the simulated spectrum of the $S=9/2$ cluster. This spectrum was generated by Dr. Ravi using an $S=9/2$ spin Hamiltonian and the following parameters: for the Fe$^{3+}$ site, $\Delta E_Q = -0.92$ mm/s, $\delta = 0.55$ mm/s, $a = 21.2$ T, and $\eta = -2$; for the Fe$^{2+}$ site, $\Delta E_Q = +2.35$ mm/s, $\delta = 1.12$ mm/s, $a = 13.2$ T, and $\eta = +2$ (Hendrich, et al., 1991; Surerus, et al., 1989).
the two outer-most lines and one of the inner lines coincide (approximately) with features in the spectrum of the unknown paramagnetic species. This partial agreement suggests that the species observed in our experiments could be the ferromagnetically-coupled Fe(II), Fe(III) cluster detected by Hendrich, et al. (1991). However, we have thus far failed to observe the EPR spectrum reported for this species. In addition, the temporal behavior of the "unknown paramagnetic species" is not what would be predicted for mixed valence R2 based on the model of Elgren, et al. (1991) and on the known instability of the Fe(II), Fe(III) cluster. The species accumulates only after formation of X is complete, and its quantity increases up to 60 s. The failure of the species to decay on the time-scale examined provides argument against its being an Fe(II), Fe(III) cluster. An objective of future work will be to determine whether the unknown paramagnetic species is a stable product or a slowly-decaying intermediate, and to ascertain whether the unknown paramagnetic species is associated with the EPR spectrum which has been reported for the S = 9/2 mixed valence cluster of R2 (Hendrich, et al., 1991).

References


Chapter 7: Time Course of the Reaction of Apo R2

with Limiting $\text{Fe}^{2+}$ and $\text{O}_2$ Monitored by Rapid

Freeze-Quench Mössbauer Spectroscopy
While the results of Chapters 3-6 construct a relatively coherent picture concerning the mechanism of the excess Fe\(^{2+}\) reaction, they leave several crucial aspects of the limiting Fe\(^{2+}\) mechanism unresolved. For example, the results of Chapter 4 indicate that the diferric-radical species (X) accumulates in the limiting Fe\(^{2+}\) reaction, but the lack of time-resolved quantitation of the intermediate cluster makes it impossible to conclude with certainty which of the postulated intermediates, I or II', contains X. In addition, the data presented thus far shed little light on the origin of the 560 nm absorption transient observed in Chapter 3. This band might arise from an additional Fe-containing intermediate (such as the \(\mu\)-peroxodiferric cluster which we originally proposed (Bollinger et al., 1991a)) or from an oxidized amino acid of R2. Likewise, the data of Chapters 3-6 provide few clues as to the origin of the broad \(g = 2.0\) EPR features (observed in Chapter 4), which also might arise either from an intermediate Fe species or from an amino acid radical. Still a third possible origin of the broad signals is interaction from multiple paramagnetic centers (as is proposed in Chapter 4).

Two significant results of Chapter 6 provide the basis for the experiments which are described in this chapter. First, the experiments of Chapter 6 demonstrate that all of the major Fe-containing reactants, products and intermediates can be detected, and that those with resolved spectral features can be quantified as functions of reaction time, by the rapid freeze-quench Mössbauer method. Second, Chapter 6 establishes the quantitative agreement of the data (the stoichiometries and rate constants) obtained by this method with those obtained by stopped-flow absorption and rapid freeze-quench EPR. The demonstration of this agreement in investigation of the kinetically simple excess Fe\(^{2+}\) reaction provides proof that the results of the three methods can be compared in order to understand the kinetically more complex reaction of apo R2 with limiting Fe\(^{2+}\).

In the experiments which are described in this chapter, the time-course of the reaction of apo R2-wt with limiting Fe\(^{2+}\) and O\(_2\) was monitored by rapid freeze-quench
Mössbauer spectroscopy. These experiments made possible the time-resolved quantitation of both \( X \) and the diferric cluster. Comparison of these data with those of Chapter 3 reveals that \( X \) forms concomitantly with development of the 560 nm absorption transient, but decays much less rapidly than the 560 nm transient. These results indicate that \( X \) is a component of both \( \text{I} \) and \( \text{II}' \). The combined data of this chapter and of Chapter 3 also indicate that \( \cdot Y_{122} \) forms significantly faster than does the diferric cluster. This observation and the fact that decay of the 560 nm absorption is faster than decay of \( X \) provide strong kinetic evidence for pathway A of Scheme 4.1, and imply the existence of the postulated intermediate \( \text{II}' \), which contains both \( X \) and \( \cdot Y_{122} \). Comparative analysis of the EPR spectra and Mössbauer spectra of intermediate time-points (1.0-3.0 s) provides additional evidence for the existence of \( \text{II}' \), as it appears that a fraction of the \( X \) which is recognizable in the Mössbauer spectrum exhibits a broadened EPR spectrum. This analysis provides the most direct evidence that the slow-decaying, broad \( g = 2.0 \) resonances which develop in the limiting \( Fe^{2+} \) reaction result from interaction of \( X \) and \( \cdot Y_{122} \). Finally, the time-resolved spectra of the reaction reveal no feature which correlates in time with the 560 nm transient. This result suggests that the absorption band does not arise from an Fe-containing intermediate, but must instead arise from a free radical.

Materials and Methods

\( ^{57}\text{Fe}^{0} \) was purchased from Advanced Materials and Technology of the former U.S.S.R. Apo R2-wt and the \( ^{57}\text{Fe}^{2+} \) stock solutions were prepared as described in Chapters 2, 3 and 6.

Time-Course of the Limiting \( Fe^{2+} \) Reaction by Mössbauer Spectroscopy

Three sets of time-course samples were prepared. In each experiment, an \( O_{2} \)-saturated solution of apo R2 in 100 mM HEPES buffered at pH 7.7 was mixed at 5 °C
with an equal volume of an O$_2$-saturated solution of $^{57}$Fe$^{2+}$ in 2.5 mM H$_2$SO$_4$. In Experiment 1, 0.59 mM apo R2-wt and 1.34 mM $^{57}$Fe$^{2+}$ were mixed, and twelve samples were quenched at reaction times which varied from 0.061 s to 60 s. In Experiment 2, 0.62 mM apo R2-wt was mixed with 1.47 mM $^{57}$Fe$^{2+}$, and four samples were quenched at reaction times which varied from 0.061 s to 0.44 s. In Experiment 3, 0.59 mM apo R2-wt was mixed with 1.36 mM $^{57}$Fe$^{2+}$, and four samples were quenched at reaction times which varied from 0.097 s to 4.01 s. (The 0.44 s time-point from Experiment 2 and the 0.23 s, 1.4 s and 4.0 s time-points from Experiment 3 have not yet been characterized.)

Mössbauer spectra of the samples were acquired at 4.2 K with a magnetic field of 50 mT applied parallel to the γ-beam. These spectra were analyzed (as described in Chapter 6) for the quantity of X and diferric cluster present in each sample. The subtraction program written by Prof. Huynh was used. The analysis was repeated independently in our laboratory.

Results

*Time-Course of the Limiting Fe$^{2+}$ Reaction Monitored by Mössbauer Spectroscopy*

Just as in the reaction of apo R2-wt with excess Fe$^{2+}$, the time-dependent Mössbauer spectrum of the limiting Fe$^{2+}$ reaction clearly reflects the progress of the reaction. At 0.061 s (Fig. 7.1A), the features of unreacted ferrous ion dominate the spectrum (solid line plotted over the data), but a significant quantity (see below for quantitation) of X has already formed (solid line plotted just above the data). The spectrum of the diferric cluster is not yet apparent. At somewhat longer reaction times (0.28 s, Fig. 7.1B), the contribution to the spectrum from ferrous ion has decreased, while the contribution due to X has increased (solid line above the data). In addition, the features of the diferric cluster are now visible (solid line plotted over data). With increasing reaction time (1.0 s, Fig. 7.1C), the contribution from X decreases, while the
Fig. 7.1: Time-course of the reaction of apo R2-wt with limiting Fe$^{2+}$ and O$_2$ as monitored by Mössbauer spectroscopy. The spectra are of samples from Experiment 1 (details are given in Materials and Methods). The reaction was quenched (A) at 0.061 s, (B) at 0.28 s, (C) at 1.0 s, or (D) at 60 s. The solid line plotted over the data in A is the theoretical reference spectrum of ferrous-R2 (see Chapter 6) scaled to 55% of the integrated intensity of the experimental spectrum. The spectrum just above the data in A-C is the theoretical reference spectrum of X (25% in A, 45% in B, and 25% in C). The solid line plotted over the data in B-D is the theoretical reference spectrum of the diferric cluster (13% in B, 35% in C, 60% in D). The experimental spectra were acquired at 4.2 K with a magnetic field of 50 mT applied parallel to the γ-beam.
contribution from the diferric cluster increases. Finally, near completion of the reaction (60 s, Fig. 7.1D), the features of the diferric cluster dominate the spectrum.

**Quantitation of X and the Diferric Cluster as Functions of Time**

Also as in the excess Fe$^{2+}$ reaction, both X and the diferric cluster can be quantified as functions of reaction time from the Mössbauer spectra of the freeze-quenched samples. Table 7.1 summarizes the quantities of the two species determined for each time-point, and Fig. 7.2 shows a plot of the data. The quantity of X present in the reaction increases rapidly without a significant lag phase, reaches its maximum value (∼0.55 equiv) at some time between 0.15 and 0.28 s, and then decreases slowly (relative to the decay phase of X in the excess Fe$^{2+}$ reaction). After an apparent initial lag phase, the quantity of diferric cluster rises smoothly.

**Comparison of the Mössbauer Quantitation with Stopped-Flow Absorption Data**

For comparison, the time-courses for formation of •Y122 (as determined by A$_{410}$,dropline) and for development and decay of the 560 nm absorption band are also shown in Fig. 7.2. These data are from a stopped-flow experiment which was carried out under reaction conditions identical to those employed in the Mössbauer time-course. The A$_{560}$-versus-time trace is scaled so that the final absorbance coincides with zero on the left-hand axis, and so that the maximum value of the trace coincides with the maximum quantity of X. It can be seen from Fig. 7.2 that the $t_{\text{max}}$ of X and that of A$_{560}$ (0.19 s in this experiment) roughly coincide. Taking into account that the rate constants measured in Chapter 6 for the excess Fe$^{2+}$ reaction ($k_1 = 7.3 \pm 1$ s$^{-1}$ and $k_2 = 1.0 \pm 0.1$ s$^{-1}$) are marginally greater than those measured in Chapter 3 by stopped-flow ($k_1 = 4.9-7.5$ s$^{-1}$ and $k_2 = 0.72-0.77$ s$^{-1}$), a discrepancy which we attribute to a slightly higher reaction temperature in the rapid freeze-quench experiments, the $t_{\text{max}}$ of X might actually be slightly greater than that of A$_{560}$. Irrespective of this minor point, it is clear from Fig. 7.2
Table 7.1: Summary of Quantitation of X and the diferric cluster as functions of reaction time. The reaction conditions employed in Experiments 1-3 are described in Materials and Methods.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Reaction Time (s)</th>
<th>% X</th>
<th>Equiv X</th>
<th>% Diferric Cluster</th>
<th>Equiv Diferric Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.061</td>
<td>25 ± 3</td>
<td>0.29 ± 0.03</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td></td>
<td>0.16</td>
<td>46 ± 3</td>
<td>0.53 ± 0.03</td>
<td>6 ± 3</td>
<td>0.07 ± 0.03</td>
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<tr>
<td></td>
<td>0.28</td>
<td>45 ± 3</td>
<td>0.52 ± 0.03</td>
<td>13 ± 4</td>
<td>0.15 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>0.44</td>
<td>40 ± 3</td>
<td>0.46 ± 0.03</td>
<td>20 ± 4</td>
<td>0.23 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>0.63</td>
<td>34 ± 4</td>
<td>0.39 ± 0.05</td>
<td>25 ± 4</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>25 ± 3</td>
<td>0.29 ± 0.03</td>
<td>35 ± 3</td>
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</tr>
<tr>
<td></td>
<td>1.8</td>
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<td>0.17 ± 0.03</td>
<td>50 ± 4</td>
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</tr>
<tr>
<td></td>
<td>3.0</td>
<td>12 ± 4</td>
<td>0.14 ± 0.05</td>
<td>55 ± 4</td>
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</tr>
<tr>
<td></td>
<td>5.0</td>
<td>8 ± 4</td>
<td>0.09 ± 0.05</td>
<td>60 ± 4</td>
<td>0.69 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>&lt; 5</td>
<td>&lt; 0.06</td>
<td>60 ± 4</td>
<td>0.69 ± 0.05</td>
</tr>
<tr>
<td>2</td>
<td>0.061</td>
<td>23 ± 3</td>
<td>0.28 ± 0.04</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>0.16</td>
<td>40 ± 3</td>
<td>0.48 ± 0.04</td>
<td>8 ± 3</td>
<td>0.10 ± 0.04</td>
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<tr>
<td></td>
<td>0.28</td>
<td>42 ± 4</td>
<td>0.50 ± 0.05</td>
<td>10 ± 4</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>3</td>
<td>0.098</td>
<td>40 ± 3</td>
<td>0.46 ± 0.03</td>
<td>5 ± 4</td>
<td>0.06 ± 0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup>We estimate the minimum detectable level to be ~3%

that X accumulates concomitantly with the development of the 560 nm absorption band which is characteristic of I. This result indicates that X is a component of I, for if X were a component only of II', its formation would exhibit a lag phase and would lag behind development of the 560 nm absorption band. The fact that decay of the 560 nm transient is faster than decay of X (again, this difference might be even greater if the reaction temperature of the freeze-quench experiments was more precisely maintained) indicates
Fig. 7.2: Kinetics of formation and/or decay of X, the diferric cluster, •Y122, and the 560 nm absorption band in the limiting Fe$^{2+}$ reaction. The quantities of X and •Y122 are from Table 7.1. The trace for •Y122 is from the experiment of Fig. 3.8. (The reaction conditions were identical to those of Experiment 1 of this chapter.) A$_{412}$,dropline was used to calculate the quantity of •Y122 as a function of reaction time. The A$_{560}$-versus-time trace is from the same stopped-flow experiment. For purposes of comparison, the right hand Y-scale, which relates to A$_{560}$ only, is adjusted so that A$_{560}$ at completion (0.0227) corresponds to zero on the left-hand axis, and the maximum absorbance (0.0543) corresponds (roughly) with the maximum quantity of X.
that X is also a component of II', since decay of the intermediate cluster should coincide with decay of A560 if X were a component only of I.

Examination of Fig. 7.2 also reveals that formation of •Y122 is initially faster than formation of the diferric cluster. For example, the Mössbauer quantitation on the 0.62 s sample indicates that 0.29 ± 0.04 equiv of the diferric cluster is present. The magnitude of A410,dropline in a stopped-flow experiment carried out under identical reaction indicates that 0.39 ± 0.03 equiv •Y122 is present at this same time. Five considerations provide argument that the apparent faster formation of •Y122 relative to the diferric cluster is real. First the maximum difference between the curves (0.1 equiv at ~0.6 s) is greater than the sum of the uncertainties which we estimate for the values. Second, the measured quantity of •Y122 is greater than the measured quantity of diferric cluster over a time range that spans five Mössbauer samples. Third, in terms of the stopped-flow results, the difference is reproducible. The equiv •Y122-versus-time curve from a second (identical) stopped-flow experiment also rises faster than the equiv diferric cluster-versus-time curve of Fig. 7.2. (Unfortunately, due to the length of time necessary to acquire the Mössbauer spectra of the time-course samples (2-4 days per sample), the diferric cluster quantitation has not yet been reproduced in the region of time in which the quantity of •Y122 most exceeds the quantity of diferric cluster. Duplicate samples have been prepared, however, for the early time-points, and it is in part on the deviation among replicate samples which we base our error estimates.) Fourth, the opposite trend is observed for the excess Fe2+ reaction, in which the equiv •Y122-versus-time curve, as determined by stopped-flow, lags behind the equiv diferric cluster-versus-time curve, as determined by rapid freeze-quench Mössbauer (see Fig. 7.3 below). (The difference in this case is most likely a trivial consequence of the aforementioned temperature difference.) Fifth, the rate constants measured in Chapter 6 by the rapid freeze-quench Mössbauer method are marginally greater than those measured by stopped-flow. Thus, the true amount by which the quantity of •Y122 exceeds the quantity of diferric cluster at
a given reaction time might actually be greater. The observation that •Y122 forms faster than the diferric cluster implies the accumulation of an intermediate (such as the postulated II') which contains •Y122 and an intermediate iron cluster.

Comparison of the stopped-flow quantitation of •Y122 and the rapid freeze-quench Mössbauer quantitation of the diferric cluster for the limiting Fe²⁺ reaction to those for the excess Fe²⁺ reaction (Fig. 7.3) provides perhaps the best evidence yet that distinct reaction pathways are operative. As described in Chapter 3, formation of •Y122 is faster in the limiting Fe²⁺ reaction than in the excess Fe²⁺ reaction. Conversely, formation of the diferric cluster is slower in the limiting Fe²⁺ reaction. These contrasting results are inconsistent with a single pathway mechanism.

Comparison of the Mössbauer Quantitation with EPR Data

Comparison of the quantity of X measured for a given time-point by Mössbauer spectroscopy with the quantity of X recognizable in the EPR spectrum of the same time-point reveals an apparent inconsistency in the data. As an illustration of this point, consider the 1.0 s time-point. The Mössbauer spectrum of this sample indicates that 0.29 ± 0.03 equiv X is present (Fig. 7.4A). The analysis of Chapter 4 suggests that an •Y122/X ratio between 78/22 and 74/26 (the ratio shown in Fig. 7.5A is 76/24) best reproduces the shape of the EPR spectrum at this time. (The EPR sample being considered was prepared under reaction conditions identical to those used in preparation of the Mössbauer time-course samples. The control experiment of Chapter 6 provides verification that no systematic difference exists between the Mössbauer and EPR samples.) This analysis would imply that 0.91 ± 0.18 equiv •Y122/R2 is present at 1.0 s. This value is impossibly high. The stopped-flow data indicate that 0.5 ± 0.04 equiv •Y122 is present at 1 s, that 0.6 ± 0.02 equiv is present after 3 s, and that only 0.74 ± 0.03 equiv has formed after 60 s. Furthermore, the EPR analysis indicates that at least 33% of the double-integrated intensity of the spectrum is contributed by the broad
**Fig. 7.3:** Kinetics of formation of •Y122 and the diferric cluster in the limiting Fe$^{2+}$ reaction compared to those in the excess Fe$^{2+}$ reaction. The data for the limiting Fe$^{2+}$ reaction are from Fig. 7.2. The diferric cluster data for the excess Fe$^{2+}$ reaction are taken from Table 6.2. The •Y122 data for the excess Fe$^{2+}$ reaction are calculated (from A412,dropline) from a stopped-flow experiment in which the reaction conditions were identical to those of Experiment 1 of Chapter 6.
Fig. 7.4: Quantitation of X in the 1.0 s (A), 1.8 s (B), and 3.0 s (C) time-points of Experiment 1. The solid line in each is the theoretical reference spectrum of X (25% in A, 15% in B, and 12% in C). The experimental spectra were acquired at 4.2 K with a magnetic field of 50 mT applied parallel to the γ-beam.
Fig. 7.5: Analysis of the EPR spectra from the reaction of apo R2-wt with limiting Fe$^{2+}$ to estimate the ratio of $\bullet$Y122/X. The reaction conditions were identical to those of Experiment 1. The outer spectra are (A) of the 1.0 s time-point, (B) of the 1.8 s time-point and (C) of the 3.0 s time-point. The inner spectrum in each is a summation of the spectra of X and $\bullet$Y122, in the proportions indicated in Table 7.2. The integrated intensity of each inner spectrum as a percentage of the outer spectrum is also listed in Table 7.2.
features. This would imply that at least 1.5 equiv of total radical (X + •Y122 + species responsible for the broad features) is present at 1.0 s, a value which is also unreasonably high. When applied to the 1.8 s time-point (Figs. 7.4B and 7.5B) and the 3.0 s time-point (Figs. 7.4 C and 7.5C), this analysis leads to similarly unreasonable values for the quantities of •Y122 and total radical present. (The analysis is summarized in Table 7.2.) It should be noted that the •Y122/X ratios deduced solely from the EPR spectra of these samples potentially suffer from a systematic error. Since these samples contain at least one other EPR active component, the unknown line-shape of the undefined component(s) could bias the apparent ratio. The conclusions of this analysis must therefore be considered tentative. Nevertheless, the Mössbauer and EPR spectra appear to be discrepant in terms of the quantity of X which they reflect. An interpretation which reconciles this apparent discrepancy is presented below.

Table 7.2: Summary of analysis comparing the quantities of X reflected by the Mössbauer spectra of three time-points to the quantity reflected by the EPR spectra. The Mössbauer samples are from Experiment 1, and the EPR samples were prepared identically, except that 56Fe2⁺ was used instead of 57Fe2⁺. The ratios of •Y122/X are those of the inner spectra of Fig. 7.5A-C.

<table>
<thead>
<tr>
<th>Reaction Time (s)</th>
<th>Equiv X from Mössbauer</th>
<th>•Y122/X from EPR</th>
<th>Equiv •Y122 (Calc.)</th>
<th>% X + •Y122 from EPR</th>
<th>% Broad Features</th>
<th>Equiv Total Radical (Calc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.29 ± 0.03</td>
<td>3.2 ± 0.3</td>
<td>0.9 ± 0.18</td>
<td>&lt; 65</td>
<td>&gt; 35</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>1.8</td>
<td>0.17 ± 0.03</td>
<td>5.3 ± 0.5</td>
<td>0.90 ± 0.25</td>
<td>&lt; 71</td>
<td>&gt; 29</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>3.0</td>
<td>0.14 ± 0.05</td>
<td>11 ± 1</td>
<td>1.5 ± 0.67</td>
<td>&lt; 77</td>
<td>&gt; 23</td>
<td>2.1 ± 0.9</td>
</tr>
</tbody>
</table>
Analysis of the Time-Resolved Mössbauer Spectra for Additional Components

The time-resolved quantitation of X (Fig. 7.2) demonstrates that the intermediate cluster is a component of I. The presence of X in I accounts for one of the two oxidizing equivalents proposed to be present in the intermediate, but leaves the identity of the second unresolved. As the results of Chapters 3, 4, and 6 establish that X does not exhibit the 560 nm absorption band, it is reasonable to expect that this band is associated with the unidentified second oxidizing equivalent of I. The Mössbauer spectra of the limiting Fe$^{2+}$ time-course samples were scrutinized for the presence of features which correlate in time with the 560 nm absorption band. No such feature could be discerned. This result indicates that the species associated with the transient absorption band, the component which stores the second oxidizing equivalent of I, is not an intermediate iron species. Its identity is discussed further below.

Although they exhibit no features which might be associated with the 560 nm absorbing species, the time-resolved spectra of the limiting Fe$^{2+}$ reaction do exhibit additional features which are potentially of interest. A partially resolved peak near 0.1 mm/s, which appears as a shoulder on the low energy line of the ferrous ion doublet (Fig. 7.6A), is seen in all the spectra. The feature is not apparent in the spectra of the excess Fe$^{2+}$ reaction (Fig. 7.6B). The solid line in Fig. 7.6A is a hypothetical reference spectrum for the species associated with the partially resolved feature. This hypothetical spectrum was generated with Mössbauer parameters ($\Delta \varepsilon_Q = 0.66$ mm/s and $\delta = 0.08$ mm/s) which are characteristic of iron in the ferryl (Fe(IV)) oxidation state (Schulz et al., 1984; Leising et al., 1991). By using this reference spectrum, the possible Fe(IV) species was quantified as a function of reaction time. The quantitation is summarized in Table 7.3. (The quantities of X and the diferric cluster are included for comparison.) The unknown species is present throughout the reaction, and its quantity varies only slightly with time. The temporal behavior of the species provides argument against its containing Fe(IV), as a ferryl species would not be expected to persist for 60 s. Moreover, the time-dependence
Fig. 7.6: Mössbauer spectral features of a "possible Fe(IV)" species which forms in the limiting Fe$^{2+}$ reaction but not in the excess Fe$^{2+}$ reaction. The data in A are the spectrum of the 0.28 s time-point of Experiment 1. The solid line in A is the hypothetical reference spectrum of the species. It was generated with Mössbauer parameters $\Delta E_Q = 0.66$ mm/s and $\delta = 0.08$ mm/s. Spectrum B is of the 0.31 s time-point from the excess Fe$^{2+}$ reaction (Experiment 1 of Chapter 6). The arrow in B is to indicate that the partially resolved feature of the "possible Fe(IV)" species is absent.
of the species (or lack thereof) suggests that it may be a mechanistically uninteresting side-product.

**Table 7.3:** Quantitation of the "possible Fe(IV)" species associated with the hypothetical reference spectrum of Fig. 7.6A.

<table>
<thead>
<tr>
<th>Reaction Time (s)</th>
<th>% &quot;Possible Fe(IV)&quot;</th>
<th>Equiv &quot;Possible Fe(IV)&quot;</th>
<th>Equiv X</th>
<th>Equiv Diferric Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.061</td>
<td>9</td>
<td>0.21</td>
<td>0.29 ± 0.03</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td>0.16</td>
<td>10</td>
<td>0.23</td>
<td>0.53 ± 0.03</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>0.28</td>
<td>14</td>
<td>0.32</td>
<td>0.52 ± 0.03</td>
<td>0.15 ± 0.05</td>
</tr>
<tr>
<td>0.44</td>
<td>12</td>
<td>0.28</td>
<td>0.46 ± 0.03</td>
<td>0.23 ± 0.05</td>
</tr>
<tr>
<td>0.63</td>
<td>12</td>
<td>0.28</td>
<td>0.39 ± 0.05</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>1.0</td>
<td>11</td>
<td>0.25</td>
<td>0.29 ± 0.03</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>1.8</td>
<td>11.5</td>
<td>0.26</td>
<td>0.17 ± 0.03</td>
<td>0.58 ± 0.05</td>
</tr>
<tr>
<td>3.0</td>
<td>11</td>
<td>0.25</td>
<td>0.12 ± 0.05</td>
<td>0.63 ± 0.05</td>
</tr>
<tr>
<td>5.0</td>
<td>8</td>
<td>0.18</td>
<td>0.09 ± 0.05</td>
<td>0.75 ± 0.05</td>
</tr>
<tr>
<td>60</td>
<td>8</td>
<td>0.18</td>
<td>&lt; 0.06</td>
<td>0.81 ± 0.05</td>
</tr>
</tbody>
</table>

None of the spectra of the limiting Fe$^{2+}$ reaction exhibit the resolved feature at ~1 mm/s which was assigned in Chapter 6 to the "fast-relaxing ferric species" (Fig. 7.6A). The 60 s time-point does, however, exhibit weak features of the type which are associated with "adventitiously bound" high-spin ferric ion (the arrows in Fig. 7.1D). Thus, it appears that the fate of a portion of the Fe$^{3+}$ which is produced when Fe$^{2+}$ provides the fourth electron may be different in the two reactions. Recall from Chapter 2 that this difference was also apparent in samples of R2 reconstituted by preforming the ferrous-R2 complex and then exposing the complex to O$_2$ (Fig. 7.7).
Fig. 7.7: Mössbauer spectra of R2-wt reconstituted (A) with 2.0 equiv of Fe$^{2+}$ or (B) with 4.0 equiv of Fe$^{2+}$. The samples were prepared by addition of $^{57}$Fe$^{2+}$ to an O$_2$-free solution of apo R2-wt, and then exposing the ferrous-R2 complex to O$_2$. Details of the preparation are described in the Materials and Methods section of Chapter 2. The spectra were acquired at 4.2 K with a magnetic field of 50 mT applied parallel to the γ-beam. The arrows in A indicate the features which are characteristic of "adventitiously bound" high-spin ferric ion. The solid line in both A and B is the spectrum of the diferric cluster scaled to 80% of the intensity of the experimental spectra.
Discussion

In Pathway A (limiting Fe\(^{2+}\)) of the mechanism which is proposed in Chapters 3 and 4 for the reaction of apo R2-wt with Fe\(^{2+}\) and O\(_2\) (Scheme 7.1), the intermediate I rapidly accumulates, and decays to give II', which contains •Y122 and a one-electron oxidized intermediate iron cluster. In the subsequent step, Fe\(^{2+}\) reduces the intermediate cluster by one electron, to convert II' to native R2. The time-course for formation and decay of X shown in Fig. 7.2 demonstrates that both I and II' contain X. Thus, conver-

Scheme 7.1: Mechanism proposed to be the predominant of two pathways in the reaction of apo R2-wt with limiting Fe\(^{2+}\). As before, apo R2 refers to one monomer of the dimer, and no implication is intended regarding the environment of the Fe\(^{2+}\) which converts II' to native R2 nor regarding the fate of the Fe\(^{3+}\) which results.

\[
\text{apo R2} + 2\text{Fe}^{2+} + \text{O}_2 \xrightarrow{k_{\text{obs}} = 5-10 \text{ s}^{-1}} \text{II} \\
\text{native R2} \xrightarrow{k_{\text{obs}} = 3.4-5.5 \text{ s}^{-1}} \text{II}^0
\]

- two oxidizing equivalents
- 560 nm absorption band
- 335 nm absorption band?
- diferric-radical species (X)?

•Y122
diferric cluster

•Y122
one-electron oxidized intermediate iron cluster (X?)
sion of I into II' must result from reduction by Y122 of the as yet unidentified (560 nm-absorbing) second oxidizing equivalent of I. The subsequent conversion of II' into native R2 must then involve reduction of X by Fe^{2+}. In Scheme 7.2, the mechanism of Scheme 7.1 is elaborated to reflect this new information.

The data of Fig. 7.2 are strikingly consistent with this mechanism. Since generation of •Y122 and destruction of the 560 nm absorbing species are proposed to occur in the same step, and since generation of the diferric cluster and destruction of X are proposed to occur in the subsequent (slower) step, formation of •Y122 should be faster than formation of the diferric cluster, and decay of the 560 nm absorbing species should be faster than decay of X. Fig. 7.2 clearly illustrates that each of these conditions is met. Moreover, taking into account the fact that the rate constants determined in the rapid freeze-quench experiments slightly exceed those measured by stopped-flow, the differences illustrated in Fig. 7.2 between the rates of •Y122 and diferric cluster formation and between the rates of A_{560} and X decay may not fully reflect the true differences. Thus, the time-resolved quantitation of X and the diferric cluster, together with the stopped-flow data of Chapter 3, provide strong kinetic evidence for the mechanism of Scheme 7.2.

The apparent discrepancy between the EPR and Mössbauer spectra in terms of the relative quantities of X and •Y122 which they reflect can be reconciled according to Scheme 7.2. In light of the hypothesis that II' contains both X and •Y122, an obvious and reasonable explanation for the apparent inconsistency is that the fraction of X in a given sample which is contained in II' is recognizable in the Mössbauer spectrum, but, because of interaction between X and the nearby •Y122, is unrecognizable (broadened) in the EPR spectrum. In this scenario, a fraction (or all) of the broad g = 2.0 features would arise from the perturbed signals of X and •Y122 in II'. A dipolar interaction of the magnitude necessary to cause the observed broadening in the EPR spectrum would not be expected to perturb the Mössbauer spectrum of X. The effect of the nearby •Y122 on the
Scheme 7.2: Scheme 7.1 elaborated to reflect the demonstration that both I and II' contain X.

\[
\text{apo R2} + 2\text{Fe}^{2+} + \text{O}_2 \xrightarrow{k_{\text{obs}} = 5-10 \text{ s}^{-1}} (\text{Fe}^{3+})_2\text{L}^*_{\text{ox560}}
\]

\[
(\text{Fe}^{3+})_2\text{L}^*_{\text{red560}} = X
\]

- \(\text{L}^*\) = ligand radical
- \(\text{ox}_{560}\) = 560 nm-absorbing oxidizing equivalent
- \(\text{red}_{560}\) = product of reduction of \(\text{ox}_{560}\)
nuclear spin levels of the Fe ions in X would be exerted only indirectly through the
electron spin of the cluster, and such second order interactions (comparable to super-
hyperfine coupling) are generally not detectable by Mössbauer spectroscopy. Thus, the
comparative analysis of the Mössbauer and EPR spectra of the reaction provides evidence
for the accumulation of II'.

The time-resolved Mössbauer spectrum of the limiting Fe$^{2+}$ reaction provides
significant insight as to the identity of the 560 nm absorbing intermediate. The
demonstration that X is a component of I indicates that the 560 nm absorbing species
contains only a single oxidizing equivalent. The observation that no obvious spectral
feature correlates in time with the 560 nm transient strongly suggests that the transient
does not arise from an additional Fe-containing intermediate. These two results rule out
our previous assignment of the 560 nm absorbing species as a μ-peroxodiferric cluster
(Bollinger, et al., 1991a). (The μ-peroxodiferric model complex on which the assignment
was based exhibits a single quadrupole doublet with Mössbauer parameters characteristic
of high spin ferric ion (Menage et al., 1990). A signal of this type would easily have been
detected in these experiments.) With iron-containing species ruled out, the only possible
identity of the 560 nm absorbing species is a free radical, which may be derived either
from O$_2$ or from the protein. Three considerations compel us to propose that the 560 nm
absorbing species is the free radical product of the one-electron oxidation of a tryptophan
(W) residue. First, a copious literature exists on the generation of W radicals (from W
itself, or in peptides or proteins containing W) by pulse radiolysis (Adams et al., 1972;
Posener et al., 1976; Solar et al., 1991). This literature indicates that the (oxidized) W
radical exhibits two absorption bands, with values of $\lambda_{\text{max}}$ which depend on its
neutral tryptophan radical (•W), which is the predominant species in solution at pH
greater than 4.3 (the reported pK$_a$ of the radical cation produced by abstraction of an
electron from W (Posener, et al., 1976; Jovanovic & Simic, 1985)), absorbs maximally at
510 nm and 325 nm, with molar absorptivities (ε_{510} and ε_{325}) of 2,300 and 3,700 M^{-1}cm^{-1}, respectively (Solar, et al., 1991). The tryptophan radical cation (•WH+) absorbs maximally at 560 nm and 335 nm, with molar absorptivities of 3,000 and 4,750 M^{-1}cm^{-1}, respectively (Solar, et al., 1991). The λ_{max} values of •WH+ match exactly with the positions of the bands detected in Chapter 3, and its ε_{560} is similar to that estimated for the 560 nm absorbing species (1,500-2,900 M^{-1}cm^{-1}). Second, related work indicates that W radicals can oxidize tyrosine residues (as in the WY dipeptide or as in proteins such as chymotrypsinogen A) to tyrosyl radicals (Prütz et al., 1980; Sloper & Land, 1980; Jovanovic et al., 1986). Thus, the proposal that the 560 nm absorbing species is a •WH+ is consistent with the data which suggest that the species generates •Y122 in the fast phase of the limiting Fe^{2+} reaction. Third, compound I of cytochrome c peroxidase (CCP) is believed to harbor a radical at W191 (Yonetani et al., 1966; Ho et al., 1983; Finzel et al., 1984; Edwards et al., 1987; Mauro et al., 1988; Erman et al., 1989; Scholes et al., 1989; Miller et al., 1992). Not only does this W191 radical exhibit an absorption band centered near 570 nm (Coulson et al., 1971; Ho, et al., 1983), but it also exhibits a broad, axial, g=2.0 EPR signal (Yonetani, et al., 1966). Recall that broad g = 2.0 features are observed in the limiting Fe^{2+} reaction of R2-Y122F (see Figs. 4.17 and 4.18), in which interaction of X and •Y122 cannot occur. The putative •WH+ might thus be the origin both of the rapidly-decaying broad features in this reaction and of those which develop early in the limiting Fe^{2+} reaction of R2-wt (see Figs. 4.16 and 4.19). Furthermore, as pointed out by Nordlund, W48 of R2 is related to W191 of CCP, inasmuch as each is part of a hydrogen bonding network which connects it to its active site iron (Nordlund, 1990). W191 of CCP is hydrogen bonded to D235, which in turn is hydrogen bonded to the proximal histidine (H175) of the heme (see Fig. 1.6) (Finzel, et al., 1984). Analogously, hydrogen bonds with D237 connect W48 of R2 to one of the two histidine ligands (H118) of the diiron cluster (see Fig. 1.5) (Nordlund et al., 1990). Thus, the characteristics deduced for the 560 nm absorbing species (that it stores a single
oxidizing equivalent, generates •Y122, exhibits absorption bands at 560 nm and 335 nm, is associated with broad g = 2.0 EPR features, and does not contain iron) are all consistent with its being a tryptophan radical cation, and the hydrogen bonding network of which W48 is a part makes this residue the obvious candidate for the W which is oxidized. As suggested by Nordlund (1990), the hydrogen bond between W48 and D237 might also explain why the radical appears to be protonated at pH 7.6, more than 3 units higher than the reported pKₐ of •WH⁺ (4.3) (Posener, et al., 1976; Jovanovic and Simic, 1985).

With the tentative identification of the 560 nm absorbing intermediate as a tryptophan radical cation, each of the oxidizing equivalents in I, II, and II' is assigned. The complete mechanistic hypothesis which results from this work is shown in Scheme 7.3. The current hypothesis is identical to that which we previously put forth, except that the µ-peroxodiferric cluster, which we previously proposed to be responsible for the 560 nm absorption band (Bollinger, et al., 1991a), is ruled out. The available data are still consistent with our previous assertion that neither of the species which generates •Y122 contains Fe(IV) (Bollinger, et al., 1991a; Bollinger et al., 1991b). However, with the current hypothesis that I contains X and •WH⁺, the participation of a fleeting Fe(IV) species prior to formation of I is not ruled out. We can only state that no evidence which suggests that a ferryl species is involved in the R2 reconstitution reaction has yet been found. The time-resolved Mössbauer spectra of the limiting Fe²⁺ reaction do exhibit features which would be consistent with the presence of a ferryl species, but the features do not decay on the time-scale of the reaction. This lack of kinetic competence suggests that the "possible Fe(IV)" species is not on the main reaction pathway.

The evidence of this chapter suggests that the manner in which Fe²⁺ delivers the fourth electron may be different in the limiting Fe²⁺ reaction from that in the excess Fe²⁺ reaction. The "fast-relaxing ferric species," which kinetic evidence suggests is the product of this process in the excess Fe²⁺ reaction, does not form in the limiting Fe²⁺
Scheme 7.3: Our current working hypothesis for the mechanism of the reaction of apo R2-wt with Fe$^{2+}$ and O$_2$. Apo R2 refers to one monomer of the dimer.
reaction. Conversely, the magnetic spectrum characteristic of "adventitiously bound" high-spin ferric ion, which is discernible in the 60 s time-point of the limiting Fe$^{2+}$ reaction, does not develop in the excess Fe$^{2+}$ reaction. These differences are observed both in the freeze-quenched samples and in those prepared by exposure of preformed ferrous-R2 to O$_2$. In the mechanism of Scheme 7.3, the order of delivery of the fourth electron relative to •Y122 formation differs in the two pathways. In pathway B, the process converts I to II in a fast step which occurs prior to •Y122 production. In pathway A, the process converts II' to native R2 in a much slower step which occurs subsequent to •Y122 production. Thus, it is not inconsistent that the Fe$^{3+}$ product which results from delivery of the fourth electron might be different in the two reactions.

References


