MECHANISTIC INVESTIGATIONS OF CLASS I RIBONUCLEOTIDE REDUCTASES AND RELATED OXYGEN-UTILIZING METALLOENZYMES

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by
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ABSTRACT

Enzymes, nature’s preferred catalysts, often effect remarkable transformations of their substrates, and the mechanistic elucidation of these catalysts has significantly advanced our understanding of biological processes. Our work is focuses on the detailed characterization of enzymes operating in an aerobic environment that utilize one or more transition metals as cofactors to facilitate transformation of their substrates. The approach of choice to facilitate the mechanistic elucidation of these enzymatic reactions involves the capture of transient species (reactive intermediates) that occur along each reaction sequence, and the kinetic and spectroscopic characterization of each captured species. A major focus of this thesis involves mechanistic investigations of a classic O₂-utilizing metalloenzyme, ribonucleotid reductase (RNR).

By catalyzing the conversion of ribonucleotides to deoxyribonucleotides [ND(T)Ps], RNRs provide all organisms with the required precursors for the de novo synthesis and repair of DNA. RNRs accomplish this chemically challenging feat with great fidelity by harnessing free-radical chemistry. To date, all RNRs characterized utilize an unstable cysteine thyl radical (C•), formed in close proximity to the bound ND(T)P to initiate ribonucleotide reduction. Class I RNRs, which all of our studies are focused on and encompass the enzymes from all mammals, aerobically-growing Escherichia coli (Ec), and the human pathogen Chlamydia trachomatis (Ct), are comprised of two non-identical protein subunits, termed α and β. The α subunit contains the oxidizable C residue and the site of nucleotide reduction, whereas the β subunit assembles a metallocofactor cofactor, ~35 Å away from the site of catalysis in α. To reduce ribonucleotides, all RNRs must transfer an oxidizing equivalent or “hole”, stored
at the metallocofactor in β, to α and generate the C• in a reversible process. The identity of metallocofactor is key distinguishing factor between the subclasses of class I RNRs, with the Ia and Ib enzymes utilizing diiron- and dimanganese-tyrosyl radical cofactors, and Ic employing a Mn\textsuperscript{IV}/Fe\textsuperscript{III} cofactor.

The first part of this thesis addresses the proper assembly of the Mn\textsuperscript{IV}/Fe\textsuperscript{III} cofactor of Ct RNR. The heterodinuclear nature of the cofactor raises the question of which metal site, 1 or 2, houses the Mn\textsuperscript{IV} that functionally replaces the Y• of the class Ia enzyme’s cofactor. This work, a collaborative effort with the laboratory of Prof. Amy C. Rosenzweig at Northwestern University, answers this question using a combination of X-ray crystallography, spectroscopy, analytical metal-content analysis, and enzymatic activity assays. Our results, presented in Appendix A, established that in vitro, Mn is not inherently selective for a particular site, and that both \textsuperscript{1}Mn/\textsuperscript{2}Fe and \textsuperscript{1}Fe/\textsuperscript{2}Mn forms of the enzyme are assembled under different conditions. However, enzyme forms containing predominantly \textsuperscript{1}Mn/\textsuperscript{2}Fe appear more active than those containing both \textsuperscript{1}Mn/\textsuperscript{2}Fe and \textsuperscript{1}Fe/\textsuperscript{2}Mn, and it is possible that \textsuperscript{1}Mn/\textsuperscript{2}Fe is the only active form.

Our work in Appendix B focuses on the mechanism by which the catalytic C• in Ct α is generated. This reaction does not occur in a single electron-transfer step via a tunneling mechanism in which the C residue transfers an electron to the Mn\textsuperscript{IV}/Fe\textsuperscript{III} cofactor. Instead, an oxidizing equivalent or “hole” is transferred in discrete electron-hopping events that may be coupled to proton transfers, which further complicates this already intricate process. This process, termed a radical-translocation (RT) process, is suggested to be mediated by several redox-competent amino acids [tyrosines (Ys) and perhaps a tryptophan (W)] along a specific pathway α and β that span the ~ 35-Å
distance. The residues are transiently oxidized, generating radical intermediates. Our work to initiate the RT process in Ct RNR with a more oxidized metal cluster provides the first capture and characterization of these radical intermediates in any class Ic RNR. Using the cofactor-assembly Mn\textsuperscript{IV}/Fe\textsuperscript{IV} intermediate, expected to have an elevated reduction potential relative to the stable Mn\textsuperscript{IV}/Fe\textsuperscript{III} cofactor, we can oxidize a Y (or multiple Ys) on the proposed RT pathway in a super-oxidized α•β complex, and use the resultant Y•(s) to effect nucleotide reduction. Remarkably, this super-oxidized complex appears to subvert, at least partially, the gating mechanism of the RT process – variants containing defective RT pathways were still capable of generating transient Y•s and actuating nucleotide reduction. Our work reported in Chapter 2 discusses our efforts to identify the precise location of the Y•(s) that accumulates in the wild-type super-oxidized α•β complex. Present results indicate that all of the observed Y• in that complex resides within the β subunit. Detailed kinetic investigations, also presented in Chapter 2, addressed the role of the lone W residue suggested to participate in RT. While the transient oxidation of the putative RT W residue (Ct β-W\textsubscript{51}) has never been observed during RT, our work revealed that the ramification of replacing W\textsubscript{51} with a phenylalanine, a defect that still leads to Y• formation with super-oxidized β, is a significantly slower formation of the Y•. Thus, the W may not be necessary for RT when using a more potent oxidant but its presence accelerates the rate by ~8-fold.

The second part of this thesis focuses on the development of a method with utility that extends beyond our studies on Ct RNR. The capture (by rapid-mixing kinetic techniques) and characterization (by spectroscopy) of fleeting, reactive intermediates is the most-favored approach to mechanistic dissection of metalloenzymes. For
metalloenzymes that also utilize molecular oxygen, O₂, this approach is often hampered by the gas’s modest aqueous solubility, which, at < 2 mM (at 1 atmosphere) limits both the effective rate constants for formation of reactive intermediates and the concentrations to which the intermediates can accumulate. Our work presented in Appendix C sought to overcome the challenge imposed by the poor solubility of O₂ by using the enzyme chlorite dismutase (Cld), for the rapid, in situ generation of O₂ at concentrations far exceeding 2 mM. Cld, a heme enzyme, efficiently converts chlorite (ClO₂⁻) to O₂ and chloride ion (Cl⁻). The method a) permits accumulation of O₂-derived complexes at concentrations well above 2 mM, b) allows greater precision in determining the O₂-dependent kinetics of enzymes that bind or react with O₂, and c) permits substantial increase in the yield of intermediates that form in a reversible, disfavored equilibrium with O₂. This means of in situ O₂ generation permits a > 5 mM "pulse" of O₂ to be generated in < 1 ms at the easily accessible [Cld] of 50 µM. It should therefore significantly extend the range of kinetic and spectroscopic experiments that can routinely be undertaken in the study of these enzymes and could also facilitate resolution of mechanistic pathways in cases of either sluggish or thermodynamically unfavorable O₂-addition steps.

Of the many applications we envisioned for the Cld/ClO₂⁻ method, the one that has advanced significantly is for the preparation of intermediates at concentrations that would afford their physical characterization by highly informative but relatively insensitive spectroscopic techniques. One of these techniques is Extended X-ray Absorption Fine Structure (EXAFS) spectroscopy, which provides structural information for systems not amenable to X-ray crystallography. EXAFS is often employed in the
characterization of metalloenzymes intermediates, and for several O₂-derived diiron protein intermediates, the characterization has proven difficult, largely due to the low concentration of the intermediates (a consequence of the poor solubility of O₂) that could be trapped. The Fe–Fe separation (d_{Fe-Fe}), by far the largest determinant factor of the core structures of these protein intermediates, has been reported as being ~ 2.5 Å. This short distance dictates unusual structures for the intermediates. One such intermediate is the high-valent cofactor-assembly Fe₂^{III/IV} intermediate in the class Ia Ec RNR. This enzyme’s reaction to generate its stable diferric-Y• radical cofactor proceeds via the Fe₂^{III/IV} intermediate, termed X, which is the ultimate oxidant of the cofactor-Y residue. The importance of X in ribonucleotide reduction, and the fact that it was the first non-heme Fe^{IV} enzyme intermediate to be discovered has made it the focus of many structural studies that seek to characterize it. The EXAFS characterization of X by Riggs-Gelasco et al. yielded a d_{Fe-Fe} of ~ 2.5 Å, a distance that demanded an unprecedented structure for which three single-atom oxo groups bridge the two Fe ions. Knowing that the EXAFS characterization of intermediate complexes benefits from increased concentration and purity of the species being investigated, we hoped that samples of X, prepared with the Cld/ClO₂⁻ method, at much higher concentrations than had ever been reported, would encourage a re-investigation into the EXAFS characterization. This work, a collaborative effort between our laboratory and that of Dr. Michael T. Green that is discussed in Appendix D, revealed that X does not contain a 2.5 Å d_{Fe-Fe}, but in fact contains a prominent 2.8 Å d_{Fe-Fe}. Computational studies predict that with this revised distance, X contains a di-µ-oxo core. This development has encouraged re-examination of the
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We are like dwarfs sitting on the shoulders of giants. We see more, and things that are more distant, than they did, not because our sight is superior or because we are taller than they, but because they raise us up, and by their great stature add to ours.

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Chapter 1:
Introduction to Class I Ribonucleotide Reductases and Related Oxygen-Utilizing Metalloenzymes
1-1. Ribonucleotide Reductases: Relevance, General Reaction, and Classification

Ribonucleotide reductases (RNRs), found in all organisms, catalyze the conversion of ribonucleotides to deoxyribonucleotides. RNRs provide organisms with the only established pathway for the de novo synthesis and repair of DNA (1, 2), and as such are therapeutic targets for abnormal growth and replication events (3). The anticancer drugs 2'-deoxy-2',2'-difluorocytidine (gemcitabine) (4, 5) and hydroxyurea (HU) (3, 6) are amongst the RNR inhibitors that have had largely successful clinical applications.

The general reaction catalyzed by RNRs involves replacement of the hydroxyl group at the 2'-position of the ribose ring of the nucleoside di- or tri-phosphate (NDP/NTP) with hydrogen (Figure 1) (1, 2, 7). This replacement is the key difference between DNA and RNA and enhances the stability of DNA’s double helical structure (2). All RNRs identified to date utilize free radical chemistry to catalyze this chemically challenging reaction (1, 2). A transient cysteine thyl radical (C•) (7), generated in an in situ fashion due to its poor stability, initiates reduction of the nucleotide by abstracting the hydrogen atom (H•) from the 3'-position of the substrate’s ribose ring (8, 9). The mechanism by which the C• is generated is the primary basis for the division of RNRs into classes I-III (Figure 2) (1, 2).

Class I RNRs, such as the enzymes from mammals, some viruses, and the well-studied prototype in aerobically-growing Escherichia coli (Ec), are comprised of two non-identical protein subunits, termed α (or R1) and β (or R2) (1, 2, 10). These subunits exist as a dimer of homodimers in the α₂β₂ active state (11-13), but can also exist in higher-order oligomers [e.g. the
human RNR has been suggested to exist as $\alpha_6\beta_6$ (14), and the Ec RNR has been shown to exist as $\alpha_4\beta_2$ (15, 16). The $\alpha$ subunit harbors the $C\cdot$ and contains the active site where nucleotides are reduced (13, 17), whereas the $\beta$ subunit is the host of a stable oxidant (10, 18, 19) capable of reversibly generating the $C\cdot$ in $\alpha$ (20). This stable oxidant is either a diiron(III/III)-tyrosyl radical cofactor in the class Ia RNRs (18, 21, 22), a dimanganese(III/III)-tyrosyl radical cofactor in the class Ib enzymes (23-28), or a manganese(IV)-iron(III) cofactor in the class Ic RNRs (29-32).

Class II RNRs, found in many eubacteria and archaeabacteria (1, 33), exist either as single monomeric proteins or homodimeric proteins (34-36). The well-studied prototype of this class is found in Lactobacillus leichmannii (37, 38), and it utilizes a 5'-deoxyadenosyl-5'-radical (5'-dA•) to reversibly oxidize the C residue (39, 40). The 5'-dA• is generated upon homolytic cleavage of the carbon-cobalt (C−Co) bond of adenosylcob(III)alamin (AdoCbl) (33, 40).

Class III RNRs are found only in anaerobic and facultative microbes (1, 41). These RNRs exist as dimers ($\alpha_2$) but require a separate activase protein ($\beta_2$) to generate the C-oxidant. The activase protein of class III RNRs is a member of the “radical SAM” superfamily of enzymes (1). All members of this family employ 5'-dA• chemistry for $H\cdot$-abstraction (42-44). These enzymes use an iron-sulfur cluster to reductively cleave the carbon-sulfonium bond of $S$-
adenosylmethionine (SAM) to generate methionine and a 5'-dA•. Some of the reactions catalyzed by the members of this family of enzymes include cofactor biosynthesis (45-47), enzyme activation (48), RNA modification (49), and DNA repair (50). They are characterized by a cysteine-rich motif that is, or closely resembles a CxxxCxxC motif (42, 43). The 5'-dA• generated by the activase of class III RNRs oxidizes a glycine residue in the α protein to form a stable glycyl radical (G•) that is the cysteine oxidant (51-53).

1-2. Class Ia: Ec RNR as a model

The class Ia RNR from aerobically-growing Ec has been studied extensively as the prototypical class Ia RNR. The functional oligomeric state is α2β2 (11-13), and as a class Ia RNR, it utilizes a diiron(III/III)-tyrosyl radical cofactor to reversibly generate the C• that initiates nucleotide reduction (18, 21, 22). Its structure and reaction mechanism is the focus of the next section.

1-2.1 Structure and function of the catalytic subunit, α.

The α (R1, Mr = 171,000 Da) subunit of Ec RNR binds nucleoside diphosphate (NDP) substrates and (deoxy)nucleoside triphosphate [(d)NTP] allosteric effectors, and is the host of the transient C• that initiates substrate reduction (1, 2). The crystal structure of Ec α was reported by Uhlin and Eklund in 1994 (13). It revealed that the protein is comprised of three domains: an α/β-barrel domain (where α and β refer to the secondary structure of the protein, not the subunits of RNR) comprising ~ 480 residues, a helical, N-terminal domain comprising ~ 220 residues, and an αβααβ domain with ~ 70 residues. The site of nucleotide reduction is presumed to be at the center of a deep cleft between the N-terminal and barrel domains.

The binding of (d)NTPs to α allosterically regulates RNR activity (54). There are two sites to which allosteric effectors bind (55, 56). The specificity site regulates the kind of substrate
that can bind in order to maintain a balanced pool of deoxyribonucleotides in the cell, and the binding of an effector to this site induces conformational changes to the protein structure that provides a signal to the active site to accommodate a particular substrate (57, 58). The second site for allosteric effector binding is the activity site. Binding of effectors at this site turns off RNR activity to prevent cytotoxic levels of dNDPs (54, 55, 57). It is now known that only dATP and ATP bind at this site, with dATP promoting higher-order oligomeric states (which inactivate RNR) and ATP reversing this action (16).

The mechanism of nucleotide reduction occurring at the active site (summarized in Figure 3) has been the focus of many studies over the years, and is now somewhat understood (59), primarily due to studies on site-directed variants of α (8, 60, 61), the structure reported by Uhlin and Eklund (13), and studies with mechanism-based inhibitors (7, 17, 62-64). In close proximity to the active site is the C residue (C439 in the prototypical class Ia from Ec) that is transiently oxidized by an oxidizing equivalent transferred from β. The resultant C439• abstracts a hydrogen atom from the 3'-position of the substrate’s ribose ring to form a 3'-nucleotide radical intermediate. The intermediate subsequently eliminates the 2'-hydroxyl group as a water molecule (after it is protonated by neighboring C462, forming a C462 anion)

**Figure 3.** Mechanism of nucleotide reduction occurring at the active site of Ec α. Taken from ref. (59).
concomitant with deprotonation of the 3'-hydroxyl group by a nearby glutamate residue (E441). This leads to the formation of a 3'-keto-2'-deoxyribonucleotide radical intermediate (1). Intermediate 1 is reduced via proton-coupled electron transfer (PCET) from the thiolate of C225 and the sulfhydryl of C462, which leads the two cysteines to form a disulfide anion. The disulfide anion is reduced when it transfers an electron to the 3'-ketone, concomitant with the transfer of a proton from E441, also to the 3'-ketone. This leads to the re-generation of the 3'-deoxynucleotide radical (2), which abstracts a H• from C439 in the final step of the reaction to re-oxidize the cofactor in β.

1-2.2 Structure and function of the cofactor subunit, β.

The β subunit (R2, Mr = 87,000 Da) of Ec RNR, first crystallized and its structure solved to a 2.2 Å resolution by Nordlund et al in 1990 (18), is mostly (70%) comprised of α-helices. Buried within the protein is the binding site for the diiron cofactor (18, 21). Each Fe is ligated by carboxylate and histidine amino acid residues in addition to O2- and/or solvent-derived ligands when the cluster is in an oxidized state (18). The metal cluster is essential for generating a potent and stable oxidant that can reversibly generate the C439• in α. This oxidant, a µ-oxo-diiron(III)-tyrosyl radical [µ-(O2•)-Fe2III/III-Y•] cofactor (18, 21, 22), is assembled in an autoactivation reaction of the Fe2II/II state of the cluster with O2 (21).

1-2.3 The ferritin-like, carboxylate-bridged di-metal enzymes

The β subunit of Ec RNR closely resembles a family of proteins referred to as the ferritin-like di-metal proteins. Structurally similar to ferritin, the iron concentrating- and storage- protein (65, 66), this family of proteins all assemble two metals (typically Fe ions) in close proximity, ligated by histidines and carboxylates to serve as electrostatic catalysts and effect transformations of their substrates (67, 68). They all activate molecular oxygen at the di-metal
center to generate an oxidized metallo-cofactor. Many (if not all) form peroxy-diferric intermediates (67), some of which have been characterized extensively. Amongst the reactions catalyzed by this family of enzymes is the oxidation of methane to methanol [by soluble methane monooxygenase hydroxylase, MMOH (69, 70)], conversion of alkane to alkene [by Δ-9 desaturase (71)] and aldehyde to alka/ene [by aldehyde deformylating oxygenase (72, 73)], amine oxidation [by AurF (74)], and amino acid oxidation (by RNR-β).

1-2.4 O2-activation at the diiron cluster of β

The mechanism of O2 activation by Ec β has been studied extensively and is described below (31). Upon addition of O2 to the diiron (II) (1FeII/2FeII) cluster in β, where 1Fe refers to the Fe ion proximal to the cofactor tyrosine (Y122), a µ-peroxo-Fe2III/III intermediate (P) is formed rather rapidly (75). While the intermediate does not accumulate significantly in the wild-type (wt) Ec β, it does form and is remarkably stable in β variants with the D84E substitution (76, 77) and in the wt β from Mus musculus RNR (78). P formed in Ec β variants with the D84E substitution have been characterized kinetically and spectroscopically (76, 77, 79). At 5 °C, its formation exhibits a first order dependence on O2, forming at a rate of ~ 2×105 M⁻¹ s⁻¹ (80). It is characterized by a broad absorption band centered at ~ 700 nm and has 57Fe Mössbauer properties (isomer shift, δ = 0.63 mm/s; quadrupole splitting, ΔEQ = 1.58 mm/s) characteristic of two high-spin FeIII ions coupled antiferromagnetically (AF) yielding a diamagnetic total electronic spin ground state (S total = 0) (76, 77). Resonance Raman (rR) characterization of P suggested it contains a peroxo moiety that

Figure 4. The diiron site of Ec β (PDB: 1MXR). Adapted from ref. (92).
bridges the two Fe ions symmetrically (81), while extended X-ray absorption fine structure (EXAFS) spectroscopic characterization reports a short Fe–Fe interaction (~ 2.5 Å) that can only be rationalized by a \( \mu-\text{(1,2)}-\text{peroxo}-\mu-\text{(-} \eta^1, \eta^2\text{-carboxylato} \) structure (77). \( \mathbf{P} \) is thought to decay to a formally \([\text{Fe}_2\text{O}_2]^{4+} \) center that is rapidly reduced by one electron shuttled to the cluster via a near-surface tryptophan, \( \text{W}_{48} \), to cleave the O–O bond. The elusive \([\text{Fe}_2\text{O}_2]^{4+} \) species, termed \( \mathbf{L} \), was captured in sequential-mixing rapid freeze quench experiments with a \( \text{W}_{48}\text{A/}Y_{122}\text{F} \) variant of \( \beta \). Mössbauer characterization of \( \mathbf{L} \) suggests that it contains at least two AF-coupled \( \text{Fe}_2^{\text{III/III}} \) species that exist in a rapid equilibrium. These are proposed of being protonated versions of \( \mathbf{P} \) (i.e., hydroperoxo-\( \text{Fe}_2^{\text{III/III}} \)) (82, 83).

The next step of the activation reaction involves the transfer of an electron from the near-surface residue, \( \text{W}_{48} \), to \( \mathbf{L} \), which yields a state containing both the high-valent \( \text{Fe}_2^{\text{III/IV}} \) cluster, termed intermediate \( \mathbf{X} \) (84, 85), and a tryptophan cation radical (\( \text{W}_{48}^{+•} \)) (80). The \( \text{W}_{48}^{+•} \) has also been kinetically and spectroscopically described (80). Its formation exhibits a first-order dependence on \( \text{O}_2 \) and \( \beta\text{-Fe}_{2}^{\text{II/II}} \) \([k_{\text{obs}} ~ 200 \text{ s}^{-1} \text{ at the highest concentration of } \text{O}_2 \text{ examined}] \) (80). Associated with its formation is a broad absorption band centered at 560 nm and a structured \( g = 2 \) Electron Paramagnetic Resonance (EPR) signal. While the \( \text{W}_{48}^{+•} \) cannot be probed by Mössbauer spectroscopy, its presence perturbs the signal of the diiron cluster due to dipolar coupling between the two paramagnetic centers (80). The \( \text{W}_{48}^{+•} \) is readily reduced by a number of natural reductants (ascorbate, thiols) (80, 86), and its reduction yields a state in which the features of \( \mathbf{X} \) are not perturbed by the nearby paramagnetic center.
Intermediate X exhibits an isotropic $g = 2$ EPR signal, which is subject to broadening when prepared with $^{57}$Fe is added to the protein (84, 85). X also exhibits $S_{\text{total}}$ of $\frac{1}{2}$, consistent with AF coupling of a high-spin Fe$^{\text{III}}$ ion to a high-spin Fe$^{\text{IV}}$ ion. The unusually large $\delta$ of the Fe$^{\text{IV}}$ site (0.26 mm/s) initially led to the mischaracterization of the cluster as an Fe$^{\text{II/III}}$-radical species (87). The initial characterization of X reported that the cluster contained a diferric-radical species with $\delta = 0.55$ and 0.36 mm/s, and isotropic hyperfine coupling constants, $A$, of -52.5 T and 24 T, respectively, for the two Fe ions. The large isomer shift reported for the $^2$Fe site of the $^1$Fe/$^2$Fe cluster, which led to the mis-identification of the Fe$^{\text{IV}}$ site as an Fe$^{\text{III}}$ site, was caused by the overlap of the high-energy line of the Fe$^{\text{IV}}$ site with the Fe$^{\text{II}}$ component that is always present in samples of X. This then led to the assignment of isotropic $A$ tensors and a large $\delta$ for the $^2$Fe site. Subsequent ENDOR results (88) that permitted a more accurate determination of the $^{57}$Fe $A$

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**Scheme 1.** Current understanding of the mechanism of cofactor generation and catalysis by the class Ia Ec RNR. The states shown in red rectangles are half-integer spin systems that are observed by EPR. The state shown in dashed rectangle has not been directly observed. Select EPR spectra are presented. Adapted from ref. (31).
tensors allowed a better simulation of the Mössbauer spectrum and the assignment of an Fe$^{IV}$-containing species in X \cite{88}. X then was the first non-heme protein-derived Fe$^{IV}$ species to be identified, and its detailed spectroscopic characterization is presented in section 1-2.6.

In the last step of the cofactor generation, X oxidizes the neighboring Y$_{122}$ as it is reduced to the $\mu$-oxo-Fe$_2^{III/III}$ cluster. The Y$_{122}^\bullet$, which forms at a rate of $\sim$ 1 s$^{-1}$ \cite{85}, is characterized by a sharp absorbance feature at 411 nm of the UV-visible spectrum \cite{89}. The X-band continuous wave (CW)-EPR spectrum of the radical is dominated by one large doublet \cite{89, 90} arising from the large A tensor of one of the hydrogens of the methylene carbon (C$_{\text{methylene}}$). The large A tensor reflects the rotational conformation of the C$_{\text{methylene}}$-H about the axis normal to the plane of the aromatic ring (i.e., the dihedral angle, $\theta$, $\sim$ 33°) and the unpaired electron spin density ($\rho$ $\sim$ 0.49) on C1 of the ring \cite{91}. The other methylene hydrogen is nearly within the plane of the aromatic ring ($\theta$ $\sim$ 90°) and therefore does not have as large of an A tensor (it interacts weakly with the C1 $\pi$ orbital). The CW-EPR spectrum also reflects a smaller triplet splitting that arises from the A tensors of the hydrogens located at C3 and C5 of the aromatic ring, where $\rho$ of C3 and C5 are $\sim$ 0.26. C2, C4, and C6 all have negative spin densities and therefore do not contribute to the hyperfine structures observed for the radical. The phenolic oxygen has significant density ($\rho$ $\sim$ 0.16), and thus an odd-alternate structure model has been proposed for the radical, where C1, C3, C5, and the O have significant spin densities and C2, C4, C6 have spin densities that are negligible \cite{91}. Recent high-frequency (W-band, 94-GHz) EPR measurements on single crystals containing the Y$^\bullet$ revealed that there is significant rotation of the radical (compared to the non-radical structure of $\beta$), particularly the phenolic oxygen, away from the diiron cluster so that the oxygen is $>$ 4 Å away \cite{92}. It is thought that moving the radical away so that it is not within proton-transfer range of the cluster and its ligands stabilizes
the radical. Mössbauer spectroscopy reports that the neighboring Fe$^{\text{III}}$ ions couple antiferromagnetically to give $S_{\text{total}}$ of 0 (21).

1.2.5 Structure of the $\alpha_2\beta_2$ complex

Currently, there is no crystal structure of the functional $\alpha_2\beta_2$ complex. Efforts to co-crystallize $\alpha$ and $\beta$ have yielded structures of higher order oligomeric states (16) or of the subunits in close proximity but not actually bound together (93). In lieu of an $\alpha_2\beta_2$ structure, a docking model has been created (13), based on shape complementarity of the structures of the individual subunits and supported by Pulsed Electron Double Resonance (PELDOR) spectroscopy (94, 95). According to this model, the $\alpha$ subunits (blue and magenta of Figure 5) sit astride the heart-shaped $\beta$ subunits (green and orange of Figure 5). The C-terminus (35 amino acid residues) of $\beta$ is disordered and not observed in the crystal structure, but is thought to become ordered and link the subunits when $\alpha$ and substrate bind. The co-crystallization of $\alpha$ with a peptide corresponding to the last 20 residues of $\beta$ yielded a structure where 15 residues of the

![Figure 5. Docking model of Ec RNR. The $\alpha$ subunits (blue and magenta) sit astride the $\beta$ subunits (green and orange). To the right is a ribbon representation to show the buried Fe site in $\beta$ and the substrates and effectors bound to $\alpha$. Adapted from ref. (13).](image-url)
peptide were inserted into a shallow groove in the helices of α (96). These peptides are also inhibitors of RNR activity, because they compete with β for binding to α (97, 98).

### 1-2.6 Development of the Cld/ClO$_2^-$ method of O$_2$ generation for the spectroscopic characterization of reactive intermediates

The capture (by rapid-mixing kinetic techniques) and characterization (by spectroscopy) of fleeting, reactive intermediates is the most-favored approach to mechanistic dissection of metalloenzymes (99-101). For metalloenzymes that also utilize O$_2$, this approach is often hampered by the modest aqueous solubility of O$_2$, which, at < 2 mM (at 1 atmosphere) (102) limits both the effective rate constants for formation of reactive intermediates the effective rate constants for formation of reactive intermediates (generally exhibiting first-order dependence on O$_2$) and the concentrations to which the intermediates can accumulate (99, 103). The low levels of accumulation often impede the spectroscopic characterization of the intermediates, as some spectroscopic techniques that are highly informative are also relatively insensitive. Thus, we set out to overcome the challenge imposed by the poor solubility of O$_2$ by using the O$_2$-evolving enzyme, chlorite dismutase (Cld), for the rapid, in situ generation of O$_2$ at concentrations far exceeding 2 mM (104).

Cld, a heme enzyme, efficiently converts chlorite (ClO$_2^-$) to O$_2$ and chloride ion (Cl$^-$), with a stoichiometry of 1 ClO$_2^-$:1 O$_2$ (105). It is essentially a detoxifying enzyme found in a number of proteobacteria that respires perchlorate (ClO$_4^-$) (106). These organisms employ a molybdopterin-dependent perchlorate reductase to sequentially reduce ClO$_4^-$ to ClO$_3^-$ and ultimately ClO$_2^-$. Without Cld, the ClO$_2^-$ would build-up to cytotoxic levels (106). This dictates that the reaction of Cld is fast and efficient, and the enzyme from Dechloromonas aromatica is one of the fastest and most efficient Clds characterized (105, 107, 108). This method of using
Cld to generate O$_2$ from ClO$_2^-$, which typically involves the addition of Cld to the oxidase/oxygenase of interest followed by mixing with ClO$_2^-$ (as illustrated in Figure 6), or doing a sequential-mixing procedure, in which Cld and the oxidase/oxygenase are kept apart and only mixed for a short period of time before being introduced to a solution of ClO$_2^-$, was applied to the study of five O$_2$-utilizing metalloenzyme systems. For the Mn/Fe-dependent RNR (vide infra) from *Chlamydia trachomatis* (*Ct*), in which the enzyme’s Mn$^{IV}$/Fe$^{III}$ cofactor forms from a Mn$^{II}$/Fe$^{II}$ complex and O$_2$ via a Mn$^{IV}$/Fe$^{IV}$ intermediate, this method was used to define the [O$_2$] dependence of the reaction at effective O$_2$ concentrations as high as ~10 mM. With a more soluble receptor, myoglobin, an O$_2$ adduct was accumulated to > 6 mM in < 15 ms. Finally, key intermediates in the reactions of taurine:$\alpha$-ketoglutarate dioxygenase, myo-inositol oxygenase, and the class Ia RNR from *Escherichia coli* (*Ec*) were all accumulated to yields more than twice those previously attained. With an estimated catalytic rate of 120,000 s$^{-1}$ for O$_2$-evolution (104, 107), Cld can generate a > 5 mM "pulse" of O$_2$ in < 1 ms at the easily accessible enzyme concentration of 50 µM. This method 1) permits accumulation of O$_2$-derived complexes at concentrations well above 2 mM, 2) allows greater precision in determining the O$_2$-dependent kinetics of enzymes that bind or react with O$_2$, and 3) permits a substantial increase in the yield of intermediates that form in a reversible, disfavored equilibrium with O$_2$. The method, presented in Appendix C, is envisioned to serve as a major technical advance to the field of enzymology.
Of all the applications anticipated for the Clδ/ClO₂⁻, the preparation of intermediates at concentrations that would make them amenable to structural characterization by relatively insensitive spectroscopic techniques is thus far most advanced. One of these techniques is EXAFS spectroscopy, which provides structural information for states not suitable for X-ray crystallographic characterization. EXAFS is often employed in the characterization of metalloenzymes intermediates, and for several O₂-derived diiron protein intermediates (77, 109-111), the characterization has proven difficult and has yielded controversial structures, largely due to constraints on the concentration of the intermediates (a consequence of the poor solubility of O₂) that could be trapped. Two of the intermediates [P (77) and X (110)] with controversial structures are found in the activation reaction of RNRs and were previously described (vide supra).

1-2.7 Structural characterization of X by EXAFS and Density Functional Theory

X, as the tyrosyl radical-generating intermediate, is significant for understanding the activation of the class Ia RNRs cofactor. Moreover, X was the first non-heme high-valent Fe intermediate to be identified in an enzyme (84, 87, 88). It is suspected of being structurally similar to a number of intermediates that accumulate in the reactions of ferritin-like di-metal carboxylate-bridged enzymes (see 1-2.3). Obtaining an understanding its electronic and geometric structure might be helpful for understanding the structural diversity amongst intermediates from the ferritin-like di-metal carboxylate-bridged family of enzymes. These enzymes activate O₂ at the reduced metal cluster (typically Fe₂^{II/II}) to generate peroxo intermediates that could effect transformations of substrates, or go on to form high-valent intermediates (67). Thus far, high-valent Fe intermediates identified in this family include the
methane hydroxylating $\text{Fe}^{IV/IV}_2$ intermediate (Q) in methane monooxygenase hydroxylase (MMOH) (70), and X in Ec RNR.

The significant role played by X during formation of RNR’s active cofactor has made it the target of studies that aim to structurally characterize it (87, 88, 110, 112-116). X was initially characterized by EXAFS spectroscopy in 1998 (110) by Riggs-Gelasco et al., who reported that X contains a 2.5 Å Fe-Fe separation ($d_{\text{Fe-Fe}}$). Based on the comparison of this distance to those obtained for various model complexes with different core structures, it was suggested that X contains three single-atom oxygen bridges: one derived from $\text{O}_2$ and two from carboxylate acid residues that would coordinate in a $\mu$-$\eta^1,\eta^2$ fashion. The assignment of this bridging mode was aided by data from $^1$H- and $^{17}$O-ENDOR experiments that detected one oxo bridge derived from $\text{O}_2$, did not detect a bridging hydron, and remained agnostic on a second bridge (112, 116). However, the short $d_{\text{Fe-Fe}}$ remained irreconcilable with calculations performed on relevant synthetic and theoretical model complexes, which predict distances $\geq$ 2.7 Å (113, 117-122). Thus, the structure of X remains enigmatic.

Knowing that the EXAFS technique benefits from high purity of the species being investigated (123-125), we used the Cl$^2$/ClO$_2^-$ method of $\text{O}_2$ generation to prepare the intermediate at $\sim$ 2.0 mM, a concentration much greater than the $\leq$ 0.76 mM used for the previous EXAFS characterization (110). The increased concentration of the intermediate in the new samples results in a significantly improved signal that allows acquisition of the Fe K-edge EXAFS data up to high values of k (the photoelection wave number), and thus permits a more rigorous investigation into the core structure of the intermediate. This work, presented in Appendix D, reports that X contains a prominent $d_{\text{Fe-Fe}}$ of 2.8 Å, not 2.5 Å. The $d_{\text{Fe-Fe}}$ fits a model that contains two $\mu$-oxo bridges.
1-2.8 Inter-subunit radical-translocation from the stable oxidant in $\beta$ to the essential cysteine in $\alpha$.

For NDP reduction to occur, the oxidizing equivalent ("hole") generated and stored in $\beta$ must be transferred, over $\sim$ 35 Å ($13, 94$), to oxidize the C residue in $\alpha$ ($Ec\ C_{439}$). The multiple rounds of dNDP formation that is required to maintain a balanced pool of DNA precursors in all organisms dictates that this "hole" translocation process is reversible. The oxidation of the $C_{439}$ residue in $\alpha$ by the cofactor $Y_{122}$ in $\beta$ does not occur in a single electron transfer reaction via a tunneling mechanism. The distance separating the two residues is estimated at 35 Å, which the Marcus-Levich equation predicts would take an electron $10^{-4} – 10^{-7}$ s to tunnel over ($10$). This rate is inconsistent with the steady state rate of product formation in the wild-type enzyme (2 – 10 s$^{-1}$) ($126$), and suggests an alternative mechanism by which $C_{439}$ is oxidized. This mechanism is thought to be the sum of several discrete electron transfer (ET) steps that may or may not be coupled to individual proton transfer step(s) that modulate the reduction potentials of the amino acids involved in ET. We recently have termed the overall process of transferring the oxidizing equivalent or "hole" as a "radical-translocation" (RT) process ($127$). The RT is proposed to be conformationally "gated" by the protein and mediated by several tyrosines and perhaps a tryptophan residue that span a specific pathway in both subunits ($128$-$134$).

Upon complexation of the $\alpha$ and $\beta$ subunits, the binding of substrate and allosteric effector induces a protein conformational change(s) that trigger(s) for radical translocation from

**Figure 7.** RT pathway of $Ec$ RNR. Location of the residues are based on the docking model of the subunits. The location of $Y_{356}$ is unknown.
the oxidized cofactor stored on β-Y$_{122}$ to α-C$_{439}$. The triggering event, referred to as “gate opening”, is now presumed to involve the transfer of a proton from the terminal water molecule coordinating $^1$Fe of the $^1$Fe/$^2$Fe cluster to the neighboring Y$_{122}•$, concomitant with an electron transfer from the proximal RT pathway residue, which may be W$_{48}$ or Y$_{356}$, also to Y$_{122}•$ (135).

The “hole”, now on one of these residues, is propagated, residue-by-residue, through α to C$_{439}$ and the bound CDP. Presumably, transient radicals are formed at W$_{48}$ and/or Y$_{356}$ in β and Y$_{731}$, Y$_{730}$ and C$_{439}$ in α (see Figure 7). These radicals have never been observed in the wt enzymes because their formation is thought to be much faster than the preceding “gate opening” step(s) and thus kinetically masked (10, 126). Moreover, the radical(s) on other tyrosines may not be spectroscopically distinguishable from the Y$_{122}•$. The reduction potentials of the proposed radical hosts have not been measured, but it is thought that the protein, through transfers of protons, tunes the potentials, making their oxidization thermodynamically favored only in the “ready” α•β•substrate•effector complex. One way by which the hindrance posed by the unfavorable kinetics (and perhaps the thermodynamics) of RT in Ec RNR has been overcome is with the substitution of the putative residues with unnatural amino acids that have altered reduction potentials. Elegant work by Stubbe and co-workers to insert 3,4 dihydroxyphenylalanine (DOPA) (133, 136), and 3-aminotyrosine (NH$_2$-Y) (134, 137-139), both with lower reduction potentials than Y, at β-Y$_{356}$, α-Y$_{731}$ and α-Y$_{730}$, introduced a thermodynamic depression on the RT pathway that allowed for the accumulation of radicals at positions other than Y$_{122}$. More recently, the insertion of 3-nitrotyrosine (NO$_2$-Y) (140), an analogue with an elevated reduction potential relative to Y, at Y$_{122}$, led to the formation of a nitrotyrosyl radical (NO$_2$-Y•) (141), which in the presence of α, substrate, and allosteric effector, decayed to produce deoxyribonucleotides and a Y•. It is thought that while the metal cluster can oxidize the NO$_2$-Y
during the activation reaction, re-oxidation of the NO₂-Y by a RT pathway tyrosine during the reverse RT (after substrate reduction) is difficult. Hence, the returning radical resides on one or more of the RT pathway tyrosine residues. PELDOR experiments show that the majority (~90%) of the Y• formed resides at Y₃₅₆ with the remainder distributed on Y₇₃₀ and Y₇₃₁ in α (142). The failure to regenerate the NO₂-Y• limits this process to a single turnover event (141). Interestingly, only half of the α₂β₂ pair of homodimers participates (141), owing to half-of-sites reactivity in Ec RNR.

1-3. Class Ic: Ct RNR as a model

McClarty and coworkers discovered a new subclass of class I RNRs in 2000 upon the characterization of the genomes of several human pathogens from the genus Chlamydiae (143). These organisms, including the obligate intracellular human parasite, Chlamydia trachomatis (Ct), contain genes encoding RNR β subunits for which the Y residue, that is in the class Ia subunits oxidized to the Y• of the cofactor, is replaced with a phenylalanine (F) – a residue not susceptible to oxidation and hence not capable of generating the C•. Furthermore, an aspartate (D) ligand to metal site 1 (the site closest to the Y₁₂₂) in the Ec β is replaced with a glutamate (E) residue. These RNRs are classified as Ic (144). To date, all putative class Ic RNRs contain both the D → E and Y → F substitutions. The crystal structure of Ct β with a diiron cluster was solved to a 1.7 Å resolution in 2004 by Högbom et al. (144). The F replacement (F₁₂₇) is retained in the structure, suggesting that the residue is not post-translationally hydroxylated to restore the Y.
Overall, the structure did not provide any insight into the reaction mechanism of this subclass of RNRS.

Upon the discovery of the Ic RNRS, Gräslund and coworkers proposed a provocative mechanism by which they might function (145, 146). Analogously to the Ec β activation, O₂ would add at the Fe_{II/II} center to generate the µ-peroxo Fe_{III/III} intermediate, which would subsequently be reduced to the Fe_{III/IV} intermediate (X_{Ct}). X_{Ct} would then directly oxidize the cofactor proximal RT residue (W₅₁ or Y₃₃₈, cognates of Ec W₄₈ and Y₃₅₆) and the resultant radical would propagate into α and eventually reduce the substrate. After substrate reduction, the returning radical would oxidize the metal cluster to regenerate X_{Ct} before commencing another cycle of RT into α (146). The accumulation of a g = 2 EPR signal that exhibits broadening with ^{57}Fe substitution in the reaction of Ct β’s Fe_{II/II} cluster with O₂ supports the formation of X_{Ct} (145). ^{57}Fe- and ^{1}H-ENDOR experiments suggest that the species contains three oxygenic ligands – two bridging oxo ligands and a terminal OH or H₂O (147). X_{Ct} is much more stable in the presence of α, substrate, and allosteric effector, which gives credence to the proposal that it is the direct oxidant of the RT pathway residue (145). While the idea of X_{Ct} functionally replacing

![Figure 9. Mechanism proposed for the reaction of Ct RNR by Gräslund and co-workers. Adapted from ref. (145).](image-url)
the Y122 is plausible, the failure of Gräslund and co-workers to demonstrate multiple turnovers of the enzyme, enzyme activity that correlated to the amount of $X_{Cl}$ present, or the show that $X_{Cl}$ was capable of 3'-H• abstraction from the substrate (using one or more mechanism-based inactivator substrate analogues that serve as reporters for H•-abstraction) presented the possibility that the proposed mechanism did not provide a satisfactory explanation of how the class Ic RNRs operate.

3.1.1 $Ct$ β: The first Mn/Fe redox enzyme

In 2007, work from the Bollinger/Krebs group provided evidence that $Ct$ RNR requires a Mn and Fe cofactor, assembled in its β subunit, for catalytic activity (29). Activity was maximized when both metals were present in a 1:1 molar ratio, suggesting that a mixed metal cofactor was operant. Activity was demonstrated by the detection of the deoxyribonucleotides by mass spectrometry, and spectroscopically with the formation of a nitrogen-centered radical (N•) when the natural substrate was substituted by the 2'-azido-2'-deoxyadenosine-5'-diphosphate (N3-ADP) substrate analogue. The analogue contains an azido group at the 2'-position of the ribose moiety, which, upon H• abstraction from the 3'-position triggers a cascade of events that culminates with the release of N2 and the formation of a N• at the 3' – position (17, 63, 148). The distinct EPR signature of this radical can serve as a reporter for a single H• abstraction event and thus, a single turnover by the enzyme.

3.2 O2-activation at the Mn/Fe cluster

![Figure 10. Metal dependence of Ct RNR activity. The 1:1 molar ratio of Mn and Fe is shown to result in maximum activity. Taken from ref. (29).](image)
The cofactor required for catalysis in Ct RNR is a stable Mn\(^{IV}\)/Fe\(^{III}\) cluster (29, 30). The Mn\(^{IV}\)/Fe\(^{III}\) cofactor is the direct oxidant of the proximal RT pathway residue (W\(_{48}\) or Y\(_{338}\)). Analogously to cofactor assembly in Ec \(\beta\), the reaction to form Ct’s Mn\(^{IV}\)/Fe\(^{III}\) cofactor begins with the reaction of the reduced Mn\(^{II}\)/Fe\(^{II}\) form of the cofactor with O\(_2\) to yield a Mn\(^{IV}\)/Fe\(^{IV}\) intermediate (149). The intermediate is characterized by an intense absorption band centered at 390 nm (\(\varepsilon \sim 4,500 \text{ M}^{-1} \text{ cm}^{-1}\)). Its formation exhibits a first-order dependence on O\(_2\) in an overall bimolecular reaction (\(k_{\text{form}} = 13 \text{ mM}^{-1} \text{ s}^{-1}\) for O\(_2\) at 5 °C) (149). The EPR spectrum reveals that the intermediate has a \(S_{\text{total}} = 1/2\), which is split into six lines due to coupling with one \(^{55}\)Mn nucleus (\(I = 5/2\)) (149). Mössbauer spectroscopy reveals that the Fe site has a low \(\delta\) of 0.17 mm/s, which is consistent with a Fe\(^{IV}\) species. Spectra collected in externally applied magnetic fields reveal that the spin projection factor of the iron site is positive. Taken together, the spectroscopic data reveals that the electronic structure of the intermediate is best described as having a \(S_{\text{total}} = 1/2\) due to AF coupling of a Mn\(^{IV}\) ion (\(S_{\text{Mn}} = 3/2\)) to a high spin Fe\(^{IV}\) ion (\(S_{\text{Fe}} = 2\)) (149). In the absence of \(\alpha\), the intermediate exhibits a protein-mediated intrinsic decay rate constant of 0.02 s\(^{-1}\) at 5 °C (150). Y\(_{222}\), a residue that has no cognate in the class I a RNRs, and W\(_{51}\) (Ec W\(_{48}\)) work to shuttle an electron to the intermediate to reduce the Fe\(^{IV}\) site in an activation-specific electron transfer pathway (blue lines of Figure 11) (150). Site-directed mutagenesis of the protein to replace these residues with redox-inert F results in a 10-fold slower decay of the intermediate, while the use of ascorbate accelerates the decay of the intermediate by 10 – 65-fold (150).

The Mössbauer spectrum of the Mn\(^{IV}\)/Fe\(^{IV}\)-decay product shows that the Fe\(^{IV}\) site is converted to a Fe\(^{III}\) site (\(\delta = 0.52 \text{ mm/s}; \Delta E_Q = 1.32 \text{ mm/s}\)) (30, 149). The sharp quadrupole doublet observed with B = 0 suggests a one-electron reduction of the cluster to give either a diamagnetic cluster, or a paramagnetic cluster with an integer value of \(S_{\text{total}}\). The application of a
a small externally magnetic field (B = 53 mT) leads to significant broadening of the quadrupole doublet, consistent with a species containing a paramagnetic integer-spin ground state. Spectra collected in externally applied magnetic fields demonstrate that the cluster has a $S = 1$ ground state, due to AF coupling of Mn$^{IV}$ and Fe$^{III}$ (29, 30). The conversion of an $S = \frac{1}{2}$ cluster to an $S = 1$ cluster is further confirmed by the loss of the EPR signal associated with the Mn$^{IV}$/Fe$^{IV}$ intermediate (149). The stable Mn$^{IV}$/Fe$^{III}$ cluster accumulates to near-stoichiometric yields, and this afforded its characterization by EXAFS spectroscopy. Samples enriched to ~ 90% of the cluster were investigated by both Mn- and Fe- K-edge EXAFS and shown to have a 2.9 Å intermetallic separation (151). Density functional theory calculations on various models of the cofactor (using the crystallographically characterized, inactive Fe$^{III}$/Fe$^{III}$ form as a starting point for the geometry optimization) revealed that the cluster contains one $\mu$-O and one $\mu$-OH-bridge.
There are two distinct EPR signals that arise from reduction of the Mn$^{IV}$/Fe$^{III}$ form of Ct β. Treatment of the holoenzyme complex ($\alpha$$\beta$•substrate•effector), either with sodium dithionite or with N$\equiv$ADP, results in the formation of a well resolved signal that can be simulated assuming an $S=1/2$ ground state with $g = 2.03$, 2.02, and 2.015; $A_{Mn} = 269$, 392, and 314 MHz; and $A_{Fe} = 64.5$, 64.5, and 64.5 MHz. The small and isotropic tensors for the Fe site are consistent with the presence of high-spin Fe$^{III}$, while the large $A$ tensors and the degree of anisotropy for the Mn is consistent with a Mn$^{III}$ ion. This well-resolved signal is in contrast to the signal emanating from treatment of β alone with sodium dithionite. This signal, also characteristic of a
Mn\textsuperscript{III}/Fe\textsuperscript{III} cluster, is broad and heterogeneous. Its Mössbauer spectrum, acquired at 190 K, reveals a quadrupole doublet with $\delta = 0.43$ mm/s and $\Delta E_Q = 0.81$ mm/s—consistent with high-spin Fe\textsuperscript{III}. The seemingly different forms of Mn\textsuperscript{III}/Fe\textsuperscript{III} observed in Ct RNR has been attributed to different conformations of the enzyme. The binding of substrate to the $\alpha$$\beta$ complex is thought to induce a conformational change to the complex that prepares it for substrate reduction, and thus reduction in this fashion results in a homogeneous signal. One the other hand, the treatment with sodium dithionite, a strong reductant, is not gated by substrate binding. Thus, it effects the reduction to result in multiple conformers of the Mn\textsuperscript{III}/Fe\textsuperscript{III} cluster.

The addition of one equivalent of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), a two-electron oxidant, to the Mn\textsuperscript{II}/Fe\textsuperscript{II} cluster, directly generates the Mn\textsuperscript{III}/Fe\textsuperscript{III} state (152). Further addition of H\textsubscript{2}O\textsubscript{2} yields the Mn\textsuperscript{IV}/Fe\textsuperscript{IV} cluster, which can be reduced by one electron to the active Mn\textsuperscript{IV}/Fe\textsuperscript{III} cluster (152). The remarkable efficiency with which the Mn/Fe cluster reacts with H\textsubscript{2}O\textsubscript{2} suggests that the oxidant might be used to reactivation the cluster if it becomes reduced to the inactive Mn\textsuperscript{III}/Fe\textsuperscript{III} form.

With all of these taken into account, a mechanism of O\textsubscript{2}-activation to yield of Ct RNR’s cofactor has been proposed (summarized in Scheme 2), and is as follows: O\textsubscript{2} adds at a Mn\textsuperscript{II}/Fe\textsuperscript{II} center to yield a Mn\textsuperscript{IV}/Fe\textsuperscript{IV} intermediate. The intermediate receives one electron, mediated by protein amino acids (Y\textsubscript{222} and W\textsubscript{51}), to reduce the Fe\textsuperscript{IV} site to Fe\textsuperscript{III}. The resultant species, a Mn\textsuperscript{IV}/Fe\textsuperscript{III} cluster, is stable and can directly oxidize a RT pathway residue. Functionally, the Mn\textsuperscript{IV} ion replaces the Y\textsubscript{122}. Upon association with $\alpha$, CDP substrate, and ATP effector, the Mn\textsuperscript{IV}/Fe\textsuperscript{III} cofactor oxidizes W\textsubscript{51} or Y\textsubscript{338}, and this initiates the radical translocation into $\alpha$ and eventually initiates substrate reduction, presumably in a manner similar to the mechanism described for Ec RNR.
1-3.3 Inactivation by hydroxyurea: Implications for inter-subunit RT

The mechanism of hole/radical translocation (RT) from Ct β to generate the essential C• in is expected to be largely similar to that proposed for Ec RNR. However, the presence of another functional ET pathway in Ct β raises the question of whether this pathway becomes operant during RT. Early insight into these RT events was obtained by studies with hydroxyurea (HU), a known RNR inhibitor (3, 6). HU works by scavenging the C oxidant. In Ec RNR-β, the Y_{122}• is irreversibly reduced in the presence of HU (3). This reduction is accelerated in the presence of α, substrate, and effector (3). It is thought that the rate acceleration of Y_{122}• decay is due to HU scavenging of the transient RT intermediates that only form in the α•β•substrate•effector complex. Studies of Ct RNR revealed two mechanisms by which HU mediates decay of the Mn^{IV}/Fe^{III} cofactor (153). Treatment of the α•β•substrate•effector complex with HU results the fast formation of a Mn^{III}/Fe^{III} cluster (likely due to HU scavenging the pathway Y•s), followed by a slower phase in which a diamagnetic cluster containing a high-spin Fe^{III} site accumulates (likely due to HU-mediated electron transfer to the buried cofactor). Studies with variant proteins showed that both ET pathways in β are operant during HU inactivation (153).

1-3.4 Location of the Mn ion in the heterodinuclear cofactor

While there have been substantial efforts to understand the mechanisms of cofactor activation and substrate reduction, the assembly of the Mn and Fe cofactor, primarily the location

![Figure 11. Orthogonal electron transfer pathways of Ct RNR. The location of Y_{338}, Y_{991}, Y_{990}, and C_{672} are unknown.](image)
of the Mn ion in the cluster, had not been investigated until the onset of my Ph.D. studies. The heterogeneous nature of the cofactor raises the question of which metal site, 1 or 2, incorporates the Mn\textsuperscript{IV} ion that functionally replaces the Ec β-Y\textsubscript{122}\textsuperscript{•}. Our postulation was that the Mn occupied site 1, primarily because of the site’s proximity to F\textsubscript{127} (cognate of Ec β-Y\textsubscript{122}) and the RT pathway. Moreover, the only metal site architectural change between the class Ia Ec β and class Ic Ct β occurs at site 1, where Ec β-D\textsubscript{84} is replaced with Ct β-D\textsubscript{84} (144). The primary motivation to provide experimental evidence for our Mn site assignment was a study by Noodleman and coworkers. In 2010, these researchers reported that site 2 had a greater affinity for Mn (154). This conclusion was based on calculations that predicted favorable energetics for Mn incorporation at site 2. Using the available structure of the inactive diiron form of the protein, and in comparison with structures of sMMOH and Ec β in different oxidation states, models of Ct β with Mn and Fe were constructed and examined with Mn at site 1 or 2, in the presence and absence of Fe. Of all the oxidation states examined, the one containing the divalent Mn and Fe would provide the most relevant information about the location of the metals, as one would expect them to become locked in their respective sites after adding O\textsubscript{2}. This model showed that the \textsuperscript{1}Fe/\textsuperscript{2}Mn form is energetically favored when Mn is added prior to, or in excess of Fe. The researchers then carried out similar calculations of energetics and spectroscopic parameters in models of the oxidized cluster and reported that models with Fe at site 1 and Mn at site 2 produced results that were closer to the experimentally-determined values.

In order to support or refute the results of the computational study, we set out to provide experimental evidence for the metal occupancy in the active form of the enzyme. Using a combination of X-ray crystallographic anomalous scattering experiments for both Mn and Fe, activity measurements to correlate the location of the Mn to the cofactor’s ability to reduce
ribonucleotides, and EPR spectroscopy to distinguish between and possibly quantify the two likely forms ($^{1}\text{Mn}^{2}\text{Fe}$ vs $^{1}\text{Fe}^{2}\text{Mn}$) of the cofactor, we showed that the incorporation of Mn is largely dependent of the manner of sample preparation. This work, which is a collaboration with the Rosenzweig group, is presented in Appendix A. We began by obtaining two crystal structures of the β reconstituted in vitro with Mn and Fe. For one preparation, in which excess Mn was added to the enzyme, followed by the slow addition of sub-stoichiometric Fe, the Mn anomalous scattering experiments showed a significant presence of Mn at both sites. For another preparation involving rapid addition of Fe in the presence of limiting Mn, the Mn anomalous scattering showed near-exclusive incorporation of Mn at site 1. Mössbauer spectroscopy revealed no differences in the spectroscopic features of the Mn$^{IV}$/Fe$^{III}$ clusters from the two preparations and suggested that all of the Mn in samples from both preparations were coupled to Fe in Mn/Fe clusters. However, activity measurements, on the basis of how much Mn was incorporated into the protein, revealed that the form of the enzyme with near-exclusive incorporation at site 1 exhibited greater activity (per Mn) than the form with Mn incorporated at both sites. The EPR spectra of samples from these two preparations, reduced by sodium dithionite to the EPR-active Mn$^{III}$/Fe$^{III}$ states revealed differences that might be attributed to having only $^{1}\text{Mn}^{2}\text{Fe}$ clusters or a combination of $^{1}\text{Mn}^{2}\text{Fe}$ and $^{1}\text{Fe}^{2}\text{Mn}$ forms of the cofactor. Our conclusion is that in vitro, Mn is not inherently selective for a particular site, and that both $^{1}\text{Mn}^{II}/\text{Fe}^{II}$ and $^{1}\text{Fe}^{II}/\text{Mn}^{II}$ forms of the enzyme may react with O$_2$. However, the form containing predominantly $^{1}\text{Mn}^{2}\text{Fe}$ are more active than those containing both $^{1}\text{Mn}^{2}\text{Fe}$ and $^{1}\text{Fe}^{2}\text{Mn}$, and that the $^{1}\text{Mn}^{2}\text{Fe}$ form may very well be the only active form of Ct RNR.

1-3.5 Detection of RT intermediates in Ct RNR
Substantial insight into the RT events of Ec RNR was obtained by Stubbe and co-workers upon the use of unnatural amino acids incorporated into the enzyme complex. Efforts to insert unnatural amino acid analogues into Ct RNR, which would provide insight into the RT events involving the novel Mn/Fe cofactor, have remained unsuccessful (155). Our approach to investigate the RT events of Ct RNR involves exploiting the natural redox cycles of the Mn/Fe cluster during cofactor formation. The reaction to generate the MnIV/FeIII cofactor proceeds via a MnIV/FeIV intermediate that accumulates to near-stoichiometric yield and is sufficiently stable to permit trapping in manual mixing experiments (149). We envisioned that the intermediate, expected to have a greater reduction potential than the stable cofactor, might work in a manner similar to the NO2-Y122 system of Ec RNR and accumulate radical intermediates.

Indeed, X-band EPR spectra of samples for which the MnIV/FeIV-β is mixed with α, substrate, and allosteric effector show the appearance of a new paramagnetic species with hyperfine structures characteristic of a Y•. The appearance of the Y• suggests that the MnIV/FeIV intermediate has been converted to a MnIV/FeIII-Y• species in a “super-oxidized” α•β complex. Site-directed mutagenesis of the protein coupled to simulation analysis of the EPR spectra acquired for samples of the wild-type and variant proteins provides evidence that the Y•(s) formed resides on the RT pathway. Remarkably, two β variants with engineered RT pathway defects that result in loss of activity with their MnIV/FeIII cofactors are capable of forming Y•s with their MnIV/FeIV states. The W51F variant of β has been shown to slow the decay of the MnIV/FeIV-activation intermediate. It is known that W51 forms part of the activation-specific ET pathway that involves the near-surface Y222 (see Figure 11), with both residues working together to shuttle a single electron to the FeIV site of the MnIV/FeIV intermediate. While β-W51F does eventually assemble the active MnIV/FeIII cofactor, the cofactor is not capable of reducing the
nucleotide substrate. The inability of this variant to function in substrate reduction might indicate that it is also utilized during RT, as opposed to only functioning during cofactor activation. Thus, the ability of this variant to support radical formation with its Mn\textsuperscript{IV}/Fe\textsuperscript{IV} presented a unique opportunity to probe the function of the accumulated radical, and the role (if any) this residue plays in RT. The second variant used to probe the efficiency of the RT pathway radical intermediate is one with a Y→F substitution at position 222, the activation-specific ET residue, and a Y→W substitution at position 338, the proposed subunit interfacial RT residue in β. These two substitutions render the variant incompetent for nucleotide reduction with its Mn\textsuperscript{IV}/Fe\textsuperscript{III} cofactor, but capable of generating a radical from its Mn\textsuperscript{IV}/Fe\textsuperscript{IV} cluster.

The work reported in Appendix B reports the ability of these two variants, in spite of their engineered defects, to use the radicals generated in their “super-oxidized” complexes to effect a single turnover of the substrate.

1-3.6 Kinetics and location of the RT pathway Y•(s) in Ctd RNR

The initial observations on the RT pathway intermediate Y•(s) generated by the “super-oxidized” state of Ctd RNR are somewhat similar to the studies by Stubbe and coworkers on the Ec NO\textsubscript{2}-Y• system, described in 1-2.7 (140-142). The NO\textsubscript{2}-Y, incorporated at β-Y\textsubscript{356} or α-Y\textsubscript{731}, is not oxidized, presumably because its oxidation requires a greater driving force than a normal Y, and hence is not oxidized by Y\textsubscript{122•} (140, 156). However, insertion of the NO\textsubscript{2}-Y at β-Y\textsubscript{122} allows it to be oxidized by the nearby Fe\textsubscript{2}\textsuperscript{IV/III} activation intermediate, X. Once formed, the NO\textsubscript{2}-Y\textsubscript{122•} decays at a rate of 40 s\textsuperscript{-1} in the absence of α, substrate, and effector. However, in the presence of the “ready” complex with β•α•substrate•effector, it exhibits biphasic decay kinetics, with the first phase decaying at ~ 300 s\textsuperscript{-1} and the second phase at 70 s\textsuperscript{-1}. Moreover, a new Y•, presumably at position β-Y\textsubscript{356}, is formed at ~ 100 s\textsuperscript{-1}, the same rate at which dNPD is generated.
Thus, one can envisage that once formed, the NO$_2$-Y$_{122}$$^•$ decays either via a productive pathway when it oxidizes β-W$_{48}$ or β-Y$_{356}$ and begin RT into α, or an via unproductive pathway where it likely oxidizes a protein amino acid residue non-specifically. The much faster rate observed for dNDP formation with the NO$_2$-Y$_{122}$ variant compared to the wt (2 – 10 s$^{-1}$) was explained as likely being due to “ungated” reduction of the NO$_2$-Y$^•$ via the RT pathway. This explanation is supported by the fact that the nitrotyrosinate, not the nitrotyrosine, is observed. The radical of the “super-oxidized” Ct RNR is not as readily reduced as the NO$_2$-Y$_{122}$$^•$ is (since the Ct RNR’s radical is trapped in manual-mixing experiments, as opposed to rapid-quench techniques that are required for trapping the NO$_2$-Y$_{122}$$^•$). Thus, obtaining a full kinetic characterization of the Y$^•$(s) observed would shed light on how these two systems differ in their RT mechanisms.

One key question the kinetic characterization of the Ct RT Y$^•$(s) addresses concerns the role of W$_{51}$ (and by analogy, Ec W$_{48}$). Although shown to be necessary for the activation reaction (the substitution with F slows the formation of the active cofactor in both systems by 10 – 65-fold) (150) there is no evidence for the transient oxidation of this residue during the RT. In Ct, the inability of the β-W$_{51}$F variant to reduce nucleotides with its Mn$^{IV}$/Fe$^{III}$ cluster (150) might suggest that it is required for RT, but the absence of any evidence for oxidation of this residue, even with the “super-oxidized” state and substitutions at Y$_{222}$F and Y$_{338}$F (127) suggests that it isn’t readily oxidized during RT. The transient oxidation of W$_{51}$ is observed during cofactor activation in β, which suggests that the residue is oxidizable. Therefore, it is plausible that the W$_{51}$$^•$ is generated in the wt-β•wt-α or in the β-Y$_{222}$F/Y$_{338}$F•wt-α with use of the more oxidized cluster, but that it exhibits kinetics that do not favor accumulation. Thus, it the wt complex, it decays on the timescale of the manual mixing experiments reported in Appendix B. It is also plausible that the W$_{51}$$^•$ does not accumulate or accumulates only to a small amount, due
unfavorable thermodynamics caused by an equilibrium between the $\beta$-Mn$^{IV}$/Fe$^{IV}$-W$_{51}$ and the Mn$^{IV}$/Fe$^{III}$-W$_{51}$ form of the enzyme. Thus, knowing the kinetics of Y•(s) formation in the wt system and the $\beta$-W$_{51}$F variant is essential for understanding the role, if any, of this residue in RT.

Our efforts to kinetically dissect these systems are presented in Chapter 2. These results show that in the wt $\beta$•$\alpha$ complex, at 25 °C, the Y• forms at a rate of ~ 14 s$^{-1}$. While this rate is lower than that observed in the studies with the NO$_2$-Y Ec RNR, it is still significantly faster than the rate of dNDP formation in the steady state (0.4 – 0.6 s$^{-1}$). Moreover, the Mn$^{IV}$/Fe$^{IV}$ intermediate may not be as potent of an oxidant as the NO$_2$-Y•, and there is no evidence that the gating mechanism is completely subverted in the super-oxidized state of Ct RNR.

The more interesting observation arises upon comparison of the rates of Y• formation in the wt complex and the $\beta$-W$_{51}$F variant. The formation of the Y• in the variant, at 1.8 s$^{-1}$, is ~ 8-fold slower than the wt. The slower rate of Y• formation suggests that W$_{51}$ does play a role in the RT. However, the mild rate enhancement for involving W$_{51}$ could be due to unfavorable thermodynamics that do not favor the oxidation of this residue. Moreover, the location of W$_{51}$ relative to Y$_{338}$ and the other RT pathway residues, which is not known, could affect the kinetics of W$_{51}$ oxidation. Being able to determine the location of the Y•(s) and knowing its distance relative to W$_{51}$ and the Mn$^{IV}$/Fe$^{III}$ cluster will be crucial for understanding the mild rate enhancement afforded by W$_{51}$ during RT.

The location of the Y•(s) generated in the super-oxidized complex is also the subject of work reported in Chapter 2. The wt complex contains three candidates that could harbor the radical: $\beta$-Y$_{338}$, $\alpha$-Y$_{991}$, and $\alpha$-Y$_{990}$. Initial insight into the location of the radical was provided by analysis of the line shape of the EPR spectra and simulations of the Y•s formed. As reported in
Appendix B, the most substantial change to the Y• occurs when \( \beta\)-Y\textsubscript{338} is replaced with F, which causes the Y• to propagate to \( \beta\)-Y\textsubscript{222} of the activation-specific pathway. This change to the observed Y• suggests that 1) the radical resides on Y\textsubscript{338}, or 2) the radical requires the presence of Y\textsubscript{338} in order to get to its host. Site-directed variants of \( \alpha\) (Y\textsubscript{991}F, Y\textsubscript{990}F, C\textsubscript{672}S) are all capable of generating Y•s that are similar in appearance to that of the wt system, yet these Y•s are not competent for substrate H• abstraction of the substrate. It is possible that, analogous to the Ec NO\textsubscript{2}-Y system, a majority of the radical is localized at Y\textsubscript{338}, and so substitutions within \( \alpha\) will have a minimal effect on the observed spectrum. The lack of activity with the \( \alpha\) variants is likely due to the disruption of RT network, which prevents generation of the C3'-H-cleaving C•. If the radical indeed resides at Y\textsubscript{338}, the use of \( \beta\)-Y\textsubscript{222}F•\( \alpha\)-Y\textsubscript{991}F variant proteins would generate a Y• that is identical to that observed in the wt system. This is because the combination of variant proteins ensures that the only RT pathway Y available to be oxidized is Y\textsubscript{338}. Surprisingly, the Y• formed with this system is significantly different than that observed in the wt system. Thus, site directed mutagenesis and EPR spectra analysis alone could not be used to identify the precise location of the Y•.

The next approach taken to identify the location of the Y• involved more manipulation of the possible host(s) to obtain different spectroscopic properties of their X-band EPR spectra. The predominant features of Y•s arise from the hyperfine coupling of the C\( \beta\)-methylene hydrons (157). Replacing the methylene protia with deuteria, which have a smaller gyromagnetic ratio (\( A_{1H}/A_{2H} \sim 6.51\)), would result in a narrowing and loss of hyperfine features of the Y•. Replacing the Ys in \( \beta\) or \( \alpha\) with \(^2\)H-Ys should result in much narrower and less structured Y• spectrum if the Y• resides within the subunit that contains the \(^2\)H-Ys.
Using a Y auxotroph, we prepared wt β labeled with \(^{2}\text{H}\)-Y at the C3 position (3,3-[\(^{2}\text{H}\)]\(_{2}\)-Y), and the EPR spectrum of the Y• formed in the super-oxidized complex is much more narrow than that of the natural abundance Y• spectrum. This confirms that a majority, if not all, of the observed radical resides on Y residue(s) in β. However, the experimental spectrum of the Y• in samples prepared with 3,3-[\(^{2}\text{H}\)]\(_{2}\)-Y-wt β does not match the simulated spectrum of the wt natural abundance Y• with a 6.51× smaller A tensor for the methylene hydrons. The difference reflects the effect of imperfect simulation of the natural abundance Y• spectrum, which is a consequence of overestimating the A tensors arising from the protia at C3 and C5 of the aromatic ring. The need to include unusually small A tensors for this protia can be a consequence of anisotropic linewidth distribution, g-strain effects, multiple conformations of a Y•, or multiple species of Y•s. Better resolution than that provided for Y•s X-band frequency will be required to deconvolute and better simulate the spectra.

1-4 Outlook

1-4.1 The Cl\(_{2}/\text{ClO}_2^−\) method of O\(_2\) generation for the preparation of the peroxo-diiron(III) intermediate in the ferroxidase reaction of the E\(_{\text{i30A}}\) variant of the bacterial ferritin from Desulfovibrio vulgaris

While the utility of the method extends to any receptor for which O\(_2\) is a ligand, we envisioned that it would be used extensively in the bioinorganic community for the capture and characterization of reactive intermediates. Guided by our discovery of the 2.8 Å Fe–Fe separation in samples containing intermediate X (section 1-2.6 and Appendix D), we are now using the method of \textit{in situ} generation of O\(_2\) from Cl\(_{2}/\text{ClO}_2^−\) to prepare several peroxo-diiron(III) complexes, which we will
attempt to characterize by relatively insensitive spectroscopic techniques like EXAFS spectroscopy.

Ferritins (Ftns) can be found nearly everywhere in nature. Their primary purpose is to concentrate and store Fe for its use as a mineral (65, 66). The initial step in Ftn’s reaction is the rapid oxidation of \( \text{Fe}^{II/II} \) by \( \text{O}_2 \) in a ferroxidase reaction. The reaction proceeds via a peroxo-diiron(III) intermediate (Ftn-P) that decays to multiple \( \text{Fe}^{III} \) species (65, 101). Ftns in vertebrates carry out this reaction at a carboxylate-bridge dinuclear site (hence the term ferritin-like dimetal enzymes (67) that has been used to describe the β subunit of class I RNRs and the hydroxylase protein of methane monooxygenase, amongst others) (65).

We became interested in interrogating the Fe-Fe distance in the Ftn-P because of the EXAFS characterization carried out by Hwang et al. (111). Like RNR-P and X, the intermediate was reported to contain a short Fe-Fe separation, at 2.5 Å. This short distance required modeling Ftn-P with at least two single-atom oxygen bridges (Figure 12). Because the similarly short structural feature of RNR-X now appears to be an artifact caused by the rather low concentration and the heterogeneity in the samples of RNR-X, we suspect that Ftn-P may also have a longer Fe-Fe separation.

While Ftns from vertebrates (including the frog M Ftn) activate \( \text{O}_2 \) at a diiron site (101), recent results have suggested that bacterial Ftns contain a trinuclear Fe-site. The Ftn from *Disulfovibrio vulgaris* (Dv) was confirmed to contain such a site (158), but the substitution of E130, a ligand exclusive to site 1, with alanine (A), greatly diminishes the affinity of that site for metal and essentially converts the enzyme into a dinuclear Fe enzyme (158). The ligand substitution also results in near-stoichiometric accumulation of a peroxo-diiron(III) intermediate.
in the ferroxidase reaction (159). This makes Dv Ftn-P an attractive system for the re-investigation of Ftn-P.

We used the Cld/ClO$_2^-$ method to prepare Dv Ftn-P in a significant amount (~ 65 % at 1.3 mM) that would afford its characterization by EXAFS spectroscopy. This study is ongoing.

4.2 The Cld/ClO$_2^-$ method of O$_2$ generation for the accumulation of the ferryl intermediate in the reaction of wild-type hydroxyethylphosphonate dioxygenase

Hydroxyethylphosphonate dioxygenase (HEPD) catalyzes the conversion of 2-hydroxyethylphosphonate (2-HEP) to hydroxymethylphosphonate (HMP) and formate (160). It is a mononuclear non-heme Fe enzyme that is ligated by two histidines and one glutamate in what is referred to as a 2-His-1-carboxylate facial triad commonly found in mononuclear Fe enzymes (161), some of which also utilize α-ketoglutarate as a co-substrate (162-165). Seminal work by the Bollinger/Krebs group captured and characterized the first reactive intermediate, an Fe$^{IV}$-oxo (ferryl), in one of these enzymes (162), which are all thought to operate via a consensus mechanism. Since then, the primary approach to capture and characterization of reactive intermediates in this family of enzymes has been isotopic substitution of hydrons that are cleaved with deuterons (D). The heavier isotope may exhibit an isotope effect (typically a slower rate of C–D cleavage) on the chemical step of the reaction, which could result in greater accumulation of the reactive species that would normally effect C–H cleavage. Sometimes coupled with isotopic substitution
is site-directed mutagenesis to the enzyme to enhance binding events, which could result in faster formation of the C–H cleaving species (99).

HEPD is intriguing because it utilizes only Fe$^{II}$ and O$_2$ to effect the cleavage of the C1–C2 bond of 2-HEP. Our collaborative work with the van der Donk laboratory using [2-$^2$H$_2$]-HEP and the E$_{176}$A variant of HEPD suggests a reaction mechanism in which the bidentate coordination of HEP to the Fe$^{II}$ center is followed by addition of O$_2$ to yield a Fe$^{III}$-superoxo complex (Scheme 3, I). This complex abstracts the pro-$S$ H• from C2 of 2-HEP (II). The resultant substrate radical (C2•) is hydroxylated to generate a ferryl (III), which then abstracts the H• from the recently-transferred OH group to from a gem-diaryl radical (IV) that can undergo $\beta$-scission, generating a radical on the methyl phosphonate and a ferric hydroxide species (V), both of which recombine to yield HMP and formate bound to the Fe$^{II}$ center (VI). This unusual mechanism is supported by the accumulation of a ferryl complex (identified by Mössbauer spectroscopy) for which there is a deuterium kinetic isotope effect on formation and decay (166). A deuterium isotope effect on the formation of the ferryl can only be explained by a mechanism that invokes H• abstraction from C2 by a species that precedes formation of the ferryl. An isotope effect on the decay of the ferryl dictates that it effects H• abstraction. The observation of kinetic isotope effects, both on formation and decay, and even with only the pro-$S$-H replaced with deuterium, suggests that the same H• is abstracted twice: first by the species preceding the ferryl (a putative Fe$^{III}$-superoxo), and again by the ferryl. Thus, the unusual mechanism is the only tenable rationalization of the data.

The reaction of wt HEPD with O$_2$ saturated buffer does not accumulate any transient species at the metal, probably due to an unfavorable on-rate of O$_2$ to the Fe$^{II}$ ion. Such unfavorable kinetics reflect a high K$_d$ for O$_2$, which can be overcome by increasing the
concentration of O₂. The ability to generate O₂ at much greater concentrations using the Cld/ClO₂⁻ method allowed us to observe the ferryl in wt HEPD and show that the mechanism proposed for the reaction of the E176A variant is also operant in wt.

The intermediate proposed to precede the ferryl is the Fe^{III}-superoxo species, which is yet to be observed. It is feasible that this intermediate forms in a disfavored equilibrium with O₂, as is the case with the superoxo-diiron(III) intermediate in myo-inositol oxygenase (vide infra). Increasing the concentration of O₂ coupled faster mixing and quenching (167) will be essential for the capture of this elusive intermediate.

1-4.3 The Cld/ClO₂⁻ method of O₂ generation for the preparation of the Fe²^{III/III}-superoxo intermediate, G, in the reaction of myo-inositol oxygenase

The enzyme myo-inositol oxygenase (MIOX) contains a mixed-valent diiron cluster (168, 169) that it utilizes to convert its substrate, cyclohexan-(1,2,3,5/4,6)-hexa-ol (or myo-inositol, MI) to D-glucuronate. The Bollinger/Krebs group in 2006 reported that MIOX adds O₂ at the Fe²^{II/III} cofactor to yield a superoxo-diiron(III) intermediate (170), termed G, that abstracts a H• from C1 of MI. G was the first enzymatic C–H cleaving superoxo-Fe intermediate to be observed and characterized. The H• abstraction from C1 of MI cascades into a series of events that culminates with the cleavage of the C1–C6 bond of MI.

Intermediate G forms in a reversible, disfavored equilibrium with the MIOX Fe²^{II/III}•MI complex (170). At 1 mM O₂ and 5 °C, G is formed at 95 s⁻¹, but decays via the reductive elimination of O₂ at 40 s⁻¹, and through the formation of the next intermediate in the reaction
sequence at 48 s\(^{-1}\). The two rate constants for the decay of \(G\) sum up to the rate for its formation at 1 mM O\(_2\). This limits the amount of \(G\) that can accumulate to \(\sim 50\%\), thus precluding its structural characterization by other spectroscopic methods that require greater purity.

Our work in **Appendix C** reported the accumulation of \(G\) at twice the purity and concentration possible with the use of O\(_2\) saturated buffer. We envisioned the ability to enrich \(G\) to greater extents would permit its characterization by other spectroscopic methods. I first prepared \(G\) via the Cld/ClO\(_2\)- method, for Mössbauer spectroscopic analysis, and determined that its 4.2 K/53 mT spectra acquired with the magnet parallel and perpendicular to the \(\gamma\)-beam are broad and difficult to simulate in the presence of residual Fe\(_{\text{II/III}}\).MI, which is also paramagnetic and broad. We hoped that obtaining the \(^{57}\text{Fe}\ \text{A}\) tensors via pulsed EPR spectroscopy would aid with the Mössbauer simulation (as was done during the characterization of \(Ec\ \text{RNR-X}\)). I have again prepared \(G\), and together with Dr. Maria-Eirini Pandelia, a post-doctoral scholar in the Bollinger and Krebs research group, have acquired the first \(^1\text{H}\)- and \(^{57}\text{Fe}\)-ENDOR, ESEEM, and HYSCORE spectra of \(G\), and the effort to obtain a complete structural characterization of \(G\) is ongoing.

1-4.4 Exploring the factors that control the proper assembly of Mn/Fe cofactor of \(Ct\ \text{RNR-\beta}\)

**Figure 13.** The metal-binding sites of \(Ct\ \beta\). Residues that serve as ligands to the metals are shown in ball-and-stick format. **A** depicts the sites in the absence of metals, while **B** depicts the sites with Mn\(^{\text{II}}\). The structure in **B** was acquired by co-crystallizing apo \(Ct\ \beta\) with Mn\(^{\text{II}}\). A Mn-specific anomalous difference map is shown in the purple mesh and the occupancy of Mn in each site ranges from 40 – 70\%, with a slight preference for site 2.
Our work reported in Appendix A demonstrated that the method by which the cofactor is assembled \textit{in vitro} determines the location of the Mn ion. We have begun to explore the factors in the reconstitution procedure that actually control the location of the metals by preparing the enzyme under different conditions and obtaining their crystal structures.

The 1.8 Å structure (Figure 13A) of metal-free (or apo) \textit{Ct} β, when compared to the reported oxidized structures, reveals there are not many changes to the ligands that normally coordinate the metals (144, 171, 172). There is, however, extra difference density close to the metal sites, which is suggestive of the presence of a disordered water molecule. Structures acquired either by soaking Mn$^{II}$ into the crystals of apo-β (not shown) or by co-crystallizing β with Mn$^{II}$ (Figure 13B) reveal that Mn is nearly equally distributed between the two sites. The most significant site architecture change is to the binding mode of E$_{227}$, which bridges the metals in a $\mu$-$\eta^1,\eta^2$ fashion instead of the monodentate binding to site 2 observed in the oxidized structures. Pre-mixing the apo β with 1 equiv of Mn$^{II}$ and adding 1 equiv of Fe$^{II}$ prior to

![Figure 14. Structures of the Mn and Fe metal-binding sites of Ct β.](image)
crystallization reveals the Mn exclusively occupies site 1, and Fe exclusively occupies site 2 (Figure 14A). In parallel to the crystallographic studies, we observed that adding pre-mixed Mn$^{II}$ and Fe$^{II}$ to apo-β prior to the introduction of O$_2$ results in greater metal occupancy and activity per β. The EPR spectra of these samples closely resemble the spectrum we previously reported to be diagnostic of Mn occupying site 1 and Fe occupying site 2. Moreover, the anomalous density acquired from the crystals of these samples (Figure 14B) confirms that Mn exclusively occupies site 1, and Fe site 2. Understanding the apparent selectivity that is conferred when the divalent metals compete for their respective sites will be crucial for understanding how the class Ic RNRs (and other enzymes utilizing heterodinuclear cofactors) assemble their cofactors while preventing mis-metallation.

1-4.5 Exploring RT in Ctn RNR with a diiron cluster

The close analogy of our RT Y• intermediates generated with the super-oxidized Mn$^{IV}$/Fe$^{IV}$ cluster of Ctn RNR to that of the Ec NO$_2$-Y$_{122}$ motivated our investigation into whether the diiron form of Ctn RNR could be used to generate RT Y•s. As described earlier, the reaction of Ctn Fe$_2^{II/II}$-β with O$_2$ yields a Fe$_2^{III/IV}$ intermediate (X$_C$) that is nearly identical to X generated in the Ec RNR.

Figure 15. X-band EPR spectra of Ctn RNR samples prepared with Fe. The left panel show spectra acquired over a wider range of the magnetic field, while the spectra shown in the right panel were acquired over a narrow range of the magnetic field to resolve the $g$=2 region. Samples were prepared from the reaction of the Fe$_2^{II/II}$ forms of β-wt (black spectra), Y$_{222}$F (red spectra), and W$_{51}$F (blue spectra) with O$_2$, α, substrate (CDP), allosteric effector (ATP). These conditions were selected to mimic the “super-oxidized” state of Ctn RNR formed with the Mn/Fe cofactor. Concentrations of reaction components and spectrometer conditions are identical to those described in Appendix B.
activation reaction (146). For the latter system, Stubbe and coworkers have reported that X is sufficiently potent to oxidize NO₂-Y₁₂₂, which then goes on to generate RT Y•s in the presence of α, substrate, and effector (141). Although Ct RNR is inactive with the diiron cluster, we have examined whether X₇ is sufficiently potent to generate RT pathway Y•s, and if these Y•s would be competent for a single H• abstraction event.

Very preliminary results show that the addition of O₂-buffer to Fe²⁺/²⁺-β wt•α•substrate•effector generates an EPR-active g=2 signal. This signal is not completely isotropic (see gold arrow in Figure 15), as would be the case if only X₇ was formed. Also, it does not appear to have significant Y• character. The spectra of the Fe²⁺/²⁺-β-Y₂₂₂F•α•substrate•effector and Fe²⁺/²⁺-β-W₅₁F•α•substrate•effector complexes were also examined. The former is similar to wt, but the latter displays the partially resolved hyperfine couplings reminiscent of a Y•. These were all then prepared in the presence of the N₃-UDP substrate analogue (Figure 16), and none formed the N• that is expected after a single H• abstraction event. While the details of the system are not very clear at the moment, the ability to form X₇, and its inability to oxidize a RT pathway Y and effect H• abstraction from the substrate may highlight a key difference in the thermodynamics of RT in Ct RNR when compared with that of Ec RNR.
Figure 16. X-band EPR spectra of Ct RNR samples prepared with N3-UDP. The samples were prepared as described for Figure 15, but with a mechanism-based inactivator substrate analogue (N3-UDP) used instead of CDP. Concentrations of reaction components and spectrometer conditions are identical to those described in Appendix B.
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Chapter 2:

Kinetics and Location of Radical-Translocation Tyrosyl Radical Intermediate(s) Generated with “Super-Oxidized” (Mn$^{IV}$/Fe$^{IV}$) Chlamydia trachomatis Ribonucleotide Reductase
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ABSTRACT

A class I ribonucleotide reductase (RNR) utilizes a tyrosyl radical (Y•) or MnIV/FeIII cluster in its β subunit to oxidize a cysteine residue ~ 35 Å away in its α subunit, generating a thyl radical (C•) that initiates turnover. C• generation is a reversible “radical-translocation” (RT) process, in which several tyrosine (Y) residues and perhaps one tryptophan (W) residue along a specific pathway that spans the subunits are transiently oxidized to radicals to relay the oxidizing equivalent. For the *Escherichia coli* (Ec) class Ia RNR, the RT intermediates were observed by the use of unnatural amino acids inserted in place of the Y• cofactor or the RT pathway Y residues. Recently, we provided the first evidence for RT-pathway tyrosyl radicals (Y•s) in the class Ic RNR from *Chlamydia trachomatis* (Ct) by using its meta-stable MnIV/FeIV cofactor-assembly intermediate to initiate nucleotide reduction in a “super-oxidized” β•α complex (Dassama, L. M. K.; Jiang, W.; Varano, P. T.; Pandelia, M.-E.; Conner, D. A.; Xie, J.; Bollinger, J. M., Jr.; Krebs, C.; *J. Am. Chem. Soc.* 2012, 134, 20498-20506). Herein we address the location of the radical(s) by incorporating 3,3-[2H2]-tyrosine throughout the β subunit and examining the effect of the substitution on the EPR spectra of its super-oxidized complex with the wild-type α protein. Analysis of the markedly narrower Y• EPR spectra reveals that the radical resides predominately in β, with Y338 the major site and possible minor delocalization onto Y222. We further report the kinetics of Y• formation and decay in the “super-oxidized” wild type β•α and β-W51F•α complexes. The formation of Y• in the latter complex requires that the oxidizing equivalent departing the metal cluster “skip” W51 to enter the intact portion of the RT pathway and initiate the reduction of substrate bound in α. Our results indicate that there is a kinetic penalty for skipping W51, which suggests the participation of this residue in the normal RT process, by contrast to what has been proposed for the archetypal class Ia RNR from Ec.
INTRODUCTION

Ribonucleotide reductases (RNRs) catalyze the conversion of ribonucleotides to deoxyribonucleotides as part of the only known *de novo* synthetic pathway to the four building blocks of DNA (1, 2). All RNRs harness free radical chemistry for catalysis, generating a transient cysteine thyl radical (C•) (3) that initiates substrate reduction when it abstracts a hydrogen atom (H•) from the 3'-carbon of the substrate (4, 5). The C• is unstable in all RNRs identified thus far, and thus is generated *in situ* by a more stable but still potent oxidant. The nature of this stable, potent oxidant is the basis for the division of RNRs into classes I-III (1, 2).

A class I RNR comprises two protein subunits (α and β). Each subunit is a stable homodimer (α₂ and β₂), and the homodimers assemble into a transient heterotetramer (α₂β₂) in the active state (1, 2, 6-9). The α subunit contains the active site for substrate reduction and the H•-abstracting C residue (9, 10). The β subunit generates and stores the stable oxidant (a metallocofactor) (6, 11) required to generate the C• (12). A class Ia RNR, such as that found in aerobically growing *Escherichia coli* (*Ec*), assembles its metallocofactor, a μ-oxo-diferric/tyrosyl radical (Fe²⁺/³⁺/Y•) cofactor (11, 13, 14), in an auto-activation reaction of the Fe²⁺/³⁺ form of the β protein with O₂ (13). The Y• of the cofactor is generated from Y₁₂₂, ~ 5 Å from the μ-oxo-Fe²⁺/³⁺ cluster. For class Ic RNRs, such as the one found in *Chlamydia trachomatis* (*Ct*), the metallocofactor is a Mn⁴⁺/Fe³⁺ cluster (15, 16), which also assembles in an auto-activation reaction of the protein’s reduced (Mn²⁺/Fe³⁺) form with O₂ (15-18).

In production of the C•, an oxidizing equivalent must travel from the cofactor in β to the C residue in α over a distance of ~ 35 Å (see Scheme 1) (6, 9, 19). This "radical translocation" (RT) process does not occur by simple electron transfer from the C residue to the cofactor. Instead, several aromatic amino acids [three tyrosines (Ys) and perhaps a tryptophan (W)] are
thought to participate in a redox-relay or electron-hopping mechanism. The presumptive Y (W) radical intermediates in the RT process do not accumulate during catalysis, because a conformational change by the protein that precedes RT is slow and subsequent events, including reduction of the substrate and the reverse RT step that culminates each turnover, are fast (6, 20). Thus, only the stable Y\textsubscript{122}\textsuperscript{•} or Mn\textsuperscript{IV}/Fe\textsuperscript{III}-containing forms of the cofactors are present during turnover. Evidence suggests that binding of the substrate triggers the gate-opening conformational change that permits the oxidizing equivalent in β to propagate into the RT pathway toward the site of nucleotide reduction in α.

In recent years, Stubbe and coworkers have provided evidence for the transient oxidation of Ys during the RT process by elegantly altering the redox landscape of the pathway in the class Ia RNR from Ec. By substituting one of the pathway Y residues (β-Y\textsubscript{356}, α-Y\textsubscript{731}, or α-Y\textsubscript{730}) with either 3,4-dihydroxyphenylalanine (DOPA) (21, 22) or 3-aminotyrosine (NH\textsubscript{2}-Y) (23-28), which both have diminished reduction potential in their radical forms relative to Y, they introduced a depression in the RT pathway that permitted accumulation of a radical residing on the Y analogue. More recently, the introduction of 3-nitrotyrosine (NO\textsubscript{2}-Y) (29, 30), an analogue with an elevated reduction potential in its radical form relative to Y, at position 122 led to the formation of a NO\textsubscript{2}-Y\textsubscript{122}\textsuperscript{•} in the activation reaction. In the holoenzyme complex, the NO\textsubscript{2}-Y\textsubscript{122}\textsuperscript{•} rapidly oxidizes the proximal RT residue (β-Y\textsubscript{356}). The NO\textsubscript{2}-Y\textsubscript{122}\textsuperscript{•} exhibited biphasic decay to the nitrotyrosinate at 280 s\textsuperscript{-1} and 70 s\textsuperscript{-1}. The formation of the nitrotyrosinate is followed by the formation of dCDP (107 s\textsuperscript{-1}) and a Y\textsuperscript{•} (97 s\textsuperscript{-1}) (29). The fast rates of NO\textsubscript{2}-Y\textsubscript{122}\textsuperscript{•} reduction suggest that the “gating” mechanism for Y\textsubscript{122}\textsuperscript{•} reduction, which is suspected to involve a proton transfer from the water ligand on \textsuperscript{1}Fe of the \textsuperscript{4}Fe\textsuperscript{III}/\textsuperscript{2}Fe\textsuperscript{III} cluster to Y\textsubscript{122}\textsuperscript{•} (6, 31), is deranged in the NO\textsubscript{2}-Y\textsubscript{122} variant. The new Y\textsuperscript{•} that is formed is thought to be the result of a turnover event in which
the very last step, reoxidation of the residue at position 122 to regenerate the resting-state cofactor, is blocked because the pathway Y_{356} is not a sufficiently potent oxidant to regenerate the NO2-Y_{122}. Indeed, pulsed electron double resonance (PELDOR) spectroscopy confirmed that the Y• produced during the single turnover with the variant β subunit resides primarily on Y_{356}, the subunit interfacial RT residue, with some minor (~10%) contribution from the α-Y_{731} and α-Y_{730} radicals (32).

Very recently we reported the unmasking of RT events in the class Ic RNR from Ct RNR (33). Using the Mn^{IV}/Fe^{IV} activation intermediate (34), we were able to generate transient Y•(s) in a “super-oxidized” Mn^{IV}/Fe^{III}/Y•-containing β-α complex. The formation of the Y• in this system is dependent on the presence of substrate and potentiated by the allosteric effector. We assigned the Y• to the RT pathway on the basis of the examination of the corresponding complexes of site-directed variants of β by EPR spectroscopy.

One question left unanswered by our J. Am. Chem. Soc. study concerns the location of the Y• generated in the wt “super-oxidized” complex. Site-directed mutagenesis of the lone RT Y residue (Y_{338}) in β did lead to a change in the line shape of the spectrum of the resultant Y•. This change in line shape required the use of different parameters (A and g tensors) to simulate the spectrum of the Y•. The F substitution at Y_{338}, when coupled with an F substitution at Y_{222}, the ET pathway residue specific to cofactor activation (see Scheme 1), completely abolished formation of the Y•. This confirmed that the Y• generated in the wt complex is confined to one of the RT pathway residues: Y_{338}, Y_{991}, Y_{990}, or perhaps distributed amongst them.

In this work we report on the location of the Y• formed in the wt “super-oxidized” complex. Using site-directed variants of β and α, coupled with deuterium isotope labeling of all
Ys in β, we have determined that a majority of the Y• giving rise to the spectrum observed with the wt complex resides in β.

One remarkable finding of our study on the Y• intermediate(s) generated by the “super-oxidized” Mn^{IV}/Fe^{IV} of Ct RNR was the ability of the β-W_{51}F variant, which is devoid of activity in its normal Mn^{IV}/Fe^{III} state (35), to generate pathway Y• and support a single turnover in its super-oxidized state. The formation of a RT pathway Y• in the W_{51}F variant raised the question as to what role, if any, W_{51} plays in RT. It has been established that the W residue shuttles an electron to the metal cluster during activation of both the Ct and Ec β proteins. In Ct β, the substitution of this residue with F results in a ten-fold slower formation of the Mn^{IV}/Fe^{III} cofactor (35). In Ec β, the electron donated by W_{48} is thought to promote cleavage of the O–O bond of a peroxo-Fe_{2}^{III/III} intermediate to generate the Fe_{2}^{III/IV} cluster, X, and the substitution of this residue causes the peroxo-Fe_{2}^{III/III} intermediate to oxidize Y_{122} directly, resulting in a state containing both Y_{122•} and X (36). However, no such definitive evidence has been provided for the oxidation of W_{51/48} during catalysis. While the inability of the W_{51}F variant to reduce ribonucleotides with its Mn^{IV}/Fe^{III} cofactor might suggest that the residue is necessary for RT, the lack of evidence for its transient oxidation, even in the “super-oxidized” complex of the β variant with both Y_{222} and Y_{338} substituted by redox inert F residues (which leaves W_{51} as the only oxidizable residue on the RT pathway), suggests that it is not readily oxidized. However, it is possible that the W_{51•} does not accumulate to a detectable level because of unfavorable thermodynamics for its oxidation by the Mn^{IV}/Fe^{IV} cluster.

One approach to assessing whether W_{51} plays an active electron-shuttling role in the RT process is to determine whether or not there is a kinetic penalty to the RT when the residue is absent. Herein we use freeze-quench EPR spectroscopy to define the kinetics of formation and
decay of the Y• in the “super-oxidized” Ct RNR complexes of the wild type (wt) and W51F β subunits. An eight-fold diminished rate of Y• formation in the W51F complex could be consistent with an active shuttling role for this residue.

EXPERIMENTAL PROCEDURES

Materials. 2-Methylbutane (iso-pentane), CDP, dCDP, ATP, DTT, and MgSO4 were purchased from Sigma-Aldrich (St. Louis, MO). A tyrosine auxotroph (CGSC Strain # 5974, WU-36) of Ec was acquired from the E. coli Genetic Stock Center at Yale University. Plasmid (pAR1219, Product # T2076) containing the gene for T7 RNA polymerase was purchased from Sigma-Aldridge (St. Louis, MO). 3H-CDP (labeled at the methylene carbon, or C3) was purchased from Cambridge Isotopes (Andover, MA). (5-3H)-CDP was acquired from ViTrax (Placentia, CA).

Over-expression and purification of proteins. Procedures for over-expressing RNR subunits in Ec strain BL-21(DE3) cultured in rich medium and purifying the proteins have been described (15, 35). For expression of Ct β in the auxotroph, the WU-36 cells were made competent by the method of Inoue (37). The competent cells were transformed with plasmids encoding the genes for both Ct β (pET-28a, conferring kanamycin resistance) and T7 RNA polymerase (pAR1219, conferring ampicillin resistance) and transferred to LB-agar plates supplemented with 50 mg/L of ampicillin and kanamycin. A single colony was grown in M9 minimal medium, prepared as previously described (38) and supplemented with 40 mg/L tyrosine. Expression of the RNA polymerase and Ct β was induced by the addition of IPTG to the medium to a concentration of 0.17 mM. The protein was purified as previously described (15).

Preparation of freeze-quench EPR samples. All samples were prepared by using an Update Instruments quenched flow apparatus by a sequential-mixing protocol setup that has been
described (39). 0.75 equiv of Mn$^{II}$ and Fe$^{II}$ were added to an O$_2$-free solution of metal-free (apo) Ct $\beta$, and the solution was loaded into the air-tight syringe of the quenched-flow instrument. This solution was mixed with 1 equiv volume of O$_2$-saturated 100 mM HEPES, 10% v/v glycerol, pH 7.6 to form the Mn$^{IV}$/Fe$^{IV}$ intermediate. After a reaction time of 2 s, this solution was mixed with 1 equiv volume of a solution containing $\alpha$, CDP, ATP, MgSO$_4$, and DTT. The reaction was allowed to proceed for the time indicated in the figure legends before being sprayed into cold iso-pentane (~ 150 °C).

**Preparation of manually-mixed EPR samples.** All samples were prepared as previously described (33).

**EPR spectroscopy and simulation.** The X-band EPR spectrometer was previously described, with the exception that a Hewlett-Packard 5350B Microwave Frequency Counter was used during data acquisition. The collection and analysis of data (including simulation analysis) has also been described (33).

**Fitting and simulation of kinetic data.** The intensity of the EPR spectrum was extracted at 336.35 mT for each sample. The intensity plotted as a function of time was fit by the equation

$$[I]_t = [R]_0[(k_1/(k_2 - k_1))(\exp(-k_1t) - \exp(-k_2t))],$$

which gives the concentration of an intermediate species, I, in a two-step R $\rightarrow$ I $\rightarrow$ P reaction sequence, where $k_1$ and $k_2$ are the effective first-order rates constants for the formation and decay of I, respectively, and $[R]_0$ is the concentration of the reactant at time zero. Simulations were carried out using KinTek Explorer (KinTek Corporation, USA).

**RESULTS**
Tracking the location(s) of the Y•(s) using variants of α. Analysis of the EPR spectra of super-oxidized holoenzyme complexes prepared with site-directed variants of β was undertaken to establish that the Y•(s) generated reside(s) on the RT pathway, either at Y$_{338}$ or requiring Y$_{338}$ (or another oxidizable residue, such as a W, at position 338) to hop to its site of residence. Furthermore, assessment of the ability of the β-W$_{51}$F and β-Y$_{222}$F/Y$_{338}$W variants to support nucleotide reduction was essential for establishing the residence of the Y• within the RT pathway intermediate. Each of the proposed RT-pathway residues of α (C$_{672}$, Y$_{990}$, and Y$_{991}$) was substituted with a non-oxidizable residue (Y→F or C→S). In all cases, spectra attributable to one or more Y• was observed, and accumulation of the Y• was seen to depend on the presence of substrate. The spectra have line shapes similar to that observed with the wt α (Figure 1A). This similarity suggests that substitutions of RT-pathway residues in α does not impact the location(s) of the Y•(s), consistent with the hypothesis that the Y• resides primarily on β-Y$_{338}$. Although the α variants can form Y•s similar to that observed in the wt complex, none of the Y•s support nucleotide reduction (Figure 1B).

Tracking the location(s) of the Y•(s) using variants of β and α. The spectra in Figure 1A suggest that the primary location of the radical is in β, as none of the engineered disruptions to the RT pathway has a significant effect on the line shape of the Y• spectrum. In light of this conclusion, we anticipated that use of the α-Y$_{991}$F and β-Y$_{222}$F variants should confine the Y• to Y$_{338}$, the only Y on the RT pathway within β. Surprisingly, the spectrum of samples prepared using these two variants is significantly different from those observed in wt and any variant of α or β (Figure 2). Formation of this distinct putative Y• still requires the presence of the substrate, suggesting that it could still be "on pathway." Samples prepared using α-Y$_{990}$F with β-Y$_{222}$F, which we anticipated to confine the oxidizing equivalent to two residues (β-Y$_{338}$ and α-Y$_{991}$),
exhibited spectra much more similar to those observed with the wt complex (Figure 2). The significant change to the line shape of the Y• when it is confined to β-Y338, much more than when there are other RT Y residues available for oxidation, suggests that the Y• observed in the wt complex may not reside exclusively on β-Y338.

**Tracking the location(s) of the Y•(s) using 3,3-[2H]2-Y in β.** To resolve the ambiguity arising from our tracking experiments employing site-directed variants, we exploited the known, geometric property provided by hydrons on the methylene carbon, C3, of the tyrosyl radical (i.e., the dependence of the 1,2H hyperfine coupling interactions of the hydrons on their rotational angles with respect to the delocalized unpaired electron). For the X-band EPR spectra of Y•s, the hyperfine interactions are the primary determinant of line shapes and line intensities relative to the centroid of the spectrum (40). Indeed, the spectrum of the Y• that accumulates in the wt “super-oxidized” complex can be well simulated (see Table S1) with A tensors [31.3, 27.3, 24 MHz] and [16.0, 15.0, 15.0 MHz] for the methylene (C3) protia, respectively, while using A tensors provided for protia on the ring that are close to values published for Y•s observed proteins (40). The substitution of these protia with deuteria should result in smaller A tensors due to the smaller gyromagnetic ratio of deuterium compared with protium (A_{1H}/A_{2H} = 6.51). Thus, replacement of all of the Ys in the protein with 3,3-[2H]2-Ys should result in a narrowing of the EPR spectrum of the Y• as a consequence of the smaller A tensors for the C3 protia.

Because the most pronounced changes to the line shapes of the Y• spectra in the “super-oxidized” complexes of Ct RNR are observed in samples of site-directed variants of β, we introduced 2H-Ys into β by expressing the protein in a tyrosine auxotroph grown in minimal medium supplemented with 3,3-[2H]2-Y. In the absence of added Y, the auxotroph does not grow in minimal medium. By contrast, supplementation of the medium by either natural abundance Y
or 3,3-[\textsuperscript{2}H\textsubscript{2}]-Y permits cell growth and production of the β protein. The protein produced in medium supplemented with 3,3-[\textsuperscript{2}H\textsubscript{2}]-Y were combined with α prepared by the conventional method, and the Y• generated by the reaction of “super-oxidized” 3,3-[\textsuperscript{2}H\textsubscript{2}]-Y-β with α, substrate, and effector, was characterized by EPR spectroscopy. The spectrum (Figure 3, spectrum ii) is markedly different and significantly narrowed, demonstrating loss of proton-hyperfine structure. This result suggests that the Y• resides within β. Samples prepared from the reaction of “super-oxidized” 3,3-[\textsuperscript{2}H\textsubscript{2}]-Y-β with α -Y\textsubscript{991}F, substrate, and effector, which effectively prevents RT into the α subunit, were analyzed, and the representative spectrum of the Y• generated (Figure 3, spectrum iv) is not significantly different from that of the Y• in the wt complex (compare ii and iv). This result shows that the Y• accumulates in β, even in the wt β•α complex.

The spectrum of the 3,3-[\textsuperscript{2}H\textsubscript{2}]-Y• can be simulated by using the theoretical spectrum of the natural abundance Y• and scaling the A tensors of the C3 protia by the protium/deuterium gyromagnetic ratio difference. The theoretical spectrum generated by doing so fits the experimental spectrum, for the most part. However, there are regions where the theoretical spectrum does not match the experimental spectrum, particularly on at the high- and low-field regions of the spectrum (see Figure S1 for the non-zero difference spectrum). This mismatch of the theoretical spectrum reflects an overestimation of the A tensors arising from the protia at positions 3 and 5 of the phenol ring of the Y•. Our simulation analysis of the natural abundance Y• restricted the A tensors from these protia to the published values (40). As a result, the overestimation of A tensors was compensated for by the introduction of an additional line broadening component. This overestimation, not apparent in the spectrum of the natural abundance Y•, can be a consequence of one or a combination of the following: anisotropic
linewidth distribution, g-strain effects, multiple conformations of the Y•, and the presence of multiple Y•s.

**Kinetics of the Y• formed with “super-oxidized” β-wt and β-W51F.** We previously established that the Y• forms in only ½ of the α2β2 complex due to half-of-sites reactivity (33). Thus, in a majority of the spectra collected, the signal of residual MnIV/FeIV intermediate is observed. This signal is partially resolved from that of the Y•, which allows accurate subtraction of its contribution to reveal and quantify the signal of the Y•. The EPR spectra of samples prepared by pre-forming the MnIV/FeIV to its maximal accumulation and then mixing with α, CDP, ATP, DTT, and MgSO4 at 25 °C show that the Y• in the wt α•β complex forms at a rate of 14 s⁻¹ and decays at 0.1 s⁻¹ (Figure 4). The spectra of samples prepared in a similar fashion but using β-W51F show that the Y• is formed at 1.9 s⁻¹ and decays at 0.14 s⁻¹ (Figure 5). Thus, the substitution of W 51 slows the RT by ~ 8-fold. Moreover, the maximum amplitude (11519, arbitrary units, Table S2) of the Y• in the W51F complex is ~ 80% that of wt (9122.8, arbitrary units, Table S2). On the basis of kinetic simulation analysis (Figure S2), the diminished rate of Y• formation and slightly greater rate of decay in the W51F variant should not result in a 20% decrease in amplitude. One explanation for the low amplitude could be that the W→F substitution results in a much more “global” change to the protein structure of dynamics that overall makes it less efficient for RT, rather than a “local” change to the RT pathway that forces the oxidizing equivalent to “skip” position 51.

**Kinetics of Y• initiated with dCDP.** We reported in our previous study (33) that dCDP also can trigger formation of the Y• in wt Ct RNR. The dCDP is expected to act as an inhibitor of RNR activity because it also binds at the substrate-binding site. While it is capable of initiating formation the formation of the RT Y•, it might be anticipated to do so less efficiently, because it
is an inhibitor of the reaction. The EPR spectra of samples prepared with wt Ct RNR and dCDP reveal that the Y• forms at 6.2 s⁻¹ and decays with the same rate constant (0.01 s⁻¹) as with CDP (Figure S2). Moreover, the Y• accumulates to only 15% of that observed for wt with CDP. This result can be interpreted by inefficient promotion of the RT by dCDP, or by poor binding of dCDP to α. If the latter is true, the amount of Y• that accumulates will exhibit a concentration dependence on dCDP, and the defect might be overcome with increasing concentrations of dCDP during RT.

DISCUSSION

A key question not answered by our first study on the super-oxidized Ct RNR enzyme concerns the location of the Y•. Tracking experiments with site-directed variants of β suggested the Y• resided at β-Y338, but the use of β-Y222F protein complexed with α-Y991 to generate the “super-oxidized” enzyme complex, which is anticipated to confine the Y• to Y338, did not seem to support that suggestion. The ability to incorporate 3,3-[^2H]2-Y into β and generate Y•s with significantly smaller A tensors informed that a large fraction of the Y• does indeed reside within β, likely at Y338. In principle, a simulated spectrum of this new Y• should be generated from the simulated spectrum of the wt complex with natural abundance Y• by scaling the A tensors arising from the C3 hydrons by 6.51. However, a simulated spectrum generated in this fashion, assuming all Y•s contain deuteria at C3, does not perfectly match the experimental spectrum acquired for samples of 3,3-[^2H]2-Y-β in a “super-oxidized” complex with wt α. This is due to imperfect simulated spectrum of the natural abundance Y• spectrum, a consequence of overestimating the A tensors of the ring protia during the simulation. The unusually small A tensors that would be required to simulate the A tensors of these protia could reflect the presence
of multiple Y•s or multiple conformers of a Y•. These issues are not resolvable in the X-band EPR spectrum. Thus, our efforts to acquire spectra at higher frequencies and or using pulsed-EPR may provide the resolution required to deconvolute the natural abundance Y• spectrum.

The rate constant of 14 s⁻¹ for generation of the Y• in the “super-oxidized” Ct RNR is considerably less than that observed with the NO₂-Y₁₂₂ system in Ec, suggesting that, by contrast to the Ec system, use of the more oxidized Mn/Fe cluster in the Ct system does not completely abolish protein gating. With the W→F substitution at position 51, the observed rate constant is ~ eight-fold less (~ 2 s⁻¹). The simplest explanation for this observation is that the W is utilized during RT. While indirect, this may be the first evidence for participation of the cofactor-proximal W residue in RT in a class I RNR. However, the observed kinetic penalty is relatively small and could result from secondary perturbations to the structure of the complex by the non-isosteric W→F substitution. Indeed, we have not yet directly observed a transient W-radical. The 20% diminution of maximal Y• accumulated with the variant, which is not attributable to the lesser rate of its formation, might suggest such a structural defect. Kinetic simulation analysis (Figure S2) suggests that a 20% loss of amplitude would be explained by an ~800-fold slower Y• formation process (compared to wt) – not the ~ 8-fold slower formation observed. This result is very similar to the observed when the kinetics of Y• formation in the wt complex with dCDP is investigated. While dCDP is sufficient to elicit Y• formation in the absence of CDP, it does so at a rate that is ~ 50% less than the rate determined for the wt complex with CDP. Furthermore, the maximal amplitude of the Y• is only ~ 15% that of the wt Y• with CDP. The slightly slower formation of the Y• that are accompanied with significant losses of amplitude in the wt β•α•dCDP•ATP and β-W₅₁F•α•CDP•ATP complexes might reflect perturbations to the enzyme conformation rather than inefficient RT. Regardless, without knowing the distance between W₅₁
and Y_{338} and between the metal cluster and Y_{338}, it is difficult to predict the kinetic penalty for a RT event that skips this residue. Our ongoing efforts to use saturation-recovery EPR and double electron-electron resonance spectroscopy to measure distances between the metal cluster and the RT residues might make calculations to predict the kinetic penalty for “skipping” W_{51} feasible.

The reverse RT with the Mn^{IV}/Fe^{III} cofactor requires oxidation of the now Mn^{III}/Fe^{III} cluster back to the Mn^{IV}/Fe^{III} state. With the “super-oxidized” Mn^{IV}/Fe^{IV} cluster, the formation of the Y• results in the Mn^{IV}/Fe^{III} state, and the Y_{338}• is unable to further oxidize the Mn. While one would expect the Y_{338}• to oxidize Y_{991} and do another forward RT, we suspect that the oxidizing equivalent must return to the cluster in order for the subunits to dissociate and release the product. Thus, if the returning oxidizing equivalent cannot return to the cluster, it resides at Y_{338} until it is reduced via an unproductive pathway. This provides a plausible explanation for why the RT pathway Y• can effect only a single turnover – it may not be sufficiently potent to oxidize the metal cluster. This is reminiscent of observations from the studies carried out with the Ec NO_{2}-Y_{122} system. There, the returning oxidant, after it has effected reduction of nucleotide, does not regenerate the NO_{2}-Y_{122}•, and instead resides on RT pathway Ys until its eventual decay via a non-productive pathway. Thus, it is possible that in the class I RNRs, the multiple rounds of RT are possible only if the initiating cofactor is regenerated after each round of substrate reduction.
SCHEMES AND FIGURES

Scheme 1. Orthogonal pathways mediating reduction of the Mn/Fe cluster in Ct RNR. The location of Y_{338}, Y_{991}, Y_{990}, and C_{672} are unknown. They are positioned based on alignment of the crystal structure of Ct β (41) within the docking model of the Ec α_2β_2 complex (9). A simple schematic to aid with the interpretation of figures is shown at the bottom.
Figure 1. EPR spectra of samples prepared with variants of α to investigate the location of the RT pathway Y•s. Shown in panel A are spectra of the Y•s formed in samples of variants proteins of α and wt β. The spectrum of the Y• formed in the wt β•α complex is shown at the top for the purpose of comparison. The ability of the variants of α to support dCDP formation are assessed in B. EPR samples were prepared as described in our previous work (33), and the activity measurements were carried out as previously described (33). Spectrometer conditions were: 100 KHz modulation frequency, 0.2 mT modulation amplitude, 0.163 ms time constant, 163 s scan time, 5 scans per spectrum, 14±0.2 K temperature, 9.47 GHz microwave frequency, 5 scans total per spectrum.
Figure 2. EPR spectra of radicals generated with variants of β and α to determine the location of the RT pathway Y•. The Y•s were generated with the “super-oxidized” β-Y222F•α-Y991F (top) and β-Y222F•α-Y990F (bottom) complexes. Samples were prepared as previously described (33). Spectrometer conditions are provided in the legend of Figure 1, with the exception that the spectra in panel A are single scans for each spectrum, and were acquired with a 1 mT modulation amplitude.
Figure 3. EPR spectra of Y•s generated in the “super-oxidized” β•α and β•α-Y991F complexes. The spectra labeled i and ii are of samples that were prepared with wt α, and those labeled iii and iv are of samples that were prepared with α-Y991F. The β proteins used for all samples were expressed in the Y-auxotrophic strain in minimal medium supplemented with natural abundance Y (i, iii) or 3,3-[2H2]-Y (ii, iv). The black spectra are experimental spectra for samples prepared and analyzed as previously described (33). The red traces are simulations of the natural abundance Y• spectra form samples of the “super-oxidized” wt complex. The blue traces are simulations for which the A tensors of C3 hydrons used to generate the red traces have been scaled by 6.51 to account for the smaller magnetic moment of the deuteria. A detailed description of the simulations can be found in reference (33), and the parameters are provided in Table S1.
Figure 4. Use of freeze-quench EPR spectroscopy to monitor the kinetics of Y• formation and decay upon mixing MnIV/FeIV-β with α, CDP and ATP. The intensity at 333.15 mT for each trace in A was plotted as a function of time (B) and the resultant data were fit to the equation provided in Experimental Procedures. The fitting result of the kinetic trace in B is provided in Table S2. Sample preparation is also described in Experimental Procedures. Final concentrations of samples were 0.2 mM β, 0.3 mM α, 0.15 mM Mn, 0.15 mM Fe, 1 mM CDP, 0.5 mM ATP, 10 mM MgSO4, 10 mM DTT. Spectrometer conditions are provided in the legend of Figure 1.
**Figure 5.** Use of freeze-quench EPR spectroscopy to monitor the kinetics of Y• formation and decay upon mixing MnIV/FeIV-β-W51F with α, CDP and ATP. A description of the sample preparation techniques is provided in *Experimental Procedures*. The data was analyzed as described for *Figure 4*, and the fitting result of the kinetic trace in B is provided in *Table S2*. The final concentrations for samples were the same as the samples used for the spectra presented in *Figure 4*. Spectrometer conditions are provided in the legend of *Figure 1*. 
ABBREVIATIONS:

RNR, ribonucleotide reductase; Y•, tyrosyl radical; C•, cysteine thiol radical; RT, radical-translocation; Ec, Escherichia coli; β, cofactor subunit of RNR; α, catalytic subunit of RNR; Ct, Chlamydia trachomatis; H•, hydrogen atom; PCET, proton coupled electron transfer; DOPA, 2,2-dihydroxyphenylalanine; NH2-Y; 3-aminotyrosine; NO2-Y; 3-nitrotyrosine; W, tryptophan; F, phenylalanine; Y, tyrosine; C, cysteine; PELDOR, pulsed electron double resonance; EPR, electron paramagnetic resonance; wt, wild-type; CDP, cytidine-5'-diphosphate; dCDP, deoxycytidine-5'-diphosphate; ATP, adenosine-5'-triphosphate; DTT, dithiotreitol; MgSO4, magnesium sulfate; IPTG, isopropyl β-D-1-thiogalactopyranoside
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manganese(IV)/iron(III) cofactor in *Chlamydia trachomatis* ribonucleotide reductase, *Science* 316, 1188-1191.


SUPPORTING INFORMATION

Kinetics and Location of Radical-Translocation Tyrosyl Radical Intermediate(s) Generated with “Super-oxidized” (Mn^{IV}/Fe^{IV}) *Chlamydia trachomatis* Ribonucleotide Reductase
Table S1. Simulated A tensors of Y•s prepared with natural abundance- and 3,3[^2]H[^2]-Y•s in β

<table>
<thead>
<tr>
<th>Position</th>
<th>Natural abundance Y• A tensors (MHz)</th>
<th>3,3[^2]H[^2]-Y• A tensors (MHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-methylene₁</td>
<td>31.3, 27.3, 24.0</td>
<td>4.8, 4.19, 3.68</td>
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<tr>
<td>H-methylene₂</td>
<td>16.0, 15.0, 15.0</td>
<td>2.46, 2.3, 2.3</td>
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</tbody>
</table>

Table S2. Fitting results for kinetic traces of Y•s presented in Figures 4, 5, and S3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>wt β•α</th>
<th>β-W51F•α</th>
<th>wt-β•α, dCDP</th>
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</thead>
<tbody>
<tr>
<td>ΔA (arbitrary units)</td>
<td>11,519</td>
<td>9,123</td>
<td>1,645</td>
</tr>
<tr>
<td>k₁ (s⁻¹)</td>
<td>14.36</td>
<td>1.87</td>
<td>6.22</td>
</tr>
<tr>
<td>k₂ (s⁻¹)</td>
<td>0.009</td>
<td>0.014</td>
<td>0.009</td>
</tr>
</tbody>
</table>

A = amplitude of intensity at 336.35 mT; k₁ = rate of formation; k₂ = rate of decay
Figure S1. Non-zero difference spectrum generated from the theoretical and experimental 3,3-[2H]2-Y• spectra. The theoretical spectrum of the 3,3-[2H]2-Y• was obtained by scaling the A tensors arising from the C3 hydrons by $6.51 \times$ the values determined for the natural abundance Y• that accumulates in the wt “super-oxidized” β•α complex ($A_{1H}/A_{2H} = 6.51$).
**Figure S2.** Kinetic simulation analysis of the Y•s generated by the “super-oxidized” wt β•α and β-W51F•α complexes. The red trace describes the Y• generated in the wt complex, and the black trace represents the reaction of the β-W51F•α complex. The rate constants used during the simulations are taken from the fitting results provided in Table S2. The blue trace is a simulation that accounts for a 20% reduction in the amplitude of the Y•. This trace is obtained with a rate constant for Y• formation that is 800-fold less than that observed for the wt complex.
Figure S3. Kinetics of Y• formation in the “super-oxidized” wt β•α•dCDP complex. The samples were prepared as described in Experimental Procedures and the data was collected and analyzed as described in Experimental Procedures and in the legend of Figure 4. The fitting results are provided in Table S2.
Appendix A:

Evidence That the β Subunit of *Chlamydia trachomatis* Ribonucleotide Reductase Is Active with the Manganese Ion of Its Manganese(IV)/Iron(III) Cofactor in Site 1

Acknowledgements

I thank Dr. Amie K. Boal, with whom I share the first-authorship of this manuscript, for her tremendous contributions to this work. Amie’s passion for science, excellence, and competence moved this project from what was just a fascinating idea to the level it is at right now. She obtained crystals and structures for every enzyme form I prepared, which was crucial for making the discovery about the non-exclusive site incorporation of Mn. In the early stages of this work Taeho Lim, an undergraduate student in the Bollinger and Krebs group, helped with the purification of proteins. My co-advisor, Prof. J. Martin Bollinger, Jr., suggested the alternative reconstitution procedure (procedure 2) and provided tremendous guidance for experiment design and data interpretation. My co-advisor Prof. Carsten Krebs oversaw all Mössbauer data analyses and interpretation of those results. Prof. Bollinger, Jr. wrote the bulk of this manuscript, with Prof. Krebs, Dr. Boal, and Prof. Amy C. Rosenzweig making significant contributions.
Evidence That the β Subunit of Chlamydia trachomatis Ribonucleotide Reductase Is Active with the Manganese Ion of Its Manganese(IV)/Iron(III) Cofactor in Site 1

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Evidence That the β Subunit of Chlamydia trachomatis Ribonucleotide Reductase Is Active with the Manganese Ion of Its Manganese(IV)/Iron(III) Cofactor in Site 1

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Supporting Information

ABSTRACT: The reaction of a class I ribonucleotide reductase (RNR) begins when a cofactor in the β subunit oxidizes a cysteine residue ~35 Å away in the α subunit, generating a thyl radical. In the class Ic enzyme from Chlamydia trachomatis (Ct), the cysteine oxidant is the MnIV ion of a MnIV/FeIII cluster, which assembles in a reaction between O2 and the MnIV/FeIII complex of β. The heterodinuclear nature of the cofactor raises the question of which site, 1 or 2, contains the MnIV ion. Because site 1 is closer to the conserved location of the cysteine-oxidizing tyrosyl radical of class Ia and Ib RNRs, we suggested that the MnIV ion most likely resides in this site (i.e., ¹MnIV/²FeIII), but a subsequent computational study favored its occupation of site 2 (¹FeII/²MnIV). In this work, we have sought to resolve the location of the MnIV ion in Ct RNR-β by correlating X-ray crystallographic anomalous scattering intensities with catalytic activity for samples of the protein reconstituted in vitro by two different procedures. In samples containing primarily MnIV/FeIII clusters, Mn preferentially occupies site 1, but some anomalous scattering from site 2 is observed, implying that both ¹MnIV/²FeII and ¹FeII/²MnIV complexes are competent to react with O2 to produce the corresponding oxidized states. However, with diminished MnIII loading in the reconstitution, there is no evidence for Mn occupancy of site 2, and the greater activity of these “low-Mn” samples on a per-Mn basis implies that the ¹MnIV/²FeIII-β is at least the more active of the two oxidized forms and may be the only active form.

In the remarkable first step in the reaction of a class I ribonucleotide reductase (RNR), an oxidizing equivalent or “hole” migrates ~35 Å from its resting location in the interior of the cofactor subunit, β, onto a cysteine residue in the active site of the catalytic subunit, α, where the nascent cysteine thiol radical abstracts hydrogen (H+) from the 3’ carbon of the substrate to initiate the reduction of its 2’ carbon.3–6 During translocation of the hole, aromatic residues in a specific pathway are transiently oxidized to radicals in shorter “electron-hopping” steps.6 In the most extensively studied class I RNR, the 1a enzyme from Escherichia coli (Ec), the resting location of the hole is Y122,7,8 which is oxidized to a tyrosyl radical (Y*) in an auto-activation reaction between the nearby non-heme FeII/II cluster and O2.9–11 The Y* is asymmetrically disposed with respect to the μ-oxo-FeIII/III co-product of the activation reaction, positioned closer to metal site 1, which, in turn, is the closer of the two sites to W48 of the hole-transfer pathway.12 Whereas the use of a Y* is conserved among class Ia10,11 and Ib13,14 RNRs, the class Ic enzyme from Chlamydia trachomatis (Ct) instead stores the hole directly on the metallocofactor, a stable, high-valent heterodinuclear MnIV/FeIII cluster that is reduced to MnIII/FeIII upon hole migration.15–19 Analogously, to the Y*-generating activation step of the Ec 1a enzyme,10,11 the Ct RNR cofactor assembles in a reaction between the reduced dimetal cluster (MnII/FeIII) and O2.20

The presence of two different metals in the Ct RNR cofactor raises a number of questions regarding its formation and function. Most importantly, must the MnIV of the active state reside specifically in site 1 or 2 or, alternatively, can it function ambiguously in either site? The initial structure of the Ct β protein did not resolve this question, because it was solved before the Mn requirement of the enzyme had been revealed and is assumed to have been of the inactive FeIII/III complex.21

Under the presumption that only one of the two possible MnIV/FeIII forms would be active, we previously suggested that the MnIV occupies site 1, putting it closer to the known location of the hole (the Y*) in the class Ia and Ib enzymes and in line with the pathway W residue (W51).18 This speculative site assignment seemed to be supported by a subsequent report on a structurally similar Mn/Fe protein, a ligand-binding putative oxidase from Mycobacterium tuberculosis, in which the location of the Mn was shown by X-ray crystallographic anomalous scattering experiments to be site 1,22 and by a computational study on the Ct RNR itself.23 However, a more recent computational study suggested that the MnIV might reside in site 2.24

In Ct cells, assembly factors (e.g., metallochaperones) could specify the location of the MnIV, but in the heterologous expression and/or in vitro reconstitution procedures employed in all studies on the Ct RNR reported to date,1,5,25,26 the location of the MnIV should be dictated by a combination of (i)
the thermodynamics and kinetics of Mn\textsuperscript{II} and Fe\textsuperscript{II} binding to the two sites (i.e., their relative metal-binding affinities, their specificities for Fe\textsuperscript{II} vs Mn\textsuperscript{II}, and their corresponding on- and off-rates), (ii) the reactivity of the two possible forms of the labile Mn\textsuperscript{II}/Fe\textsuperscript{II} cluster, \( ^{1}\text{Mn}^{II}/\text{Fe}^{II} \) and \( ^{1}\text{Fe}^{II}/\text{Mn}^{II} \) (denoting Mn in site 1 or 2, respectively), toward O\textsubscript{2}, and (iii) the conditions under which the Mn/Fe cofactor is assembled. For example, in the activation procedure that we have employed in previous studies\textsuperscript{26} (procedure 1), Mn\textsuperscript{II} is added to apo \( \beta \) at a Mn/\( \beta \) ratio (1.5) similar to the experimentally determined stoichiometry of functional metal sites (\( \sim 1.4 \)); as for the \( \epsilon \) \( \beta \) protein, it is not clear why the number of functional sites is less that the theoretical value of 2). Half this quantity of Fe\textsuperscript{II} (0.75 equiv) is subsequently slowly infused over several minutes in the presence of excess O\textsubscript{2}, and weakly bound or unbound metal ions are then removed by dialysis of the sample against EDTA. The expectation is that Mn\textsuperscript{II} should initially fill all or nearly all of the metal sites, forming the O\textsubscript{2}-inert \( \text{Mn}^{II} \beta \) complex. Upon addition of limiting Fe\textsuperscript{II}, it should (if Fe\textsuperscript{II} on-rates for the C\textsubscript{t} protein) rapidly bind to sites vacated by the labile Mn\textsuperscript{II}, forming either \( ^{1}\text{Mn}^{II}/\text{Fe}^{II} \beta \) or \( ^{1}\text{Fe}^{II}/\text{Mn}^{II} \beta \). The O\textsubscript{2}-reactive form(s) should then be trapped to produce the corresponding oxidized state(s).

A sample prepared by this procedure \textbf{1} \( [1.50 \text{ Mn}/\beta \) added and 0.55 retained, as determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) after removal of unbound metal ions, Table S1\] was crystallized (PDB accession code 4D8F) in preparation for X-ray diffraction experiments to address the location of the Mn. In contrast to the crystals previously used to determine the structure of the Fe\textsubscript{II}/III protein, which had only one \( \beta \) monomer per asymmetric unit (ASU),\textsuperscript{21} these Mn/Fe forms of the protein crystallized with either one or two dimers (two or four \( \beta \) monomers) in the ASU (Table S2). Thus, the diffraction data on these crystals afford six independent views of the cofactor site. Diffraction data collected at the Mn absorption edge show that Mn preferentially occupies site 1 of the cluster site. Figure 1A shows the Mn anomalous difference density at the active site in a representative monomer. In all six views of the cofactor site, the Mn anomalous scattering peak at site 1 is more intense than that at site 2 (Table 1). However, in two separate data sets, Mn occupancy of site 2 is evident in at least one monomer in the ASU (anomalous density > 6.0\( \sigma \)).

To further investigate this apparent preferential occupancy of site 1 by Mn, a second sample was prepared by the initial addition of limiting Mn\textsuperscript{II} followed by the rapid addition of excess Fe\textsuperscript{II} in the presence of excess O\textsubscript{2} (procedure 2; PDB accession code 4D8G). In this protocol, low Mn\textsuperscript{II}/\( \beta \) ratios should maximize differential site occupancy arising from any difference between the intrinsic affinities of the two sites. The corresponding data taken on crystals of this sample (0.3 Mn/\( \beta \) added and 0.22 retained, Table S1), yield a Mn anomalous difference map indicative of far less occupancy of site 2 and increased contrast between sites 1 and 2 (Figure 1B and Table 1). The Mn anomalous scattering data thus imply that site 1 is preferentially occupied by Mn following both in \textit{vitro} reconstitution procedures and that this preference is greater when the reaction is performed using limiting Mn\textsuperscript{II}.

To gain information about the Fe content of each site, data sets were also collected at 7.2 keV. In the sample from procedure \textbf{1}, anomalous difference amplitude maps\textsuperscript{27} (Figure S1) suggest significant Fe occupancy only of site 2. In the procedure \textbf{2} sample with less Mn and excess Fe, the Fe scattering is still more intense at site 2, but significant occupancy of site 1 is also observed (Figure S1). Thus, the Fe edge data suggest a preference for site 2, particularly when site 1 is initially fully occupied by Mn\textsuperscript{II} in the activation process.

The highest resolution data sets for each procedure were solved and fully refined (Tables S2 and S3). In addition to the crystal-packing differences noted above, minor differences from the published structure of the Fe\textsubscript{II}/III form could be noted (Figure S2). Although the data sets are of sufficiently high resolution (1.75—2.40 Å) to visualize structural detail at the cofactor sites (Figure S3), the resulting models must necessarily be interpreted with caution. Because of heterogeneity in the occupancy of Mn vs Fe in site 2 in the procedure \textbf{1} structures and low overall metal occupancy in the procedure \textbf{2} structure (Figure S3), it is difficult to definitively model the positions of side chains and bridging ligands. Additionally, the oxidation states of the metals in the final structures are unknown because the Mn\textsuperscript{IV}/Fe\textsuperscript{III} cluster has been shown to undergo both adventitious reduction on the time scale of crystallization and X-ray photoreduction during data collection.\textsuperscript{28}

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**Table 1. Anomalous Peak Heights at the Mn Absorption Edge**

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<td>C</td>
<td>10.2</td>
<td>5.2</td>
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<tr>
<td></td>
<td>D</td>
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<td>2.0</td>
</tr>
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<td>7.3</td>
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<tr>
<td></td>
<td>D</td>
<td>8.7</td>
<td>5.0</td>
</tr>
</tbody>
</table>

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![Figure 1. Anomalous difference electron density maps for procedure 1 (A) and procedure 2 (B) samples. Maps representing data collected at the Mn absorption edge (6.68 keV) are shown in purple mesh (contoured at 5.5\( \sigma \)).](image-url)
Figure 2. Mössbauer spectra of the procedures 1 (A–C) and 2 (D–F) samples. Spectra were collected at 4.2 K with externally applied magnetic fields of 0 mT (A,D) or 53 mT (B,E). (C,F) The corresponding difference spectra. The red and blue solid lines are *reference spectra* of the Fe_{II/III} and Mn_{IV/III} forms, respectively. The solid black lines represent the summed contribution of these two components. The ~10% contribution of the Mn_{II/II} form has been removed from the spectrum shown in spectrum E (see Figures S4 and S6). The solid line in spectrum C is the difference spectrum of the procedure 2 sample, multiplied by 3.5 and shown for comparison.

To investigate the origin of the crystallographically observed differences in the Mn and Fe occupancies, replicate samples were prepared by procedures 1 and 2, the quantities of Mn and Fe retained in the samples were determined by ICP-AES (Table S1), and the various cluster forms were quantified by a combination of Mössbauer and EPR spectroscopies. The Mössbauer spectra of the procedure 1 sample (Figure 2A, B) exhibit the field-dependence typical of the stable heterodinuclear Mn_{IV}/Fe_{III} cluster. Analysis of the spectra reveals that the sample contains ~75% of total Fe in Mn_{IV}/Fe_{III} clusters. In addition, the sample contains small quantities of Fe_{II/II} and Mn_{III}/Fe_{III} clusters (~11% of total Fe in each). The magnetically split features of the Mn_{III}/Fe_{III} clusters are seen in the 4.2 K/53 mT spectrum collected over a wider range of Doppler velocities (Figure S4) and the X-band EPR spectrum of this sample (Figure S5A). The spectroscopic analyses reveal the presence of 0.45 equiv Mn_{IV}/Fe_{III} 0.07 equiv Mn_{III}/Fe_{III}, and 0.03 equiv of Fe_{II}/II. The Mn_{IV}/Fe_{III} and Mn_{III}/Fe_{III} forms thus account for all of the Mn in the procedure 1 sample, implying that the Mn anomalous scattering from site 2 arises from the Fe_{III}/Mn_{III} system and, therefore, that both the Fe_{III}/Mn_{III} and Mn_{III}/Fe_{III} forms of Ct β may be reactive toward O₂.

The Mössbauer spectra of the procedure 2 sample (Figure 2D, E) are dominated by the quadrupole doublet associated with the Fe_{II/II} complex (78 ± 3% of total Fe, red lines), as expected in view of the excess of Fe_{II} over Mn_{II} used in activating the protein. The remaining 22 ± 3% of spectral intensity exhibits the dependence on the magnitude of the externally applied magnetic field that characterizes the S = 1 Mn_{II}/Fe_{III} state (Figure 2E). The fact that a lesser fraction of the intensity exhibits this dependence implies that the sample contains a smaller fraction of Mn_{IV}/Fe_{III} clusters. The X-band EPR spectrum of this sample (Figure SSB) further reveals a small quantity, less than the detection limit of Mössbauer spectroscopy, of the Mn_{IV}/Fe_{III} form. Thus, the sample contains 0.25 equiv (78% of 0.65 equiv Fe/2 Fe per cluster) Fe_{II/II} clusters and 0.15 equiv of Mn_{IV}/Fe_{III} clusters. The quantity of Fe in the form of Mn_{IV}/Fe_{III} clusters determined by Mössbauer spectroscopy agrees well with the quantity of Mn determined independently by ICP-AES (Table S1), thereby demonstrating that ~95% of the Mn is in the oxidized state.

These findings are consistent with the observation of Fe in both sites (Figure S1).

The ICP-AES, X-ray anomalous diffraction, Mössbauer, and EPR data on these samples together suggest that both possible forms of the Mn_{IV}/Fe_{III} cluster (with the Mn_{IV}/Fe_{III} and Fe_{II/II}/Mn_{IV} are produced from the corresponding reduced states by reaction with O₂, with the Mn_{IV}/Fe_{III} cluster being formed preferentially. In principle, these two oxidized forms might be distinguishable spectroscopically, but the Mössbauer data do not suggest even partial resolution of features attributable to the two different forms. However, treatment of these two samples with an excess of dithionite reduces the Mn_{IV}/Fe_{III} cluster(s) to the S = 1/2 Mn_{III}/Fe_{III} state(s) (Figure S5C, D). The EPR difference spectra showing the changes resulting from reduction of the procedures 1 and 2 samples (Figure SSE) are markedly different. Specific attribution of EPR features to different forms of the Mn_{III}/Fe_{III} will require more extensive correlation of spectral differences with differences in activation method. Nevertheless, the documented differences correlated to activation procedure lend credence to the notion that both possible Mn_{IV}/Fe_{III} clusters can form. This conclusion raises the question of which form(s) is (are) active.

![Figure 3](image-url)
least more active than the $^{57}$Fe$^{III}/^{57}$Mn$^{IV}$ form. Definitive determination of whether the latter form is merely less active or is completely inactive will require both further optimization of reconstitution procedures to maximize yields of either form and identification of specific spectral features that can be used to assess the product distribution more quantitatively.

A very recent study by Högbom and co-workers also addressed the location of the Mn ion by X-ray crystallographic anomalous diffraction measurements. Their overall conclusion that Mn occupies site 1 is consistent with our results. However, the use of protein prepared by Ec over-expression in Mn-supplemented medium without any further treatment to enrich in the active state, the absence of any assessment of the nature and distribution of cluster forms, and, most importantly, the relatively low activity/Mn of the samples ($<35\%$ of the minimum value in our study) underscores the need for the correlation of site occupancy to catalytic activity. In addition, their conclusion that Mn resides exclusively in site 1 would imply a remarkable specificity imposed either intrinsically by the C$\beta$ protein or by fortuitous metal loading in the heterologous expression host. By contrast, our conclusions suggest a more modest selectivity, perhaps hinting that the native host might possess specialized assembly factors to direct formation of the correct cluster form. Regardless, experimentally addressing the magnitude and basis of the Mn$^{III}$ vs Fe$^{III}$ selectivity of the cluster sites will be critical to understanding how the class Ic C$\beta$ protein properly assembles its novel cofactor.

## ASSOCIATED CONTENT

### Supporting Information

Description of reconstitution procedures; metal content of all samples; general crystallographic methods and refinement statistics for both structures; additional anomalous difference maps; diagram of crystal packing interactions between the two C$\beta$ dimers; electron density maps at the cofactor sites of a representative C$\beta_2$ Mössbauer spectrum of the procedure 1 sample collected over a wider range of Doppler velocities; X-band EPR spectra of the procedures 1 and 2 samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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## REFERENCES

Evidence that the β Subunit of Chlamydia trachomatis Ribonucleotide Reductase is Active with the Manganese Ion of its Manganese(IV)/Iron(III) Cofactor in Site 1

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**EXPERIMENTAL PROCEDURES**

**Over-expression and purification of wt Ct α and β.** The Ct RNR α subunit with the N-terminal 248 residues truncated and a 20 residue His$_6$-containing metal-ion affinity tag appended to the new N-terminus (denoted simply as α in the text) was prepared as previously described. Preparation of the N-terminally metal-ion-affinity-tagged apo β protein was carried out as previously described. The protein was further purified by anion exchange chromatography on a Q-sepharose fast flow column (2.6 × 63 cm, GE Life Sciences). The protein was loaded onto the column in 50 mM Tris-chloride buffer (pH 7.6 at 4 °C) containing 10% v/v glycerol and eluted with a linear gradient of 0 to 0.70 M NaCl (in the same buffer) over 700 min. Fractions that contained the β protein were pooled and concentrated, and the NaCl was removed from the protein by dialysis against 100 mM sodium HEPES (Sigma-Aldrich) buffer (pH 7.6 at 4 °C) containing 10% v/v glycerol.

**Reconstitution of β and preparation for crystallization and spectroscopy.** Samples of the Mn$^{IV}$/Fe$^{III}$ form of Ct β used in this study were prepared by addition of Mn$_{II}$SO$_4$ and Fe$^{II}$SO$_4$ (either natural abundance or ~95% enriched in $^{57}$Fe) to air-saturated solutions of apo β. For all samples, a concentrated solution of Mn$_{II}$SO$_4$ was added at 0 °C to air-saturated 0.15-0.20 mM β protein (at Mn$^{II}$/β ratios given in Table S1), followed by addition of 5 mM ascorbic acid. In procedure 1, 0.75 equiv Fe$^{II}$ was then slowly dialed in over 20 min to the stirring solution of β; in procedure 2, 1 equiv Fe$^{II}$ was added rapidly to the stirring β solution. After addition of both metals, the protein solution was stirred for an additional 20 min. Excess metals were removed by dialysis against 100 mM Na-HEPES buffer (pH 7.6) containing 10% v/v glycerol and 10 mM EDTA; excess EDTA was removed by further dialysis in the same buffer, lacking EDTA. Samples used for crystallization were concentrated to 0.70-1.8 mM β. For EPR and Mössbauer characterization, samples were concentrated to 2.4 mM β and 2.5 mM β for the procedure-1 and procedure-2 samples, respectively. They were subsequently transferred to Mössbauer cups or EPR tubes and frozen in liquid nitrogen. After X-band EPR spectra of the two samples had been collected, both were incubated with 20 mM sodium dithionite for 10 min at ambient temperature to reduce the EPR-silent Mn$^{IV}$/Fe$^{III}$ clusters to the EPR-active Mn$^{III}$/Fe$^{III}$ form(s). They were subsequently re-frozen in liquid N$_2$ for acquisition of the EPR spectra of the reduced samples.

**Determination of metal content by inductively coupled plasma atomic emission spectroscopy (ICP-AES).** ICP-AES samples were prepared from Mn- and Fe-reconstituted protein to contain 0.1-1 ppm of Mn and Fe. Proteins were denatured with 5% nitric acid (trace metal grade, Fisher Scientific) and centrifuged at 1,500 × g for 10 minutes at room temperature to pellet the precipitate. The metal content was then determined by using a Perkin-Elmer Optima 5300 Inductively Coupled Plasma Emission Spectrometer at the Pennsylvania State University Materials Characterization Laboratory. Single-element standards of Mn and Fe (High Purity Standards, Charleston, SC) were used for calibration. The experimentally determined Fe/β and Mn/β ratios (reflecting metals retained through the dialysis procedure) are given in Table S1.
**Determination of catalytic activity.** Samples of reconstituted Mn^{IV}/Fe^{III}-β were tested for catalytic activity. At 37 °C and pH 7.6 (Na-HEPES), 10 μM β was incubated with 0.10 mM α, 1 mM CDP, 0.5 mM ATP, 10 mM MgSO₄, and 10 mM DTT. Aliquots were removed from the assay mixture at the appropriate times, and the reaction terminated by the addition of HCl to a final concentration of 100 mM. Samples were centrifuged in 3 kDa centrifugal filter units (Millipore) at 12,000 × g for 10 min. The flow-through was analyzed by mass spectrometry (direct injection, negative ion mode), and CDP and dCDP were quantified ratiometrically, as previously described.³ The average dCDP/β produced as a function of time from three repetitions are shown as points in Figs. 3A and S7A. The lines represent linear fits through the points, and the error bars represent the standard deviations from the mean. Velocities per β for each repetition were obtained, and the standard deviations of those velocities were used as the error bars for Figs. 3B and S7B.

**EPR and Mössbauer spectroscopic measurements.** The spectrometers and the Spin-Hamiltonian used to simulate the Mössbauer spectra were previously described.⁴,⁵

**General crystallographic methods.** For each structure, a solution of the Mn^{IV}/Fe^{III}-containing Ct β protein in 100 mM HEPES pH 7.6, 10% glycerol was diluted to 10 mg/mL in 20 mM HEPES pH 7.6, 5% glycerol prior to drop setup. Diffraction data sets were collected at the Life Sciences Collaborative Access Team (LS-CAT) and General Medicine and Cancer Institutes Collaborative Access Team (GM/CA-CAT) beamlines at the Advanced Photon Source (APS) and processed using the HKL2000 package.⁶ Structures were solved by molecular replacement using the program PHASER with the coordinates for the Fe^{II/III}-containing Ct β (PDB accession code 1SYY)⁸ as a search model. Model building and refinement were carried out with Coot and Refmac5⁹,¹⁰ Ramachandran plots were calculated with PROCHECK and MolProbity.¹¹-¹³ Figures were prepared with PyMOL.¹⁴ Anomalous difference maps were created with FFT.¹⁵ Iron-specific anomalous difference maps were created as described previously using SFTOOLS and FFT.¹⁶,¹⁷

**Structure of sample reconstituted by procedure 1.** Rectangular prism-shaped crystals were obtained at 20 °C using the hanging drop vapor diffusion method, in which the drop of the protein solution (see Table S1 for metal content) was equilibrated against a well solution of 10% (w/v) PEG 3000, 200 mM sodium acetate, 100 mM HEPES pH 7.6. The crystals were overlaid with cryoprotectant solution [30% (w/v) glycerol, 10% (w/v) PEG 3000, 200 mM sodium acetate, 100 mM HEPES pH 7.6] for less than 5 min, mounted on rayon loops, and flash frozen in liquid nitrogen.

Data collection and refinement statistics are found in Table S2. The final model consists of residues 1-320 for chain A with an additional seven residues visible at the N-terminus attributed to the His₆-containing affinity tag, residues 1-324 for chain B with ten additional N-terminal residues, residues 1-318 for chain C with six additional N-terminal residues, residues 1-328 for chain D with two additional N-terminal residues, four manganese ions, four iron ions, four acetate molecules, and 528 water molecules. Ramachandran plots indicate that 99.9% of residues are in allowed and additionally allowed regions.
**Structure of sample reconstituted by procedure 2.** Rectangular prism-shaped crystals were obtained at 20 °C using the hanging drop vapor diffusion method, in which the drop of the protein solution (see Table S1 for metal content) was equilibrated against a well solution of 5% (w/v) PEG 3000, 200 mM sodium acetate, 100 mM HEPES pH 7.4. The crystals were overlaid with cryoprotectant solution (50% (v/v) PEG 400, 5% (w/v) PEG 3000, 200 mM sodium acetate, 100 mM HEPES pH 7.6), mounted on rayon loops, and flash frozen as described for the procedure-1 structure.

Data collection and refinement statistics are shown in Table S3. The structure was solved by molecular replacement using the coordinates for the Fe$_{2}^{III/III}$-containing Ct β (PDB accession code 1SYY) as a starting model. The final model consists of residues 1-318 for chain A with eight additional N-terminal residues, residues 1-324 for chain B with ten additional N-terminal residues, residues 1-318 for chain C with six additional N-terminal residues, residues 1-329 for chain D with two additional N-terminal residues, four manganese ions, four iron ions, and 447 water molecules. Ramachandran plots indicate that 100% of residues are in allowed and additionally allowed regions.
Table S1. Metal stoichiometries (added and retained) for Ct β samples used for crystallography, RNR activity assays, and EPR and Mössbauer spectroscopic characterization.

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<th>Mn added</th>
<th>Mn retained</th>
<th>Fe added</th>
<th>Fe retained</th>
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<th>Activity</th>
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*Prepared with $^{57}$Fe
*Sample used for crystallographic analysis
Table S2. Data collection and refinement statistics for the structure of the procedure 1 sample.

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Figure S1. Anomalous difference maps from procedure 1 (A) and procedure 2 (B) data sets collected at Mn and Fe absorption edges. Maps representing Mn absorption (6.68 keV) are shown in purple mesh (contoured at 5.5σ). A difference DANO map calculated from the iron anomalous scattering data (7.2 keV) with the Mn contribution subtracted is shown as orange mesh (5.5σ).
Figure S2. A diagram (left) of the crystal packing interactions between the two Cr $\beta_2$ dimers in the asymmetric unit (ASU) of crystal form 1. A crystal contact that may be important for expansion of the ASU is highlighted by the black box and shown in the inset. The contact is composed of an acetate molecule from the crystallization solution, residues associated with an N-terminal affinity tag from chain B, and residues on the surface of chain D near the C-terminus. The final thirty residues at the C-terminus are necessary for the long-distance inter-subunit hole transfer that generates the essential cysteine thyl radical in $\alpha$, but have not typically been observed in crystal structures of class I RNR $\beta$ subunits. The interaction between the affinity tag and the C-terminus in chain D results in electron density for ten additional residues (319-328). The involvement of a crystallization reagent and the affinity tag makes it difficult to ascribe significance to the specific interactions observed. Nevertheless, the new electron density provides an unprecedented view of how the C-terminus might pack into the surface near the $\beta$ subunit cofactor site.
Figure S3. Electron density maps for the cofactor sites in a representative dimer in the ASU for the procedure 1 (A) and procedure 2 (B) structures. A $2F_o - F_c$ map is shown in gray (contoured at $1.5\sigma$ in A and $1.0\sigma$ in B) and a $F_o - F_c$ difference map is shown contoured at $3\sigma$ (green) or $-3\sigma$ (red). The difference map was calculated after refinement without solvent ligands. Due to the modest resolution (2.2 Å) of the procedure 1 structure, exogenous solvent ligands were not included in the final model but they are included in the procedure 2 structure (1.75 Å resolution). In this structure, the metals are modeled at 0.5 occupancy at each site. In both structures, weak or absent density for bridging ligands, long metal–metal and metal–OH$_2$ distances, and difference density near the active site all imply significant structural heterogeneity in the first coordination sphere of the cofactor.
Figure S4. 4.2-K/53-mT Mössbauer spectrum of the sample prepared by procedure 1 (see SI text for preparation and Table S1 for metal content) collected over a wider range of Doppler velocities (vertical bars). This spectrum was used to quantify the intensity of the sub-spectrum associated with Mn$^{III}$/Fe$^{III}$ clusters contained in the sample. This component exhibits a broad, magnetically split spectrum, because of the cluster’s half-integer $S = 1/2$ ground state. The presence of this component is evident from the broad absorption lines at $\sim 4$ mm/s and $\sim 4$ mm/s. The Mn$^{III}$/Fe$^{III}$ form is estimated to contribute $\sim 12\%$ to the intensity of the experimental spectrum, as determined by comparison to a “reference spectrum” of the Mn$^{III}$/Fe$^{III}$ state collected under identical conditions (green line).
Figure S5. X-band EPR spectra of the $^{57}$Fe-labeled samples prepared identically to those used for Mössbauer analysis (see SI text for preparation and Table S1 for metal content). A shows the spectrum of the procedure-1 sample prior to dithionite reduction. The signal centered at $g = 2$ arises from the Mn$^{III}$/Fe$^{III}$ cluster, which has an $S = 1/2$ ground state due to antiferromagnetic coupling of the Fe$^{III}$ ($S_{Fe} = 5/2$) and Mn$^{III}$ ($S_{Mn} = 2$) sites. The intensity of the EPR signal associated with the Mn$^{III}$/Fe$^{III}$ form in this sample is ~3-fold more than that of the sample prepared by procedure 2 (panel B); Mössbauer spectra of the former sample reveal that it contains 12% of total Fe (0.07 equiv) in the form of Mn$^{III}$/Fe$^{III}$ clusters (see Figures S4 and S6). Both samples were treated with 20 mM sodium dithionite for 10 min at ambient temperature to reduce the EPR-silent Mn$^{IV}$/Fe$^{III}$ clusters to the EPR-active Mn$^{III}$/Fe$^{III}$ form(s). The resulting spectra are shown in panels C (procedure-1 sample) and D (procedure-2 sample). The difference spectra C-A (panel E, blue line) and D-B (panel E, red line) correspond to the spectra of the Mn$^{III}$/Fe$^{III}$ species produced by reduction, which are markedly different. Spectrometer conditions were: $v = 9.50$ GHz microwave frequency, 20 mW microwave power, 100 kHz modulation frequency, 10 G modulation amplitude, 14 ± 0.2 K temperature, 0.167 s time constant, 167 s scan time.
**Figure S6.** 4.2-K/53-mT Mössbauer spectrum of the sample prepared by procedure 1 (see SI text for preparation and Table S1 for metal content) collected over a narrower range of Doppler velocities to allow for better quantification of the the Mn$^{IV}$/Fe$^{III}$ and Fe$_2^{III/III}$ clusters. The spectrum of the Mn$^{III}$/Fe$^{III}$ form is plotted as solid green line. Because a significant portion of the spectrum of the Mn$^{III}$/Fe$^{III}$ cluster(s) is outside this range of Doppler velocities, its fractional contribution to the total spectrum estimated here (4%) is an underestimate. The contribution of the spectrum of the Mn$^{III}$/Fe$^{III}$ form was determined visually by varying its intensity until there was no absorption on the baseline of the resultant difference spectrum. Removal of this contribution gave the 4.2-K/53-mT spectrum of the procedure-1 sample shown in Figure 2B in the main manuscript.
Figure S7. RNR activities of samples reconstituted with $^{57}$Fe analyzed by Mössbauer and EPR spectroscopies. Samples were prepared by procedure 1 (black) or procedure 2 (red). The assay procedure is described above. (A) dCDP produced per β. (B) activity on a per-Mn basis.
Supporting References


Appendix B:

Radical-Translocation Intermediates and Hurdling of Pathway Defects in “Super-oxidized” (Mn^{IV}/Fe^{IV}) Chlamydia trachomatis Ribonucleotide Reductase

Dassama et al., J. Am. Chem. Soc. 2012, 134, 20498 - 20506
Acknowledgements

I thank Dr. Wei Jiang, who, during her tenure as a graduate student at the time in the Bollinger and Krebs research group, discovered the ability of the “super-oxidized” MnIV/FeIV to generate tyrosyl radicals in the presence of a. Dr. Jiang also trained me in the general procedures for expressing and purifying RNRs, preparing samples, and using the EPR spectrometer. She became my mentor, instructing me in the proper way to design and carry out experiments, for which I remain indebted to her. Jiajia Xie, a graduate student, and Paul Varano, an undergraduate student, both working with Dr. Jiang, provided expression plasmids of the β variants. Denise Conner, also a graduate student in the Bollinger and Krebs lab, synthesized the 2′-adizo-2′-deoxyuridine-5′-diphosphate substrate analogue used in this study. Dr. Maria-Eirini Pandelia, a post-doctoral scholar in the Bollinger and Krebs group, carried out the EPR simulations for the tyrosyl radicals and provided (and has continued to provide) me with helpful instructions on the use of MATLAB and the EasySpin simulation program. My co-advisors, Profs. J. Martin Bollinger, Jr. and Carsten Krebs, provided tremendous guidance during the course of this work, and both made significant and extensive revisions to the manuscript to prepare it for publication.
Radical-Translocation Intermediates and Hurdling of Pathway Defects in “Super-oxidized” (Mn^{IV}/Fe^{IV}) Chlamydia trachomatis Ribonucleotide Reductase

Laura M. K. Dassama,†,‡ Wei Jiang,†,‡,§ Paul T. Varano,‡ Maria-Eirini Pandelia,‡ Denise A. Conner,‡ Jiajia Xie,† J. Martin Bollinger, Jr.,*†,‡ and Carsten Krebs*†,‡

†Departments of Biochemistry and Molecular Biology and §Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802, United States

ABSTRACT: A class I ribonucleotide reductase (RNR) uses either a tyrosyl radical (Y^*) or a Mn^{IV}/Fe^{III} cluster in its β subunit to oxidize a cysteine residue ∼35 Å away in its α subunit, generating a thyl radical that abstracts hydrogen (H^+) from the substrate. With either oxidant, the inter-subunit “hole-transfer” or “radical-translocation” (RT) process is thought to occur by a “hopping” mechanism involving multiple tyrosyl (and perhaps one tryptophanyl) radical intermediates along a specific pathway. The hopping intermediates have never been directly detected in a Mn/Fe-dependent (class Ic) RNR nor in any wild-type (wt) RNR. The Mn^{IV}/Fe^{III} cofactor of Chlamydia trachomatis RNR assembles via a Mn^{IV}/Fe^{IV} intermediate. Here we show that this cofactor-assembly intermediate can propagate a hole into the RT pathway when α is present, accumulating radicals with EPR spectra characteristic of Y^*s. The dependence of Y^* accumulation on the presence of substrate suggests that RT within this “super-oxidized” enzyme form is gated by the protein, and the failure of a β variant having the subunit-interfacial pathway Y substituted by phenylalanine to support radical accumulation implies that the Y^*(s) in the wt enzyme reside(s) within the RT pathway. Remarkably, two variant β proteins having pathway substitutions rendering them inactive in their Mn^{IV}/Fe^{III} states can generate the pathway Y^*(s) in their Mn^{IV}/Fe^{IV} states and also effect nucleotide reduction. Thus, the use of the more oxidized cofactor permits the accumulation of hopping intermediates and the “hurdling” of engineered defects in the RT pathway.

INTRODUCTION

By catalyzing conversion of ribonucleotides to 2’-deoxyribonucleotides, ribonucleotide reductases (RNRs) provide all organisms with the precursors for the de novo synthesis and repair of DNA. All known RNRs use a free-radical mechanism that is initiated by the abstraction of the hydrogen atom (H^+) from the 3’-position of the nucleotide substrate by a transient cysteine thyl radical (C^*). The mechanism by which the C^* is generated is the primary basis for the division of RNRs into classes I–III.

A class Ia RNR, such as the most extensively studied orthologue from aerobically growing Escherichia coli (Ec), comprises two subunits, α and β. Both subunits are homodimers, and they form a 1:1 complex (α2β2) in the active state. The α subunit binds substrates and effectors and contains the cysteine residue (C439 in the Ec enzyme discussed hereafter) that forms the H^+-abstracting C^* during turnover. The C439^* is generated in situ by a stable oxidant in β. This potent oxidant, a μ-oxo-Fe^{II/III} tyrosyl radical (Y^*) cofactor, is the product of an auto-activation reaction, during which the reduced (Fe^{II/III}) form of the diiron cluster reacts with O_2. The activation reaction proceeds via a μ-peroxo-Fe^{II/III} intermediate to a state containing both a Fe^{II/IV} complex, designated X, and a cation radical residing on the near-surface residue, tryptophan (W48). The W48 cation radical (W48^*) can undergo rapid reduction by a number of natural compounds (e.g., ascorbate, thiols), leaving X to oxidize the nearby (∼5 Å away) Y122 to the Y122^* as X is reduced to the μ-oxo-Fe^{II/III} cluster of the active β subunit. The net effect of the transient oxidation of W48 by the diiron–O_2 adduct and the subsequent reduction of the W48^* from solution is the shuttling of a single electron to the buried diiron cofactor.

The catalytic cycle of nucleotide reduction begins with the one-electron oxidation of C439 in α by the long-lived Y122^* ∼35 Å away in β upon formation of the α2β2 complex having the nucleoside S’-diphosphate (NDP) substrate and (deoxy)nucleoside triphosphate allosteric effector bound in α. The long-distance, inter-subunit radical-translocation (RT) process that generates the C439^* does not occur by a simple electron-transfer (ET) step via a tunneling mechanism. Rather, ET...
proceeds in multiple “hopping” steps involving a chain of aromatic amino acid residues (Y_{356} and perhaps W_{48} in \( \beta \), and Y_{731} and Y_{950} in \( \alpha \)), which are conserved across all class I RNRs. The stable Y_{122}^* is proposed to acquire an electron from the closest pathway residue (W_{48} or Y_{356}), and the resultant “hole” is then propagated, residue-by-residue, via a series of similar hopping steps. It is thought that the individual ET steps are coupled to local proton transfers (PT), and, consequently, the term “proton-coupled electron transfer” (PCET) has been used to describe either the overall RT process or its constituent steps. [Note: In this article, we denote the net transfer of the hole in \( \beta \) to the conserved C_{459} in \( \alpha \) as “radical translocation” (RT). “Hole hopping”, “hole translocation”, “electron relay”, and “radical transfer”, which have previously been used by us and others, have the same meaning. We disfavor “radical transfer” because the process invariably involves multiple ET events that interconvert different oxidized species (in most cases pathway radicals) rather than actual transfer of a free radical per se. We also disfavor the acronym PCET to denote the entire RT process or its constituent steps.]

Although the mechanisms of cofactor generation and substrate reduction have been studied extensively, the crucial RT step that initiates and terminates each turnover had, until very recently, remained enigmatic, owing, in part, to the occurrence of a substrate-dependent conformational change that allows the RT process to occur along the mediating pathway. This substrate dependence has been termed conformational “gating”, and the change that occurs in the \( \alpha_2\beta_2 \) complex when substrate binds as “opening of the gate”. Because the gate-opening step is slow and subsequent events are fast, the wild-type (wt) enzyme does not accumulate RT intermediates during turnover. In the last several years, Stubbe and co-workers have elegantly unveiled these intermediates by rational alterations to either the RT pathway or the cofactor. They replaced key Y residues in \( Ec \) RNR with three different unnatural Y analogues, 3,4-dihydroxyphenylalanine (DOPA), 8,28,31 3-aminotyrosine (NH\(_2\)-Y), 8,29,32–35 and 3-nitrotyrosine (NO\(_2\)-Y). The two former analogues are more easily oxidized than Y (i.e., their radicals have reduction potentials less than that of Y\(^+\)). Effectively, their inclusion in the pathway introduces a thermodynamic depression in the hopping pathway, and the radical can then reside on this residue to a significant extent. The latter analogue was incorporated at position 122 in \( \beta \), and the resultant NO\(_2\)-Y_{122}^* has an elevated reduction potential relative to the natural Y_{122}^*. The presence of the more potentely oxidizing initiator appeared to subvert the gating mechanism, permitting the much more rapid propagation of the radical into the pathway and the accumulation of hopping intermediates.

The class Ic RNR from \( Chlamydia trachomatis \) (\( Ct \)) differs from \( Ec \) RNR in that its \( \beta \) subunit lacks the stable, radical-initiating Y\(^+\). The Y is replaced structurally by a redox-inert phenylalanine (F_{127}) and the function of the Y\(^+\) is assumed by the Mn\(^{IV}\) ion of a Mn\(^{IV}/Fe^{III}\) cofactor. All RT pathway residues are conserved, suggesting that, aside from the identity of the C\(^+\)-generating cofactor, the mechanisms of RT and nucleotide reduction might also be conserved. Analogously to cofactor assembly in \( Ec \) RNR, the Mn\(^{IV}/Fe^{III}\) cofactor in \( Ct \) RNR-\( \beta \) assembles in a reaction of the fully reduced cofactor form (Mn\(^{II}/Fe^{II}\)) with O\(_2\). The reaction proceeds via a Mn\(^{IV}/Fe^{IV}\) intermediate that accumulates to near-stoichiometric yield and is sufficiently stable to permit its efficient trapping even in manual-mixing experiments. The intermediate is subsequently reduced by one electron at the Fe\(^{IV}\) site. The electron is shuttled through an activation-specific electron-relay pathway (shown in Scheme 1) that is, to the best of our knowledge, unique to the \( Ct \) enzyme. This pathway comprises the surface-exposed Y_{222} (the cognate in \( Ec \) RNR-\( \beta \) is a redox-inert leucine) and W_{51} (the cognate of W_{48} in the \( Ec \) protein). As is the case with \( Ec \) RNR, no pathway RT intermediates are observed during turnover in \( Ct \) RNR. While efforts to incorporate unnatural amino acids into the \( Ct \) enzyme have thus far been unsuccessful, the longevity [\( t_{1/2} \) of tens of seconds at \( 5\,^\circ\text{C} \)] of the Mn\(^{IV}/Fe^{III}\) cofactor assembly intermediate, itself expected to be a more potent oxidant than the native Mn\(^{IV}/Fe^{III}\) cofactor, led us to consider the...
possibility that it could be used to alter the redox-potential landscape of the RT pathway to favor accumulation of radical intermediates.

Indeed, we show herein that mixing the MnIV/FeII-β complex of Ct RNR with an O2-containing solution of α subrate (cytidine 5′-diphosphate, CDP), and allosteric effecter (ATP) to form the MnIV/FeIII activation intermediate in the presence of all other reaction components results in accumulation of EPR-active species with hyperfine couplings characteristic of Y′s. The data imply the conversion of the MnIV/FeIII intermediate to a MnIV/FeIII-Y′ species in a “super-oxidized” α/β complex. Site-directed mutagenesis experiments demonstrate that the Y′ resides within the RT pathway. Remarkably, two β variants with engineered RT pathway defects that render them inactive in their MnIV/FeIII states are, in their MnIV/FeIV states, capable of both generating Y′s and promoting a single turnover of the substrate. Thus, with the more oxidized cofactor, intermediates of the RT process can be accumulated in Ct RNR, and engineered defects in the RT pathway can effectively be “hurdled” to rescue nucleotide reduction in the disabled variants.

**EXPERIMENTAL PROCEDURES**

**Materials.** Restriction enzymes and calf intestinal phosphatase were purchased from New England Biolabs (Ipswich, MA). 2-Methylbutane (iso-pentane), CDP, deoxycytidine 5′-diphosphate (dCDP), ATP, DTT, MgSO4, Dowex-1 resin, and potassium tetraborate tetrahydrate (K2B4O7·4H2O) were purchased from Sigma-Aldrich (St. Louis, MO). (S)-H3-CDP was purchased from Vitrax (Placentia, CA). Ecoscent scintillation fluid was purchased from National Diagnostics (Atlanta, GA).

**Overexpression and Purification of Ct β and Ct α.** Construction of the plasmid vectors pET28a-α (Δ1–248) and pET28a-β expressing the wt subunits and the modified vectors expressing all the variants used in this study (except for the β-Y222F/Y338W variant) has been described elsewhere. The vector for β-Y222F/Y338W was constructed by applying a polymerase chain reaction (PCR) with the pET28a-β′-Y222F as the template and primers 1 (5′-TGA CCG TTG CAT ATT CAA GCA GAT ATT TTA GAT GG-3′) and 2 (5′-GGT GGT GCT CGA GGT ACC AAG TTA AGC TTG CTG CAT GTT GG-3′) to introduce the desired substitution (underlined). The PCR fragment was purified by agarose gel quantification and ligated with the larger (vector) fragment of Ndel- and XhoI-restricted pET28a-CT β-wt. The sequence of the entire coding region of the vector was verified by ACGT, Inc. (Wheeling, IL). Purification of the N-terminally His-tagged proteins has also been described.

**Synthesis of 2′-Azido-2′-deoxyuridine-5′-diphosphate Analogue (N3-UDP).** 2′-Azido-2′-deoxyuridine was prepared as previously described. The 5′-diphosphate was appended by the method of Besada and co-workers. The quantity of tributylammonium phosphate was reduced from 6 to 3 equivalents to limit formation of the iso-triphosphate byproducts. The identity and purity of the product were verified by comparison of its 1H, 13C, and 13N NMR spectra to published data for the compound.

**Preparation of Samples for EPR Spectroscopy.** All samples (except in cases otherwise indicated) were prepared by adding 0.75 equiv of MnII and FeII to O2-free solutions of ~2 mM apo-Ct β and subsequently mixing with an air-saturated solution (hereafter referred to as “α mix”) containing α, CDP (or dCDP or N3-UDP), ATP, DTT, and MgSO4. Concentrations after mixing were 0.40 mM α, 0.20 mM β, 0.15 mM Mn, 0.15 mM Fe, 1 mM CDP (or dCDP or N3-UDP), 0.5 mM ATP, 10 mM DTT, and 10 mM MgSO4. Samples made with CDP and dCDP were frozen in cold (−150 °C) 2-methylbutane 5–10 s after mixing (unless otherwise indicated), whereas samples with N3-UDP were frozen 1 min after mixing.

**EPR Spectroscopy.** EPR spectra were acquired on an ESP300 spectrometer equipped with a 4102ST X-band resonator and an ER 041 MR microwave bridge, all from Bruker (Billerica, MA) and described elsewhere. Cryogenic temperatures were maintained with an Oxford Instruments (Oxfordshire, UK) ESR 900 cryostat. Spectrometer conditions are provided in the figure legends. Simulation of EPR spectra of the Y′(s) was carried out with the program EasySpin. The simulation parameters were constrained as illustrated by Svitunenko and described in more detail in Supporting Information. The parameters are provided in Table S1.

**“Spin” Quantification by EPR Spectroscopy.** The extent of accumulation of the pathway Y′(s) was determined from analysis of the EPR spectra of duplicate samples prepared in a sequential-mix freeze–quench experiment. An O2-free solution of MnIII/FeII-β was first mixed with an O2-saturated buffer solution, and this solution was allowed to react for 2 s to form the MnIV/FeIII intermediate. The pre-formed intermediate was mixed either with a solution of α, MgSO4, and DTT or with a solution of α, CDP, ATP, MgSO4, and DTT. The resultant solution was allowed to react for 1 s (ongoing studies suggest that the concentration of Y′(s) reaches its maximum value at this reaction time) before the reaction was terminated by freeze-quenching in cold (−120 K) 2-methylbutane. Final concentrations after mixing were 0.2 mM β, 0.15 mM Mn, 0.15 mM Fe, 0.3 mM α1, 1 mM CDP (when present), 0.5 mM ATP (when present), 10 mM MgSO4, and 10 mM DTT. The signal intensities attributable to the MnIV/FeIV intermediate and the RT pathway Y′(s) were determined from the analysis of the EPR spectra as previously described. The results are summarized in Table S2.

**Measurement of Product Formation with 3H-CDP.** (S)-H3-CDP with specific activity >15 Ci/mmol was acquired from Vitrax (Placentia, CA). The assay procedure was adapted from that reported by Steeper and Steuart. Samples were prepared so as to be identical to those used for EPR spectroscopy, with 1 mM (S)-H3-CDP (~5000 cpm/nmol) substituted for CDP. The samples were incubated at 37 °C. Aliquots were removed at the desired reaction times, diluted 5-fold with reaction buffer (100 mM Na-HEPES, 10% v/v glycerol, pH 7.6), and quenched by incubation at 100 °C for 5 min. Quenched samples were centrifuged at 14000g for 10 min. The cleared supernatant was transferred to a clean tube, and 1 μL of 10 U/μL calf intestinal phosphatase, 5 μL of 20 mM deoxycytidine, and 50 μL of 500 mM Tris-HCl (pH 8.5) were added. The resultant solutions were incubated for 1 h at 37 °C, and each was then loaded onto a column containing 1.5 mL of Dowex-1 resin pretreated with 0.9 M potassium tetraborate (K2B4O7·4H2O). The dCDP product was eluted by washing with 9 mL of distilled, deionized water. A 1 mL aliquot of the eluate was mixed with 5 μL of Ecoscent scintillation fluid; radioactivity was quantified by counting for 5 min per sample on a Beckman LS-6500 scintillation counter.

**RESULTS**

**Initial Observation of a Possible Tyrosyl Radical (Y′) and Requirements for Its Formation.** The well-characterized activation reaction of the Mn- and Fe-dependent Ct RNR β subunit begins with addition of O2 to its MnIV/FeII cluster, which produces a long-lived, high-valent MnIV/FeIII intermediate that decays by reduction of the FeIV ion to FeIII. This intermediate has unique Mössbauer- and EPR-spectroscopic features (Figure 1A, spectrum i) reflecting, first, antiferromagnetic coupling of the S = 2 FeIV and the S = 3/2 MnIII to give Stotal = 1/2 and, second, strong hyperfine coupling of the I = 5/2 57Mn nucleus to the electron spin. The spectra of samples prepared by mixing a solution of MnIII/FeII-β with an air-saturated solution containing either α only (Figure 1A, ii) or α with the allosteric effector ATP (Figure 1A, iii) both reveal the presence of only the MnIV/FeIV intermediate. By contrast, when the activation reaction is initiated by mixing the MnIII/FeII-β solution with an air-saturated solution of α and CDP...
FeIII-Y*-containing forms, reflecting reversibility of RT between the cluster and the Y residue(s); (2) incomplete complex formation between α and the MnIV/FeIV-containing β, which would prevent the fraction of the MnIV/FeIV cluster present in unbound β from generating a Y*; and (3) half-of-sites reactivity in RT, which would prevent one of the two MnIV/FeIV clusters present in each β subunit from generating a Y*. Explanation 1 was ruled out on the basis of the kinetics of MnIV/FeIV cluster and Y* decay following formation of the super-oxidized enzyme form (Figure 2A). Whereas two species in rapid equilibrium should develop and decay in constant proportion as a single kinetic entity, the residual MnIV/FeIV complex (squares) decays considerably (~6-fold) faster than the Y* (circles) in these samples. As a test of explanation 2, the ratio of α:β was varied from 1:1 to 6:1, and the relative proportions of MnIV/FeIV cluster and Y* were assessed by EPR (Figure 2B). No significant change could be discerned with increasing α:β, implying that the β subunit is almost fully saturated by α even at a 1:1 molar ratio. The results of Figure 2 thus imply that explanation 3, half-of-sites reactivity within the α:β complex, is the most likely reason for the incomplete decay of the MnIV/FeIV cluster in its generation of the putative Y*.

To test this hypothesis further, we prepared duplicate sets of freeze-quenched EPR samples by a double-mixing protocol. A solution of MnII/FeII-β was first mixed with O2-containing buffer to generate the MnIV/FeIV complex, and the intermediate was then reacted for 1 s with either α, MgSO4, DTT, CDP, and ATP in the experiment or only α, MgSO4, and DTT in the control. Comparison of the EPR spectra of the complete-reaction samples to those of the no-nucleotide control samples (Figure S2 and Table S2) reveals that the intensity of the EPR signature of the MnIV/FeIV intermediate decays by an average of 45%, very similar to the 50% loss expected under the assumption of half-of-sites reactivity.8,28,31,58,59 This lost...
The spin quantification is thus completely consistent with the hypothesis that the $Y'(s)$ is (are) generated by the Mn$^{IV}$/Fe$^{IV}$ complex on one side of each $\alpha_{1}\beta_{2}$-ATP-CDP complex.

Evidence That the Radical(s) Reside(s) within the RT Pathway. Our previous work provided evidence that C$t$ RNR possesses two functionally orthogonal pathways for reduction of the Mn/Fe cluster: the activation-specific pathway, comprising Y$_{338}$ and W$_{51}$ of $\beta$ (blue lines in Scheme 1), mediates reduction of the Fe$^{IV}$ of the Mn$^{IV}$/Fe$^{IV}$ intermediate; the catalysis-specific RT pathway, comprising Y$_{356}$ and W$_{51}$ of $\beta$ (in addition to Y$_{991}$ and Y$_{990}$ of $\alpha$; red lines in Scheme 1), mediates propagation of the oxidizing equivalent from the Mn$^{IV}$ ion of the functional Mn$^{IV}$/Fe$^{III}$ cofactor to the essential C$_{672}$ in $\alpha$ (the cognate of C$_{459}$ in Ec $\alpha$) during turnover. The primary basis for identification of the latter pathway was the undetectable catalytic activities of the W$_{51}$F and Y$_{338}$F variants, but the importance of Y$_{338}$ is also strongly supported by its alignment with Y$_{166}$ of Ec $\beta$, which the work of Stubbe and colleagues has convincingly shown to undergo substrate-dependent, protein-gated, transient oxidation during catalysis.

To ascertain whether the putative Y$'$ detected in Figure 1 resides within the catalytic RT pathway, we substituted Y$_{338}$ with the redox-incompetent F and tested for loss of F, or changes to, the signal of the Y$'$. The EPR spectrum of a sample prepared by mixing Mn$^{IV}$/Fe$^{II}$-W$_{51}$F with an air-saturated solution containing C$_{6}$, CDP, and ATP again has a radical signal at $g = 2.00$, suggestive of a Y$'$ (Figure 3, i). The signal is, however, markedly different from that observed with wt $\beta$ (Figure 3, i), suggesting that the radical resides, at least in part, on a different Y residue. Previous work examining reactions of the C$t$ holoenzyme with hydroxurea (HU) had suggested that, with Y$_{338}$ replaced by F, substrate-dependent propagation of the oxidizing equivalent from the Mn$^{IV}$/Fe$^{III}$ factor onto Y$_{338}$ can occur, initiating HU reduction of the cofactor to the Mn$^{III}$/Fe$^{III}$ state. We therefore suspected that the different signal observed in the super-oxidized complex with $\beta$-Y$_{338}$F might result from propagation of the oxidizing equivalent into the activation-specific pathway and onto Y$_{338}$. To test this possibility, we interrogated the double-variant $\beta$ protein with substitutions of both Y$_{338}$ and Y$_{356}$ with F. The EPR spectrum of a sample prepared with this double variant lacks any signal for an organic radical (Figure 3, v). By contrast, the Y$_{356}$F variant supports formation of a radical with an EPR spectrum very similar to that observed with wt $\beta$ (Figure 3, vii). The simplest interpretation of the change in the radical EPR spectrum upon substitution of Y$_{338}$ alone, abolition of the spectrum upon substitution of both electron-relaying Y residues in $\beta$, and absence of any effect of the Y$_{356}$F substitution by itself is that the Y$'$ observed with the wt subunit resides within the catalysis-specific RT pathway (on $\beta$-Y$_{338}$ $\alpha$-Y$_{991}$ $\alpha$-Y$_{990}$ or distributed among these residues) and that the Y$_{356}$F substitution causes aberrant propagation onto Y$_{356}$ of the activation-specific RT pathway. It is noteworthy that formation of this off-pathway (Y$_{356}$) radical again requires the presence of substrate. Thus, even the aberrant propagation into the activation-specific pathway still behaves (as it did in the HU study) as if gated by substrate binding. As previously noted, this conclusion has implications for the location and nature of the conformational gate of the RT process.

Hurdling Engineered Defects in the RT Pathway. In comparison to Ec Y$_{356}$ and C$t$ Y$_{338}$, the roles of W$_{51}$ of Ec $\beta$ and W$_{51}$ of C$t$ $\beta$ in catalysis are less clear. It has been established that W$_{51}$ functions in electron relay during activation of Ec $\beta$; it is oxidized to a cation radical concomitantly with formation of the Mn$^{IV}$/Fe$^{III}$ complex with O$_{2}$ in the presence of $\alpha$, CDP, ATP, DTT, and MgSO$_{4}$. A schematic of the electron-relay pathways is provided at the left, with the non-native cluster redox state and substituted amino acid residues highlighted in red. Sample preparation was as described in the legend of Figure 1. For samples containing the Mn$^{III}$/Fe$^{III}$ states, each $\beta$ protein was pretreated by adding air-saturated 100 mM HEPES, 10% (v/v) glycerol buffer to the Mn$^{IV}$/Fe$^{IV}$-CDP, and ATP, DTT, and MgSO$_{4}$. The EPR spectrum of a sample prepared with this variant protein is fully competent for radical formation complexes with O$_{2}$ in the presence of $\alpha$, CDP, ATP, MgSO$_{4}$, and DTT and frozen after ~5 s. (A) The “raw” experimental spectra. (B) The contribution of the Mn$^{IV}$/Fe$^{III}$ intermediate has been removed to resolve the spectra of the radical components. The red and blue dashed spectra plotted over the data in i and iii are simulations with parameters provided in Supporting Information. Spectrometer conditions are provided in the legend of Figure 1.
function in RT but with an altered redox potential. This substitution was combined with Y222F in Ct \( \beta \) so that the ability of W338 to support pathway radical accumulation could be assessed without interference from the signal of the off-pathway Y222\(^*\). Not surprisingly, the \( \beta \)-Y222F/Y338W protein lacks detectable catalytic activity in its MnIV/FeIII state (the double variant is fully competent to generate this form in the reaction of its reduced enzyme form with O\(_2\)). Thus, the Y338W substitution results in loss of proper RT with the stable MnIV/FeIII cofactor. However, in the reaction of MnIV/FeIII-\( \beta \)-Y222F/Y338W with O\(_2\) in the presence of \( \alpha \), CDP, and ATP, a radical signal does indeed develop (Figure 3, xi). The shape of the spectrum is again suggestive of a Y\(^*\), which, if on the RT pathway, would in this case necessarily be located within \( \alpha \). The super-oxidized complex of this \( \beta \) double variant generated with the \( \alpha \)-Y991F variant (which readily supports radical accumulation in complex with wt \( \beta \); see Figure S1, black in the Supporting Information) lacks a significant radical signal (Figure S1, green), providing additional evidence that the radical in the \( \beta \)-Y222F/Y338W-\( \alpha \)(wt) complex resides within \( \alpha \) on either Y991 or Y990 (or perhaps distributed between them).

Functional Competence of the Y\(^*\)(s) in the Super-oxidized Ct RNR Complexes. The raison d’être of forward (\( \beta \)-\( \alpha \)) RT within the RNR holoenzyme complex is to initiate nucleotide reduction by abstraction of H\(^*\) from the 3’ carbon of the substrate. If the Y\(^*\) or Y\(^*\)’s observed in super-oxidized Ct RNR do, as the above evidence suggests, reside within the RT pathway, they might be expected to function in initiation of turnover. The substrate analogue, 2’-azido-2’-deoxyuridine-3’-diphosphate (N3-UDP), can serve as a reporter of 3’H\(^*\) abstraction. (A) Raw experimental spectra acquired over a wide range of the magnetic field. (B) Spectra acquired over a narrow range of the magnetic field and processed by subtraction of the contributions from either the MnIII/Fe\(^{2+}\) complex (i) or the MnIV/FeIV complex (ii, iv, and vi) to resolve the spectra of the organic-radical components. The method of sample preparation and the spectrometer conditions are the same as for Figures 1 and 2.

states of these RT-pathway-defective variants with successful 3’-H\(^*\) abstraction. A control reaction employing the \( \beta \)-Y338F variant, which is incompetent for accumulation of RT-pathway Y\(^*\)(s) but instead supports formation of the off-pathway Y222\(^*\), shows that N3-UDP can replace CDP in the gating function, thereby supporting development of the EPR signal assigned to Y338\(^*\), but, as expected, does not undergo conversion to the N\(^*\) in the holoenzyme complex with this \( \beta \) variant (Figure 4, vi).

The functional competence of the RT-pathway Y\(^*\)(s) was further assessed by testing directly for CPD reduction to dCDP. A published assay\(^{56}\) employing tritium-labeled CPD substrate (3H-CDP, labeled at C5 of cytidine) was used to quantify the product. Background levels of radioactivity were observed in the product fractions from reactions initiated by mixing the stable MnIV/FeIII forms of the \( \beta \)-Y338F, \( \beta \)-Y222F/Y338W, and \( \beta \)-W51F with \( \alpha \), ATP, and 3H-CDP (Figure 5, orange, black, and gray, respectively). By contrast, reactions initiated by mixing the MnIV/FeIII forms of the variants with O\(_2\)-containing solutions of the other reaction components (so as to generate the super-oxidized enzyme form) produced ~0.5 equiv of dCDP per \( \beta \) for the two variants [\( \beta \)-Y222F/Y338W (red) and \( \beta \)-W51F (blue)] shown above to support 3’H\(^*\) abstraction from N3-UDP, but gave no significant product-associated radioactivity in excess of the background for the uniformly inactive \( \beta \)-Y338F variant (green). The activity profile of the variants is entirely consistent with that deduced both by EPR [accumulation of pathway Y\(^*\)(s) or absence thereof] and by use of the radical-trapping N3-UDP analogue (production of the N\(^*\) or failure thereto). The correlation implies that the radicals observed in the super-oxidized enzyme forms are functionally competent. The quantity of dCDP observed with the conditionally active \( \beta \)-Y222F/Y338W and \( \beta \)-W51F variants suggests that only a single turnover can occur and that the known half-of-sites reactivity limits the super-oxidized forms to a single-turnover within just one side of the hetero-tetrameric \( \alpha \_1\beta \_1\beta \_2\) complex. The conclusion of a single turnover suggests, in turn, that the bi-directionality of RT between \( \beta \) and \( \alpha \) that is required for catalysis may be deranged as the enzyme operates with the
more oxidized cofactor and RT pathway defects, thus precluding subsequent turnovers. The conclusion of a single turnover is also consistent with observations by Stubbe and co-workers on RT-pathway variants of the class Ia Ec RNR.28,37

**DISCUSSION**

The characteristics of free-radical accumulation upon generation of the MnIV/FeII activation intermediate in Ct RNR-β in the presence of α, a substrate (CDP), substrate analogue (N3-UDP), or product (dCDP), and an allosteric effector (ATP) provide convincing evidence for the location of the radical(s) within the RT pathway. Radical formation requires the substrate and is potentiated by the effector, implying gating in the same manner previously demonstrated for both Ct and Ec RNR.28–30,35,37 The line shape of the g = 2.00 EPR signal is consistent with that of a Y* (with appropriate dihedral angles between the ring and Cβ−H bonds), and Ct RNR has three Y residues (β-Y122, α-Y99, and α-Y99) that align in sequence with those shown by Stubbe and co-workers to undergo transient oxidation during catalysis by Ec RNR.27–31,37 The Y138F substitution in β changes the EPR spectrum and thus the identity of the accumulating radical (putatively to Y222*), and the combination of this substitution with the Y222F substitution in β, which disables the activation-specific RT pathway unique to the Ct enzyme, abolishes radical accumulation altogether. In the context of the β-Y122F/Y338X double variant, swapping the completely non-functional F for the potentially oxidizable W at position 338 restores radical accumulation, showing that a redox-active “stepping stone” is required at this position but need not be the native Y when the cofactor is in its super-oxidized state. By far the most compelling evidence for the location of the accumulating radicals within the RT pathway, however, is their association with 3'-H* abstraction: the variants that are competent for radical accumulation (β-W51F and β-Y122F/Y338W) also support both N* formation from the N7-UDP analogue and a single turnover (reduction) of CDP substrate in their super-oxidized states, despite being essentially devoid of either activity in their stable MnIV/FeIII oxidation states.

The above observations on the super-oxidized state of Ct RNR have some analogy to recent studies by Stubbe and co-workers examining RT and turnover in the Ec RNR holoenzyme complex of a β variant with the radical-harboring Y122 replaced by 3-nitrotyrosine (NO2-Y).37 The NO2-Y radical (NO2-Y*) is a more potent oxidant (by ~200 mV) than Y*.37 As in our study, the “hot oxidant” (NO2-Y122*) was formed in situ in β, in this case immediately before mixing the subunits, because it is stable only for tens of seconds (half-life of ~40 s at 25 °C). Whereas turnover by the wt Ec enzyme initiated by Y122* normally occurs at <10 s−1,30 owing to a rate-limiting conformational change that is probably part of the opening of the gate for Y122* reduction and RT, both reduction of NO2-Y122* and CDP production were found to be much faster in the variant [bi-phase kinetics with observed first-order rate constants of ~300 and ~70 s−1 for NO2-Y122* reduction and ~100 s−1 for dCDP production].37 The authors concluded that the more potent oxidant essentially permits hurdling of the conformational gate in a manner analogous to the hurdling of engineered pathway defects in the super-oxidized Ct enzyme we report in this manuscript. Their results are also consistent with the hypothesis that the gating mechanism involves dynamic control of PT to Y122, because the rapid, putatively “ungated” reduction of the NO2-Y122* in the variant is not associated with protonation of the radical (i.e., the 3-nitrophenolate form is produced). Apparently, even without coupled PT, the initial ET from W51 α or Y356 to the more potently oxidizing NO2-Y122* radical is still favorable, permitting forward RT into α. In addition, one or more new Y* was shown to accumulate at nearly the same rate constant as for dCDP production. More recent electron double resonance spectroscopic experiments have provided evidence that the oxidizing equivalent actually becomes distributed over Y356 of β, Y731 of α, and Y730 of α, suggesting that the reduction potentials of the individual Y*’s are similar. The kinetics of RT-pathway Y* accumulation and the location(s) of the radical(s) within the pathway in the Ct RNR super-oxidized state and variants thereof are actively being pursued and may shed light on the extent to which the class Ia and class Ic enzymes behave similarly in this key step.

Even without knowledge of the kinetics and precise location of the pathway radical(s), the observations on the class Ic enzyme and its super-oxidized state speak to two outstanding questions regarding the key RT step. One key question concerns the role of W48/W51. There is no evidence to date that either undergoes transient oxidation in RT during catalysis. The inactivity of the Ct β-W51F variant might be taken as evidence that W51 has such a role, but the absence of any evidence for a W48/W51-derived radical in the super-oxidized holoenzyme complex with the β-Y222F/Y338F double variant, in which radical propagation beyond W51 is blocked, suggests that W51 is not readily oxidized, even by the more oxidized MnIV/FeIII cofactor form. Coupled with our earlier evidence that W51 is oxidized to a radical during the reaction of the Fe5/II/III complex of the Y222F variant of Ct β with O2,47 the apparent absence of any W51 oxidation by the MnIV/FeIII activation intermediate can be viewed as argument against its transient oxidation during propagation of the oxidizing equivalent from the less potent MnIV/FeIII cluster toward α during catalysis. A caveat to this conclusion is the possibility that oxidation of W51 by the Mn/Fe cofactor (in either oxidation state) might be thermodynamically.
cally disfavored, causing very little W51-derived radical to accumulate, even in the super-oxidized complex of β-Y222F/Y338F. Indeed, recent work by Gray, Winkler, and co-workers has shown that electron hopping can proceed even with an element that is thermodynamically uphill by as much as 200 mV,\textsuperscript{65–68} which would correspond to an equilibrium between the Mn\textsuperscript{IV}/Fe\textsuperscript{IV}-W\textsubscript{51} and Mn\textsuperscript{IV}/Fe\textsuperscript{III}-W\textsubscript{41} forms of the super-oxidized complex favoring the W\textsubscript{51}-reduced (non-radical) form by more than 1,000-fold. Determination of the kinetics of Y\textsuperscript{*} formation in super-oxidized C\textsubscript{t} RNR and the impact of the W\textsubscript{51}F substitution thereupon might provide a quantitative assessment of the importance of this residue in the process.

A second outstanding question concerns the nature and location of the gate for RT. In previous work, we interpreted data on the reduction of the C\textsubscript{t} RNR cofactor from the Mn\textsuperscript{IV}/Fe\textsuperscript{III} state to the Mn\textsuperscript{III}/Fe\textsuperscript{III} state by HU and the dependence thereof on the presence of the substrate and certain key electron-relaying residues as implying that the gate is adjacent to the cofactor and largely independent of the aromatic residues of the RT pathway, involving, at most, W\textsubscript{51} of the pathway.\textsuperscript{57} The observation here that the W\textsubscript{51}F variant supports radical accumulation, again dependent on the presence of the substrate, suggests that W\textsubscript{51} is dispensable at least for the apparent gating being manifested by the super-oxidized enzyme. This observation would imply that the gating function is contained within the immediate vicinity of the cofactor. Intriguingly, in addition to signaling the cofactor-proximal physical gate to open, the conformational change upon substrate binding in α must have additional impact on the RT pathway in β that allows the hole to propagate outward from the cofactor into α once the gate has opened. In the absence of α, the Mn\textsuperscript{IV}/Fe\textsuperscript{IV} cluster in β must propagate a hole into the activation-specific pathway involving Y\textsubscript{338} more efficiently than it does into the catalysis-specific pathway involving Y\textsubscript{338}, because replacement of the former residue by F slows reduction of the Fe\textsuperscript{IV} site by 10–65-fold,\textsuperscript{47} whereas substitution of the latter has no effect on the kinetics of Fe\textsuperscript{IV}-site reduction.\textsuperscript{57} Conversely, in the holoenzyme complex, it seems that Y\textsubscript{338} is oxidized to a radical in great preference to Y\textsubscript{222} and only with the former residue replaced by the redox-incompetent F does Y\textsubscript{222} undergo oxidation. Thus, the conformational change in some way switches the relative reactivities of Y\textsubscript{222} and Y\textsubscript{338} toward one-electron oxidation.

On the basis of these observations and those by Stubbe and co-workers on their Ec β-Y\textsubscript{112}NO\textsubscript{2}-Y variant,\textsuperscript{57} our working hypothesis is that the key event in opening of the RT gate in both class Ia and class Ic RNRs is a conformational change, driven by binding of the substrate in α and propagated across the subunit interface into β, that engages the mechanism by which a proton is transferred to or within the initial oxidant (Y\textsubscript{112}\textsuperscript{*} or the Mn\textsuperscript{IV}/Fe\textsuperscript{IV(III)} cluster). This PT is coupled to ET from the cofactor proximal RT residue (W\textsubscript{148/5}, or Y\textsubscript{356/338}) and makes this redox step sufficiently favorable to initiate the cascade of hopping events. For the case of Ec RNR, our recent collaborative study with the Stubbe group has provided evidence in support of their prior suggestion that the water bound to Fe\textsubscript{5} of the μ-oxo-Fe\textsubscript{112} cluster is the proton donor to the Y\textsubscript{112}\textsuperscript{*} oxidant.\textsuperscript{69} Thus, it is likely that the D\textsubscript{84} ligand to Fe\textsubscript{5} in Ec β, which is (i) completely conserved among class Ia orthologues but invariably replaced by E in the class Ic orthologues and (ii) in hydrogen-bonding distance from both Y\textsubscript{112} and the Fe\textsubscript{5} water ligand in crystal structures of Ec β\textsubscript{112},\textsuperscript{70} mediates the crucial PT. Therefore, the gate-opening transition could well involve re-orientation of this carboxylate ligand or enhancement of its conformational dynamics in a manner that enables its PT mediation. In consideration of this hypothesis, the behavior of the Ec β-D\textsubscript{84}E variant, which we previously showed is fully competent for cofactor assembly,\textsuperscript{71} in the holoenzyme complex becomes an intriguing issue for future examination, because it is expected that even subtle re-orientation of the carboxylate group by the presence of the additional methylene unit in the Ec α side chain could either completely disable the crucial PT pathway or derange its conformational modulation, rendering the variant protein inactive, ungated, or both. Additionally, we\textsuperscript{45} and others\textsuperscript{72} previously have speculated that the gate-opening step in C\textsubscript{t} RNR may involve protonation of either the o xo- or hydroxo-bridge of the μ-oxo-μ-hydroxo-bridged Mn\textsuperscript{IV}/Fe\textsuperscript{III} cofactor.\textsuperscript{73} Whether the cognate residue in C\textsubscript{t} β, E\textsubscript{89}, might also mediate the gatekeeping proton transfer is an open question.

### ASSOCIATED CONTENT

#### Supporting Information

Description of and parameters used in the simulation of the tyrosyl radicals reported in Figure 3, a schematic drawing of the phenol ring of a tyrosine residue with numerical assignments used for the simulation of the radicals, a figure demonstrating the competence of the β-wt-α-Y\textsubscript{99}F complex but not the β-Y\textsubscript{122}F/Y\textsubscript{338}F-α-wt-Y\textsubscript{99}F complex to form a radical, and a figure used for quantitative determination of the concentrations of the Mn\textsuperscript{IV}/Fe\textsuperscript{IV} and Y\textsuperscript{*}(s) are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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#### Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Radical-translocation Intermediates and Hurdling of Pathway Defects in “Superoxidized” (Mn^{IV}/Fe^{IV}) Chlamydia trachomatis Ribonucleotide Reductase

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Simulation of Tyrosyl Radicals. Simulation of the X-band EPR spectra of the radicals shown in Figure 3 was carried out by using EasySpin (www.easyspin.org).\(^1\) A total electronic spin \((S_{\text{total}})\) of \(\frac{1}{2}\) was assumed. Hyperfine interactions with six nonequivalent nuclei, two \(C_\beta\) methylene protons and the four protons on the phenyl ring, were considered. The principal \(g\)-values of tyrosyl radicals have a polynomial dependence on the spin density of the C1 carbon, with the \(g_x\) component being the most sensitive one.\(^2\) The \(g_y\) and \(g_z\) principal values vary less among the different radical signals studied.\(^2\) For both radicals, the line widths were assumed to be isotropic and pseudo-Voigtian, with an additional anisotropic line broadening contribution being introduced for the radical in the wt \(\alpha\cdot\beta\) complex. The hyperfine coupling constants of the ring protons are quite conserved among different tyrosyl radicals.\(^2\) Therefore, their values in the simulations were allowed to vary within a narrow range of 2 MHz of the reported, typical values.\(^2\) The Euler angles between the \(A_x\) tensor component of the ring protons and the \(g_x\) direction were assumed to be similar to those previously reported.\(^2\) Analysis of various tyrosyl radicals by ENDOR spectroscopy has revealed that the hyperfine tensors for the methylene protons are nearly axial, with the largest component being \(A_x\) \((A_{\text{parallel}})\) and the other two components being nearly equal \([A_y \approx A_z\) \((A_{\text{perpendicular}})\)].\(^2\) The axial symmetry was an additional constraint for the determination of the hyperfine couplings of the methylene proton by simulations. Overall, the difference between the \(g_x\) values obtained for the \(\beta\)-wt\(\cdot\alpha\)-wt and the \(\beta\)-Y338F\(\cdot\alpha\)-wt complexes are indicative of different spin densities on the C1 carbon, whereas the different hyperfine couplings and their degree of anisotropy \((A_{\text{parallel}}/A_{\text{perpendicular}})\) reflect a different dihedral angle, \(\theta\), between the \(C_\beta\)-H
bond and the axis normal to the plane of the aromatic ring. The simulation parameters are provided in Table S1.

**Spin Quantification of EPR Signals.** The quantification of duplicate samples of Ct β Mn\textsuperscript{IV}/Fe\textsuperscript{IV} and the RT pathway Y•(s) (see Materials and Methods for preparation of samples) were carried out as previously described.\textsuperscript{3} The results are presented in Table S2. Samples were prepared as described in the legend of Figure S2.

| Table S1: Simulation parameters of RT pathway radicals in Ct RNR |
|------------------|------------------|------------------|
| Species          | β-wt•α-wt         | β-Y\textsubscript{338}F•α-wt |
| A\textsubscript{H1} (MHz) | 31.3, 27.3, 24.0 | 56.0, 49.0, 49.0 |
| A\textsubscript{H2} (MHz) | 16.0, 15.0, 15.0 | 2.0, 1.0, 1.0 |
| A\textsubscript{H3} (MHz)a | -24.7, -8.0, -22 | -24.7, -8.0, -20.0 |
| A\textsubscript{H5} (MHz)b | -26.7, -8.0, -22.4 | -25.5, -8.0, -20.4 |
| A\textsubscript{H2} (MHz)c | 5.0, 7.5, 1.5 | 5.0, 7.5, 1.5 |
| A\textsubscript{H6} (MHz)d | 5.0, 7.5, 1.5 | 5.0, 7.5, 1.5 |
| Line width (mT) | 0.24, 0.25, 0.26 | 0.15 |
| g                | 2.0104, 2.0045, 2.0016 | 2.0074, 2.0046, 2.0018 |

\(a\) A was rotated into the frame of \(g\) around \(A_z\) using Euler angles of \(\alpha = 22^\circ\), \(b\) \(\alpha = -22^\circ\), \(c\) \(\alpha = 10^\circ\), and \(d\) \(\alpha = -10^\circ\).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Area (arbitrary units)</th>
<th>Relative amount of Mn\textsuperscript{IV}/Fe\textsuperscript{IV} (%)</th>
<th>Relative amount of Y• (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>− CDP, ATP (trial 1)</td>
<td>30</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>+ CDP, ATP (trial 1)</td>
<td>37</td>
<td>54</td>
<td>46</td>
</tr>
<tr>
<td>− CDP, ATP (trial 2)</td>
<td>28</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>+ CDP, ATP (trial 2)</td>
<td>29</td>
<td>56</td>
<td>44</td>
</tr>
</tbody>
</table>
Scheme S1: Schematic drawing of the phenol ring of a tyrosine residue with numerical assignments used for the simulation of the radicals.
**Figure S1.** X-band EPR spectra of samples prepared with the *Ct* α-Y991F variant demonstrating the generation of an organic radical when complexed with *Ct* β-wt (black spectra), but failure to form any significant organic radical when in complex with *Ct* β-Y222F/Y338W (green spectra). The “raw” spectra are shown in panel A, whereas spectra in panel B have had the contribution of the MnIV/FeIV states removed. Sample preparation and spectrometer conditions are identical to those described in the legend of Figure 1.
Figure S2. Representative X-band EPR spectra demonstrating loss of signal intensity attributable to the MnIV/FeIV intermediate upon formation of the Y•. MnII/FeII-β was first mixed with O2-saturated buffer to form the MnIV/FeIV intermediate, and after 2 s, the resultant solution was mixed either with α, MgSO4, and DTT (red spectrum) or with one α, CDP, ATP, MgSO4, and DTT (blue spectrum). After mixing with the α-containing solution, the reaction was quenched after 1 s by spraying the reaction mixture into cold (~120 K) 2-methylbutane. The intensity attributable to the MnIV/FeIV intermediate is ~45% less in the spectrum of the sample in which the Y• is formed than in the sample in which no Y• accumulates (green spectrum). This loss of intensity correlates well to a ~45% increase in signal intensity in the fourth line of the six packets of resonances observed. This increased intensity is attributable to the Y•. Final concentrations after mixing were: 0.2 mM β, 0.15 mM Mn, 0.15 mM Fe, 0.3 mM α, 1 mM CDP (when present), 0.5 mM ATP (when present), 10 mM MgSO4, 10 mM DTT. The spectrometer conditions were: T = 14 ± 0.2 K, microwave frequency = 9.47 GHz, microwave power = 20 µW, modulation
frequency = 100 KHz, modulation amplitude = 10 G, time constant = 167 ms, scan time = 167 s.

REFERENCES


Appendix C:

O$_2$-Evolving Chlorite Dismutase as a Tool for Studying O$_2$-Utilizing Enzymes

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Acknowledgements

This work is the “brainchild” of my co-advisor, Prof. J. Martin Bollinger, Jr., who first had the idea for the in situ generation of O₂ after hearing Dr. Jennifer DuBois give a talk on the Cld enzyme. I am very thankful that he shared his vision with me. Dr. Bennett Streit and Béatrice Blanc, both graduate students at the time with Prof. Jennifer DuBois, provided some Cld protein, and eventually, the expression plasmid. Dr. Timothy H. Yosca, a graduate student at the time in Prof. Michael T. Green’s laboratory, provided us with the $^{57}$Fe-myoglobin protein used in this study. Denise Conner and Michael Lee, both graduate students at the time in the Bollinger and Krebs group, provided proteins and helped with the execution of experiments. Both of my co-advisors, Profs. J. Martin Bollinger, Jr. and Carsten Krebs, guided me through the design of experiments, at times aided me with the execution of experiments, and helped with the interpretation of the data. Prof. Bollinger, Jr. wrote the majority of the manuscript, with Prof. Krebs making significant contributions, and with some help from Prof. DuBois.
O2-Evolving Chlorite Dismutase as a Tool for Studying O2-Utilizing Enzymes


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O₂-Evolving Chlorite Dismutase as a Tool for Studying O₂-Utilizing Enzymes

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Supporting Information

ABSTRACT: The direct interrogation of fleeting intermediates by rapid-mixing kinetic methods has significantly advanced our understanding of enzymes that utilize dioxygen. The gas’s modest aqueous solubility (<2 mM at 1 atm) presents a technical challenge to this approach, because it limits the rate of formation and extent of accumulation of intermediates. This challenge can be overcome by use of the heme enzyme chlorite dismutase (Cld) for the rapid, in situ generation of O₂ at concentrations far exceeding 2 mM. This method was used to define the O₂ concentration dependence of the reaction of the class Ic ribonucleotide reductase (RNR) from Chlamydia trachomatis, in which the enzyme’s Mn⁴⁺/Fe³⁺ cofactor forms from a Mn²⁺/Fe⁴⁺ complex and O₂ via a Mn⁴⁺/Fe⁴⁺ intermediate, at effective O₂ concentrations as high as ~10 mM. With a more soluble receptor, myoglobin, an O₂ adduct accumulated to a concentration of >6 mM in <15 ms. Finally, the C–H bond-cleaving Fe⁴⁺-oxo complex, J, in taurine:α-ketoglutarate dioxygenase and superoxo–Fe²⁺/III complex, G, in myo-inositol oxygenase, and the tyrosyl-radical-generating Fe³⁺/IV intermediate, X, in Escherichia coli RNR, were all accumulated to yields more than twice those previously attained. This means of in situ O₂ evolution permits a >5 mM “pulse” of O₂ to be generated in <1 ms at the easily accessible Cld concentration of 50 μM. It should therefore significantly extend the range of kinetic and spectroscopic experiments that can routinely be undertaken in the study of these enzymes and could also facilitate resolution of mechanistic pathways in cases of either sluggish or thermodynamically unfavorable O₂ addition steps.

Aerobic organisms are replete with proteins and enzymes that react with O₂ for such purposes as cellular and organismal respiration,1 oxidation reactions of primary and secondary metabolism,2–4 catabolism of drug and xenobiotic compounds,5,6 biosynthesis of enzyme cofactors,7–9 neurotransmitters,8 and natural products (e.g., neurotransmitters,8 and natural products)9 regulation of transcription,10–13 and uptake and storage of inorganic nutrients.14 Many of these enzymes employ reduced cofactors, consisting of one or more reduced transition metal (typically Fe²⁺15–18 or Cu¹19–21) or a reduced flavin,22 that combine with O₂ to form potently oxidizing intermediates that directly or indirectly transform their substrates. Rapid-mixing transient kinetic studies have contributed greatly to our understanding of the mechanisms of these enzymes by permitting the direct detection, kinetic tracking, and spectroscopic characterization of fleeting intermediates in their catalytic cycles.15–18 The fleeting nature of the intermediates formed (typical half-lives of <1–10 s) has, with a few spectacular exceptions,23–25 precluded their three-dimensional structural characterization by X-ray crystallography. The alternative approach has been (i) to trap the intermediates at their maximal extents of accumulation by the freeze-quench method and (ii) to subject them to a suite of spectroscopic methods. In combination with density functional theory (DFT) calculations,26 these methods can afford local, high-resolution structural information about transient species, thereby providing “snapshots” along the reaction coordinate.27–29

In the study of these enzymes, the physical properties of O₂ impose certain challenges to the elucidation of reaction kinetics and the direct characterization of intermediates. Its gaseous nature makes systematic variation of its concentration more challenging and introduces greater uncertainty into concentration values than for nonvolatile substrates. More importantly, the modest solubility of the gas imposes a very constraining upper limit of ~2 mM on the O₂ concentration that can be achieved without specialized apparatus. O₂ has often been found to combine with the reduced enzyme cofactors with second-order rate constants of 10⁴–10⁷ M⁻¹ s⁻¹, which, at the routinely accessible O₂ concentration of 1 mM, are sufficient to
give effective first-order rate constants of $10^3$–$10^4$ s$^{-1}$.31–34 In many cases, these formation rate constants have proven to be comparable to or greater than the first-order rate constants for decay of the key intermediates. In these cases, accumulation of the intermediate states for detailed characterization has been possible.34–40 In other cases, isotopic or chemical modification of the substrate or mutagenesis of the protein has been used to slow decay of intermediates to permit their accumulation and characterization.41–43 In still other cases, however, the obstacle presented by the modest solubility of O$_2$ has not been overcome, and many intriguing O$_2$-dependent enzyme reactions have thus far proven to be resistant to this powerful approach to mechanistic dissection.44

Several spectroscopic methods that can reveal important structural details for intermediates demand very concentrated samples that are highly enriched in the desired state. For example, application of extended X-ray absorption fine structure (EXAFS) spectroscopy, which in ideal cases can provide very precise metal–ligand and metal–metal distances for reactive intermediates, to dilute or heterogeneous freeze-quenched samples is notoriously problematic and in several notable cases has provided distances that cannot be reconciled with those in DFT-derived structures and inorganic model complexes. Other methods, such as the developing technique of nuclear resonance vibrational spectroscopy (NRVS), which can reveal structural details of iron complexes, require that targets be present at concentrations exceeding the solubility of O$_2$. Long-lived complexes may be generated at such high concentrations by direct treatment of precursors with gaseous O$_2$ but the sluggishness of transport across the gas–liquid interface makes such an approach impractical for complexes with half-lives of less than ~1 min. A rapid-mixing method permitting reaction with O$_2$ at greater concentrations without the need for specialized equipment could, therefore, open doors to new experiments in this area of biochemistry.

The heme enzyme chlorite dismutase (Cld) rapidly converts chlorite (ClO$_2^-$) to chloride (Cl$^-$) and O$_2$, suggesting a simple approach to overcoming both the technical difficulties in the systematic variation of O$_2$ concentration and its modest solubility. A number of proteobacteria have been shown to catalyze this reaction in conjunction with perchlorate (ClO$_4^-$) respiration. ClO$_2^-$ is sequentially reduced to ClO$_3^-$ and ClO$_2^-$ by a membrane-bound molybdenopterin-dependent perchlorate reductase, which couples the reductions to the generation of a proton gradient. The resulting ClO$_2^-$ would accumulate and kill the organism in the absence of the detoxification reaction catalyzed by Cld. Accordingly, the reaction must be fast to serve its biological function: the homopentameric Cld from $D$. aromatica (Da) is one of the fastest and most efficient Clds yet studied, with a $k_{cat}$ value of $(2.0 \pm 0.6) \times 10^5$ s$^{-1}$ (per heme) at 4 °C and pH 5.2.31,53,54 This rate constant suggests that Cld could support the generation of tens-of-millimolar O$_2$ on the millisecond time scale. Importantly, the enzyme is not significantly inhibited by millimolar concentrations of either of its two products, Cl$^-$ and O$_2$. It is, moreover, capable of approximately 1.7 $\times$ 10$^4$ turnovers per heme before undergoing irreversible inactivation because of oxidative damage to the heme. We therefore reasoned that catalytic concentrations of Cld could be used to initiate the reaction of an O$_2$-utilizing (metallo)enzyme by rapid mixing with the highly soluble, nonvolatile ClO$_2^-$ rather than with the sparingly soluble, gaseous O$_2$. Here, we demonstrate that this approach can indeed simplify the experimental variation of O$_2$ concentration, expand the range of O$_2$ concentrations that can be interrogated with commonly available equipment, and permit preparation of O$_2$-dependent intermediate states at concentrations and purities not accessible by conventional rapid mixing with O$_2$-containing aqueous solutions.

### EXPERIMENTAL PROCEDURES

**Materials.** Sodium chlorite (NaClO$_2$), α-ketoglutaric acid, sodium ascorbate, and horse heart myoglobin (Mb) were purchased from Sigma-Aldrich. $D$. aromatica enzymes 1,1,2,2,-[${}^{3}$H$_2$]$_2$-Aminoethane-1-sulfonic acid (d$_c$-taurine) and 1,2,3,4,5,6-[${}^{3}$H$_4$] cyclohexan-(1,2,3,5/4,6)-hexa-ol (d$_c$-myo-inositol or d$_c$-MI) were purchased from C/D/N Isotopes.

**Preparation of Proteins.** Methods for overexpression and purification of the $\beta_2$ subunit of *Chlamydia trachomatis* (Ct) ribonucleotide reductase (RNR), D. aromatica Escherichia coli taurine-α-ketoglutarate (αKG) dioxygenase (TauD), *E. coli* (Ec) RNR-β$_2$, and *Mus musculus* myo-inositol oxygenase (MIOX) have been presented previously.7,35,36,51,55 To prepare myoglobin (Mb) containing heme with natural abundance iron (56Fe-Mb), 400 mg of lyophilized horse heart Mb (Sigma-Aldrich product no. M1882) was dissolved in 2 mL of 100 mM potassium phosphate buffer (pH 6.8). The protein was loaded onto a 50 mL anion exchange column (DE-52, Whatman) and eluted by gravity flow with the same buffer. Fractions with $A_{400}$/ $A_{280}$ Ratios (R$_c$) of at least 5 were pooled and concentrated to a hemoglobin concentration of ~10 mM. For 57Fe-enriched heme (57Fe-Mb), the synthesis of metallorhaphin was adapted from that of Adler et al. for 57Fe heme enrichment. Apoprotein, generated using Teale’s method, was reconstituted at pH 7.5. Excess heme was removed by anion exchange chromatography using Whatman DE-52 resin, as described above for 56Fe-Mb. Fractions with R$_c$ > 5 were pooled and concentrated to a heme concentration of ~10 mM.

**Stopped-Flow Absorption and Freeze-Quench EPR and Mössbauer Experiments.** Procedures for the stopped-flow and freeze-quench experiments and the spectrometers for the stopped-flow, EPR, and Mössbauer measurements have been described previously.

**Analysis of the Stopped-Flow Absorption Kinetic Data.** $A_{390}$ versus time traces reflecting accumulation and decay of the MnIV/FeIV activation intermediate in Ct-RNR-β$_2$ were analyzed by nonlinear regression according to the equation

$$A_t = A_0 + \Delta A_1[1 - \exp(-k_1t)] + \Delta A_2[1 - \exp(-k_2t)]$$

which gives absorbance as a function of time ($A_t$) for two irreversible first-order reactions in terms of the rate constants ($k_1$ and $k_2$), the amplitudes associated with each reaction (Δ$A_1$ and Δ$A_2$), and the initial absorbance ($A_0$) at time zero. The formation of the intermediate and its decay are sequential processes, but their well-resolved rate constants make the assumption of parallel reactions acceptable. Simulation of these traces was conducted using KinTek Explorer (KinTek Corp.). The kinetic mechanism for Cld and the $K_{cat}$ for the Cld-ClO$_2^-$ Michaelis complex that were assumed in the simulations are given in Results. Kinetic constants for the formation and decay of the intermediate acquired from the regression fits in Figure 1B were assumed in the simulations. $k_{cat}$ values of 30,000, 60,000, 120,000, and
200,000 s⁻¹ (panels A–D of Figure S2 of the Supporting Information, respectively) were assumed for Cld.

**Analysis of EPR and Mössbauer Spectra for Quantifying the Mn⁴⁺/Fe⁴⁺ Intermediate in Ct RNR-β₂.** Double integration of first-derivative EPR signals was conducted using the graphing and analysis program KaleidaGraph (Synergy Software). Comparison to the corresponding double integral for the spectrum of a Cu(II)(ClO₄)₂ standard with correction for the different g-values permitted calculation of absolute spin concentration. The spectral contribution of mononuclear MnII was subtracted out by using the spectrum of a sample of apo β₂ to which a known amount of MnII had been added. The contribution from the Fe₃⁴/IV complex (X) was quantified by individually integrating the six peaks of the sextet signal of the Mn⁴⁺/Fe⁴⁺ intermediate. The double integrals of five of the peaks (all except for the fourth, with which the spectrum of X overlaps) are identical within error. The difference between the area of the fourth peak and the average area of the other five peaks represents the contribution of X to the experimental spectrum, which corresponds to 6% of the total spin. For samples prepared by the freeze-quench method, the absolute spin concentration in the reaction solution depends also on the “packing factor”, which is the fraction of the packed material that consists of the actual solution (the fraction not contributed by the frozen cryosolvent). The spin concentration determined from comparison of the double integral of samples to that of the standard is divided by this packing factor to account for dilution of the frozen sample by the cryosolvent. In our extensive experience using isopentane as the cryosolvent, we have repeatedly measured packing factors of 0.52. The narrower range of 0.50–0.60. 

Table S1 of the Supporting Information provides concentrations of the paramagnetic species determined over a range of packing factors of 0.50–0.60. The narrower range of 0.54–0.56, which agrees with the mean packing factor of 0.55 that we have determined over many years, gives ranges of 1.24–1.16 and 0.13–0.11 mM for the concentrations of the Mn⁴⁺/Fe⁴⁺ complex and X, respectively. These ranges are in good agreement with the values determined by analysis of the Mössbauer spectra.

The multiple Fe-containing species present in the freeze-quench samples all contribute to the experimental Mössbauer spectra, requiring that the spectra be “deconvoluted” into their components to extract the concentrations of species. However, the field orientation dependence of the predominant species, the Mn⁴⁺/Fe⁴⁺ intermediate, provides an alternative means of accurate quantification. This analysis was conducted as previously described. The slightly different spin Hamiltonian parameters used herein are provided in Table S2 of the Supporting Information and compared to the published values.

**RESULTS**

**Activation of the β₂ Subunit of Ct RNR by Mixing Its Mn⁴⁺/Fe⁴⁺ Complex with ClO₄⁻ in the Presence of Cld.** We selected the activation reaction of the manganese- and iron-dependent class Ic Ct RNR as an ideal test case for the *in situ* generation of O₂ by the Cld/ClO₄⁻ system. Previous studies showed that reaction of the Mn⁴⁺/Fe⁴⁺ complex of the enzyme’s β₂ subunit with O₂ results in formation of the catalytically functional Mn⁴⁺/Fe⁴⁺ cofactor via a novel Mn⁴⁺/Fe⁴⁺ activation intermediate. The reaction is a kinetically well-behaved, two-step sequence, in which the first step, formation of the Mn⁴⁺/Fe⁴⁺ intermediate, is cleanly first-order in O₂. Both steps in the sequence are associated with absorbance changes, with the reactant complex being essentially transparent and the intermediate absorbing maximally at 390 nm with a molar absorptivity (ε₃₉₀ ~ 4500 M⁻¹ cm⁻¹) approximately twice that of the Mn⁴⁺/Fe⁴⁺ product. These characteristics permit convenient monitoring in stopped-flow absorption experiments.

Rapid mixing of a solution containing the Ct β₂ protein (200 μM dimer), Mn³⁺ (3 equiv relative to β₂), Fe²⁺ (1 equiv), and Cld (10 μM heme) with an equal volume of a 20 mM ClO₄⁻ solution (100 equiv relative to β₂, 2,000 equiv relative to Cld heme) results in a rapid increase in the absorbance at 390 nm (A₃₉₀) followed by its slower decay to approximately half of the maximal value (Figure 1A, black trace). The trace is qualitatively similar to that obtained by mixing the Mn⁴⁺/Fe⁴⁺--β₂ complex with O₂-saturated buffer (green trace). Analogous traces from control reactions from which either the Mn⁴⁺/Fe⁴⁺--β₂ reactant (blue trace), the ClO₄⁻ reactant (orange trace), or the Cld catalyst (red trace) was omitted do not show the characteristic behavior, suggesting that the transient behavior of the complete reaction reflects formation and decay of the Mn⁴⁺/Fe⁴⁺ intermediate specifically as a result of the evolution of O₂ from ClO₄⁻ by Cld.

To verify that the complete reaction including the Mn⁴⁺/Fe⁴⁺--β₂ complex, Cld, and ClO₄⁻ produces the expected Mn⁴⁺/Fe⁴⁺ activation intermediate, freeze-quench EPR and Mössbauer samples were prepared from a concentrated Mn⁴⁺/Fe⁴⁺--β₂ reactant solution (giving a final β₂ concentration of 1.88 mM with 1 equiv of ṭFe(II) and 2 equiv of Mn(II)). The Mössbauer and X-band EPR spectra of identical samples that were allowed to react for 1 s (near the time of maximal A₃₉₀ in the black trace in Figure 1A) before being freeze-quenched are shown in Figure 2, and quantitative analysis of these spectra is summarized in Tables S1 and S2 of the Supporting Information. The spectra are dominated by the features of the Mn⁴⁺/Fe⁴⁺ intermediate, confirming that it is formed in high yield [1.2 ± 0.3 mM by EPR and 1.2 ± 0.2 mM (63 ± 8% of total Fe) by Mössbauer]. A small fraction of the Fe₃⁴/IV intermediate, X, resulting from reaction of O₂ with Fe₃⁴/III centers formed in competition with the desired Mn⁴⁺/Fe⁴⁺--β₂ reactant complex, is also detected [0.2 ± 0.1 mM by EPR and 0.2 ± 0.1 mM (13 ± 5% of total Fe) by Mössbauer]. The spectrosopic results thus establish that the Cld/ClO₄⁻ system does indeed support formation of the expected intermediate.

The stopped-flow absorption kinetic traces of Figure 1A suggest that the Mn⁴⁺/Fe⁴⁺ intermediate forms much faster in the Cld/ClO₄⁻ reaction than in the O₂-saturated buffer reaction (compare black and green traces), consistent with a greater O₂ concentration in the former case. To evaluate the effective O₂ concentration more quantitatively, experiments were conducted with varying ClO₄⁻ concentrations (Figure 1B). Effective first-order rate constants (kₗₒₒ) for intermediate formation extracted by regression analysis of the A₃₉₀ kinetic traces are linearly dependent on ClO₄⁻ concentration at ≤ 4 mM ClO₄⁻. The slope of the line, corresponding to the effective second-order rate constant, agrees precisely with that obtained by direct variation of O₂ concentration by mixing with O₂-containing buffer (gray diamonds in Figure 1C). This result indicates that, at ≤ 4 mM ClO₄⁻, the Cld completely converts ClO₄⁻ to O₂ and Cl⁻ sufficiently rapidly that it does not impose a lag phase on Mn⁴⁺/Fe⁴⁺ intermediate formation (which would tend to diminish the...
At greater ClO$_2^-$ concentrations, deviation from this strict first-order dependence is observed. Doubling the Cld concentration from 5 to 10 μM had no significant effect on the values of $k_{\text{obs}}$ for ≤4 mM ClO$_2^-$ but gave greater values (deviating less from the first-order dependence) at >4 mM ClO$_2^-$ (Figure S1 of the Supporting Information). This observation suggests that the deviation is not intrinsic to the Cld reaction but rather reflects failure of the Cld reaction to rapidly reach completion at the higher ClO$_2^-$ concentration. This conclusion is consistent with the chlorite-dependent destruction of the heme previously shown to limit turnover in the steady state.

Figure 1. (A) The 390 nm absorbance vs time traces following rapid mixing at 5 °C of a solution containing 0.2 mM β$_2$, 0.6 mM Mn$^{II}$, 0.2 mM Fe$^{II}$, and 0.01 mM Cld with an equal volume of either 20 mM ClO$_2^-$ (black trace), O$_2$-saturated 100 mM HEPES buffer (pH 7.6) (green trace), or O$_2$-free buffer (orange trace). Traces from control reactions, from which either Cld or β$_2$ was omitted, are colored red and blue, respectively. (B) Delineation of the O$_2$ concentration dependence of the Cld activation reaction by variation of ClO$_2^-$ concentration. Reactions were conducted as described for the black trace in panel A, but with the concentration of the ClO$_2^-$ reactant solution varied to give the final ClO$_2^-$ concentrations noted in the figure. Traces were analyzed by nonlinear regression using the equation for two exponential phases (solid lines through data (see the Supporting Information for analysis)) to extract observed first-order rate constants for formation of the Mn$^{IV}$/Fe$^{IV}$ intermediate ($k_{\text{obs}}$). (C) Plot of these observed first-order rate constants vs ClO$_2^-$ or O$_2$ concentration. The points at ≤4 mM ClO$_2^-$ were fit by the equation for a line ($\bullet$). Extrapolation of the $k_{\text{obs}}$ for the reaction with 16 mM ClO$_2^-$ to the linear fit line (---) in this case gave an effective O$_2$ concentration of 9 mM (arrow). The gray diamond points are values of $k_{\text{obs}}$ obtained after mixing with either O$_2$-saturated buffer (as in panel A, green trace) or buffer prepared by diluting O$_2$-saturated buffer 2- or 4-fold with O$_2$-free buffer, which has been done in the past to define the O$_2$ concentration dependence of the reaction.

Figure 2. 4.2 K/53 mT Mössbauer and EPR spectra of Ct $\beta_3$ samples enriched in the Mn$^{IV}$/Fe$^{IV}$ activation intermediate. The preparation of the samples is described in the text. (A) Experimental Mössbauer spectra were recorded with the magnetic field oriented parallel (top) or perpendicular (middle) to the γ beam, and the difference spectrum (bottom) was obtained mathematically. The solid blue and red lines are theoretical spectra of the Mn$^{IV}$/Fe$^{IV}$ and Fe$^{III}$/IV complexes, plotted at 63 and 13% of the total intensity, respectively. The theoretical spectrum of the Fe$^{III}$/IV intermediate was generated with published parameters. The parameters used to generate the theoretical spectrum of the Mn$^{IV}$/Fe$^{IV}$ intermediate are slightly different from the previously published ones (see Table S2 of the Supporting Information). The spectrum shown matches the experimental difference spectrum more precisely than that generated with the published parameters and therefore permits more precise quantification. We attribute the need for these slight adjustments to the facts that the previously published parameters were obtained by “global” simulation of multiple spectra and the new spectra have a significantly better signal-to-noise ratio because they were collected on a more concentrated sample. (B) EPR spectrum showing that the predominant EPR-active species is the Mn$^{IV}$/Fe$^{IV}$ intermediate. The spectrum of the contaminating Fe$^{II}$/II complex with O$_2$ overlaps with the fourth line of the sextet and contributes 6% of the total spin (quantitative analysis of the spectrum is described in Experimental Procedures). Spectrometer conditions were as follows: 9.5 GHz microwave frequency, 20 μW microwave power, 14 ± 0.2 K temperature, 100 kHz modulation frequency, 10 G modulation amplitude, 167 s scan time, and 167 ms time constant.

not intrinsic to the Cld reaction but rather reflects failure of the Cld reaction to rapidly reach completion at the higher ClO$_2^-$ concentration. This conclusion is consistent with the chlorite-dependent destruction of the heme previously shown to limit turnover in the steady state.° Competition between
ClO\textsubscript{2}\textsuperscript{-}/Cl\textsuperscript{−} system for in situ generation of O\textsubscript{2} and to extract an estimate of k\textsubscript{cat} for ClO\textsubscript{2}\textsuperscript{-}/Cl\textsuperscript{−} under these reaction conditions (Figure S2 of the Supporting Information). A simple rapid equilibrium binding kinetic model (ClO\textsubscript{2}\textsuperscript{-}/Cl\textsuperscript{−} \rightleftharpoons Cl\textsuperscript{−}O\textsubscript{2}/Cl\textsuperscript{−}) with a value of K\textsubscript{D} equal to the published value of K\textsubscript{D} for ClO\textsubscript{2}\textsuperscript{-} (215 μM) was assumed. The value of the rate constant for the single-step conversion of the bound substrate to free Cl\textsuperscript{−} and O\textsubscript{2} (equivalent to k\textsubscript{cat} in this minimal kinetic scheme) was allowed to vary. k\textsubscript{cat} values of ≤60,000 s\textsuperscript{-1} gave simulated traces with excessively pronounced lag phases and insufficiently rapid rises in A\textsubscript{380} compared to the experimental traces (Figure S2 of the Supporting Information). k\textsubscript{cat} values of ≥120,000 s\textsuperscript{-1} gave more acceptable agreement (Figure S2 of the Supporting Information) and are consistent with those measured in the steady state at pH ≤7. This extremely high turnover rate confirms that it should be possible to generate a >5 mM pulse of O\textsubscript{2} in <1 ms at the easily accessible ClO\textsubscript{2}\textsuperscript{-}/Cl\textsuperscript{−} system.

Verification of a High Yield of O\textsubscript{2} from the ClO\textsubscript{2}\textsuperscript{-}/Cl\textsuperscript{−} System by Monitoring Conversion of Fe\textsuperscript{III}-Myoglobin to Oxymyoglobin. To demonstrate the potential of using ClO\textsubscript{2}\textsuperscript{-}/Cl\textsuperscript{−} as a very soluble and efficient O\textsubscript{2} receptor (Figure 3, bottom) reveals essentially minimum of 6.5 mM O\textsubscript{2} in sample indicates that the 25 μM myoglobin (Mb) as a very soluble and efficient O\textsubscript{2} receptor. Extrapolation of the values of k\textsubscript{obs} obtained at the highest ClO\textsubscript{2}\textsuperscript{-} concentration tested (16 mM) to the fit line describing the first-order regime (dashed lines in Figure 1C and Figure S1 of the Supporting Information) indicates that effective O\textsubscript{2} concentrations of 7−11 mM were achieved in the stopped-flow experiments.

The kinetic traces of Figure 1B were simulated to assess limitations of the ClO\textsubscript{2}\textsuperscript{-}/Cl\textsuperscript{−} system for at an Unprecedented Concentration. As an additional demonstration of the utility of the approach for preparing O\textsubscript{2}-dependent reactive intermediates, we targeted the high-spin Fe\textsuperscript{III}-oxo (ferryl) intermediate, J, which accumulates during O\textsubscript{2} activation by Ec TauD. J cleaves the C1−H bond of the substrate, taurine, with a rate constant of 13 s\textsuperscript{-1} at 5 °C and is stabilized significantly (k\textsubscript{cat} = 0.35 s\textsuperscript{-1}) by inclusion of the deuterium-containing substrate, because of a large deuterium kinetic isotope effect. Even with this increased half-life of ~2 s, the complex is still sufficiently short-lived that it must be prepared by rapid mixing methods. Thus, although J has been prepared at a high purity (~80%) and interrogated by several spectroscopic methods, the maximal concentration of ~0.95 mM that has been obtained has precluded application of methods that require very high purity and concentration (e.g., NMR). The ability to make more concentrated samples would make application of these methods feasible and afford the opportunity for further insight into the structure of J.

Preparation of the Ferryl Intermediate, J, in Ec TauD at a Concentration. As an additional demonstration of the utility of the approach for preparing O\textsubscript{2}-dependent reactive intermediates, we targeted the Fe\textsuperscript{IV} \rightleftharpoons Fe\textsuperscript{III} system supports preparation of the intermediate at more than twice the maximal concentration achieved in previous studies and at a comparable purity.

Figure 3. 4.2 K/53 mT (parallel field) Mössbauer spectra demonstrating conversion of ferrous Mb to oxy-Mb by the ClO\textsubscript{2}\textsuperscript{-}/Cl\textsuperscript{−} system. A solution of 10 mM Mb (2.5 mM 57Fe-Mb and 7.5 mM 56Fe-Mb) was reduced with stoichiometric sodium dithionite (top spectrum). The Fe\textsuperscript{III}-Mb reactant was mixed with 0.25 equivalent volume of 0.125 mM ClO\textsubscript{2} and the complete reaction was freeze-quenched after 15 ms (bottom spectrum). The solid lines are quadrupole doublet simulations with parameters nearly identical to those previously published: δ = 0.91 mm/s, and ΔE\textsubscript{Q} = 2.23 mm/s (top); δ = 0.27 mm/s, and ΔE\textsubscript{Q} = 2.29 mm/s (bottom).

Preparation of the Ferryl Intermediate, J, in Ec TauD at an Unprecedented Concentration. As an additional demonstration of the utility of the approach for preparing O\textsubscript{2}-dependent reactive intermediates, we targeted the Fe\textsuperscript{IV}/Fe\textsuperscript{III} system for...
complex, X, that accumulates during activation of class Ia RNRs, including the most extensively studied ortholog from Ec.\(^{27,28,35,45,69–72}\) X oxidizes a conserved tyrosine residue by one electron to a tyrosyl radical (which is essential for the activity of these RNRs\(^{73}\)) as the diiron cluster is reduced to the \(\mu\)-oxy-Fe\(_{II}/III\) product.\(^{53,57,71}\) Previous studies established that the complex has a half-life of \(\sim 1\) s at 5 °C and can be stabilized by \(\sim 5\)-fold by substitution of the tyrosine that it oxidizes (Y122) with a redox inert phenylalanine.\(^{35,71}\) Even with this increased lifetime in the Y122F variant, the complex is still sufficiently short-lived that it must be prepared by rapid mixing methods. As a result, the best samples yet reported have had a concentration of \(\leq 0.77\) mM at a purity of \(\leq 68\%\).\(^{45}\) Characterization of these optimized samples by EXAFS spectroscopy resulted in the report of an Fe–Fe distance of 2.5 Å,\(^{45}\) much shorter than those reported in currently favored structural models.\(^{27,28,69}\) The availability of more concentrated or purer (or both) samples would motivate re-examination of this crucial structural metric either to confirm it with renewed confidence or to revise it upward to a distance more compatible with structural models.\(^{48,49}\)

The Mössbauer spectrum of a sample prepared by mixing a solution containing Ec RNR-β\(_2\)-Y122F (2.6 mM dimer), 7.41 mM \(^{57}\)Fe\(_{II}\), 10 mM ascorbate, and 12.5 \(\mu\)M Cld with 0.25 equivalent volume of 80 mM \(^{55}\)ClO\(_2\)\(^{−}\) and freeze-quenching after 0.30 s (Figure 5, vertical bars) is dominated by the magnetic features of X. The solid line plotted over the data is the theoretical spectrum of X (generated with published parameters\(^{72}\)) plotted at 70% of the total absorption area of the experimental spectrum. This contribution corresponds to a concentration of X of 2.0 mM. Thus, the Cld/ClO\(_2\)\(^{−}\) system preparation of the intermediate at more than twice the concentration achieved in previous studies and at a comparable purity.

**Use of the Cld/ClO\(_2\)\(^{−}\) System To Drive the Reversible O\(_2\) Addition Step Generating the Superoxo–Fe\(_{II}/III\) Complex, G, in MIOX.** A growing number of O\(_2\)-utilizing non-heme metalloenzymes are thought to employ midvalent metal–superoxo complexes, formed by the one-electron oxidative addition of O\(_2\) to the reduced cofactors, to cleave C–H or C–C bonds (or both).\(^{18,20,21,44,74–76}\) For only two such cases have the postulated superoxo complexes been directly detected.\(^{45,77}\) For the unusual di-iron enzyme, "myo-

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**Figure 4.** 4.2 K/53 mT (parallel field) Mössbauer spectrum of a freeze-quenched sample from the reaction of the TauD-Fe\(^{II}\)-αKG-dL-taurine complex with the Cld/ClO\(_2\)\(^{−}\) system (see the text for details). The red line is a simulation of the spectrum of unreacted ferrous component (23%), the blue line is a simulation of the quadrupole doublet spectrum of J (\(\delta = 0.29\) mm/s, and \(\Delta E_Q = 0.90\) mm/s) accounting for 77% of the total intensity of the spectrum, and the solid black line is the summed contribution of both.

**Figure 5.** 4.2 K/53 mT (parallel field) Mössbauer spectrum showing accumulation of the Fe\(_{II}/III\) activation intermediate, X, in Ec RNR-β\(_2\)-Y122F mediated by the Cld/ClO\(_2\)\(^{−}\) system. A solution containing 2.6 mM Ec RNR-β\(_2\)-Y122F dimer, 7.41 mM \(^{57}\)Fe\(_{II}\), 10 mM ascorbate, and 12.5 \(\mu\)M Cld was mixed with 0.25 equivalent volume of 50 mM ClO\(_2\)\(^{−}\), and the reaction was freeze-quenched after 0.30 s. The solid line is the theoretical spectrum of X generated with published parameters\(^{72}\) and plotted at 70% (2.0 mM) of the total absorption area of the experimental spectrum. The contributions from the Fe\(_{II}/III\) and Fe\(_{II}/III\) complexes represent 15% (each) of the total absorption area.
correlate the changes observed by EPR with formation of MI-derived intermediates and products. Irrespective of the answer, the greater accumulation of G and diminution of residual Fe^{III}/MIOX-MI reactant should facilitate further characterization of G. More generally, the results illustrate the capacity of the Cld/ClO$_2^-$ system to overcome the obstacle presented by an O$_2$ addition equilibrium that is unfavorable at the O$_2$ concentrations that can be accessed by conventional methods and equipment.

**DISCUSSION**

The Cld/ClO$_2^-$ system can be used for rapid generation of concentrations of O$_2$ exceeding the normally achievable value of 2 mM to drive accumulation of metalloenzyme intermediates and surmount reversible and disfavored equilibria in the O$_2$ addition steps that initiate the reactions of some enzymes in this class. In addition to allowing for preparation of intermediate complexes that have already been identified, such as the Mn$^{IV}$/Fe$^{IV}$ activation intermediate in Ct RNR and the H$^+$-abstracting ferryl- and superoxo-Fe$_{III}/III$ complexes in TauD and MIOX, respectively, at concentrations and purities required for well-established and developing approaches to structural characterization (e.g., EXAFS and NRVS), this method could permit identification of previously undetected precursors to known complexes, thus further resolving the complex reaction pathways of these enzymes. For example, the reactions of the αKG-dependent oxygenases and the pterin-dependent aromatic amino acid hydroxylases are known to proceed through ferryl complexes that form rapidly without demonstrated accumulation of precursor complexes.

In the former enzymes, ferryl formation involves addition of O$_2$_2 cleavage of both the O−O bond of O$_2$ and the C1−C2 bond of αKG, and formation of a new C2−O bond in the succinate coproduct. Similarly, in the pterin-dependent enzymes, ferryl formation requires O$_2$ addition, O−O bond cleavage, and formation of a new C4a−O bond to the pterin. The kinetic masking of precursors leaves the pathways to ferryl formation experimentally unresolved. With O$_2$ concentrations greater by as much as 10-fold, addition of O$_2$ should occur 10 times more rapidly, perhaps permitting accumulation and identification of ferryl precursors. Alternatively, the failure of precursors to accumulate might reflect the reversible addition of O$_2$ to produce adducts with relatively high dissociation constants (≥1 mM). This situation is also likely in cases for which the initial adducts are proposed to effect difficult H$^*$ abstraction steps, as in MIOX. Here again, the ability to access ~10 mM O$_2$ could permit this obstacle to be overcome. In many characterized reactions, including those of the αKG-dependent oxygenases, acceleration of the initial step would necessarily move the reaction times at which precursors accumulate to <10 ms, a regime that cannot be accessed by conventional cryosolvent-based freeze-quenching. In these cases, the recently developed "microsecond freeze-hyperquenching" technique should permit the reactions to be terminated at these shorter times.

Two crucial requirements for the successful application of the system are the extraordinarily high efficiency of the Cld catalyst and the modest reactivities of target enzymes to ClO$_2^-$.

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**Figure 6.** EPR spectra showing accumulation of the C1−H bond-cleaving superoxo–Fe$_{III}/III$ intermediate, G, in the MIOX reaction with $d_6$-MI initiated by the Cld/ClO$_2^-$ system. A solution containing 1.13 mM Fe$_{III}/III$-MIOX (3 mM total MIOX protein), 60 mM $d_6$-MI (blue spectrum), and 30 μM Cld was mixed either with 2 equivalent volumes of O$_2$-saturated 50 mM Bis-Tris chloride (pH 6.0) buffer (green spectrum) or with 0.5 equivalent volume of a 48 mM ClO$_2^-$ solution (red spectrum), and the reaction was freeze-quenched at a reaction time of ~10 ms. The spectrometer conditions were as follows: 9.5 GHz microwave frequency, 100 kHz modulation frequency, 10 G modulation amplitude, 10 ± 0.2 K temperature, 100 μW power, 0.167 s time constant, and 10 scans per spectrum. The spectra were scaled as indicated to account for dilution and packing factor (the fraction of the sample not contributed by the cryosolvent). The black, dashed line overlaid with the red spectrum is the published spectrum of G, acquired under similar spectrometer conditions. The green and black arrows indicate the g values of G (2.05, 1.98, and 1.91), whereas the red arrow indicates the organic radical signal present in the spectra of both reaction samples but featured more prominently in the Cld/ClO$_2^-$ sample.
coordination to its Fe$^{III}$-heme cofactor. The potential for this complication is minimized by a sequential mixing protocol, which ensures that the Cld catalyst is exposed to the components of the target enzyme reaction for only a few milliseconds before the Cld is exposed to its substrate. In cases for which the target enzyme is more reactive to ClO$_2^-$ or components of the reaction inhibit Cld, it may be important to increase the Cld concentration to maintain the homogeneity of the reaction pathway. Additionally, the catalyst concentration must be elevated in the presence of a very high ClO$_2^-$ concentration to keep the ClO$_2^-$:heme ratio less than ~1,500 to prevent deleterious competition between chlorite-mediated degradation of the Cld heme and generation of O$_2$. This is likely to be an issue primarily when delivery of exact quantities of O$_2$ is desirable (for example, in kinetic studies). The Da Cld used herein is soluble to concentrations of at least 500 μM, so even with use of high target enzyme:Cld volume ratios (e.g., 4:1 in the Mb experiment) to minimize dilution of the target enzyme, Cld concentrations of >100 μM are readily accessible. Given estimates of the $k_{\text{cat}}$ of Cld (>100,000 s$^{-1}$) and the reasonably low $K_M$ for ClO$_2^-$ of 215 μM, this Cld concentration is theoretically capable of generating a 10 mM pulse of O$_2$ in 1 ms. With these impressive parameters, it seems likely that the system will be robust and widely applicable.

## ASSOCIATED CONTENT

* Supporting Information

Figure showing $k_{\text{obs}}$ for formation of the Clt RNR-β$_{-}$−Mn$^{IV}$/Fe$^{IV}$ intermediate as a function of ClO$_2^-$ concentration in three different experiments at two different Cld concentrations, simulations of the stopped-flow kinetic traces reflecting formation and decay of the Mn$^{IV}$/Fe$^{IV}$ intermediate, tables summarizing analysis of Mössbauer and EPR spectra for quantification of the Mn$^{IV}$/Fe$^{IV}$ intermediate in freeze-quenched samples, and the Mössbauer spectrum of a control freeze-quenched sample prepared by mixing Fe$^{II}$-Mb with ClO$_2^-$ in the absence of Cld. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest. The pET41a-cld construct used in this study (protein accession/PDB ID code 3Q08) has been submitted to and can be obtained from the PSI Biology Materials Repository (http://psimr.asu.edu/index.html). This plasmid encodes a truncated form of the Cld from *Dechloromonas aromatica* (full protein sequence accession number YP_285781.1) with improved solubility and heme-incorporation. Please contact Dr. Catherine Cormier (Catherine.Cormier@asu.edu) for use of this plasmid for research purposes. Please address inquiries for the lyophilized protein to be used for research purposes to J. DuBois at jduboisnd@gmail.com. A patent for Cld-mediated dioxygen generation is pending.

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This paper is dedicated to our friend Vincent Huynh, with whom we have often agonized over the low solubility of O$_2$.

### ABBREVIATIONS

RNR, ribonucleotide reductase; Clt, *Chlamydia trachomatis*; Ec, *Escherichia coli*; Da, *Dechloromonas aromatica*; TauD, taurine-$\alpha$-ketoglutarate dioxygenase; αKG, $\alpha$-ketoglutarate; MIOX, myo-inositol oxygenase; DPT, density functional theory; EXAFS, extended X-ray absorption fine structure; NRVS, nuclear resonance vibrational spectroscopy; Cld, chlorite dismutase; EPR, electron paramagnetic resonance; $d_{\text{f}}$, taurine, 1,1,2,2-[2H$_4$]-2-aminoethane-1-sulfonic acid; MI, myo-inositol or cyclohexane-(1,2,3,5,4,6)-hexa-ol; $d_{\text{f}}$-MI, 1,2,3,4,5,6-[2H$_6$]-cyclohexane-(1,2,3,5,4,6)-hexa-ol.

### REFERENCES


O₂-evolving Chlorite Dismutase as a Tool to Study O₂-Utilizing Enzymes†

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**Figure S1.** Observed first-order rate-constant for Mn^{IV}/Fe^{IV} intermediate formation as a function of ClO$_2^-$ from three independent experiments employing two different concentrations of Cld. The onset of the deviation from the first-order dependence on [ClO$_2^-$] occurs at a greater value of [ClO$_2^-$] with the greater [Cld] (10 µM), suggesting that the deviation is associated with inefficiency or inactivation of the Cld catalyst at high concentrations of ClO$_2^-$. 
Figure S2. Kinetic simulations to assess the $k_{\text{cat}}$ of Cld under the reaction conditions described in Fig. 1B. The simplest two-step kinetic model for Cld, in which ClO$_2^-$ binds non-covalently with a $K_D$ of 215 μM [the reported $K_M$ of the reaction (1)] and is converted in a single step to free O$_2$ and Cl$^-$ with a phenomenological rate constant of $k_{\text{cat}}$, was assumed, and $k_{\text{cat}}$ was varied. Simulated traces with the same range of [ClO$_2^-$] used in Fig. 1B and with $k_{\text{cat}}$ values $\leq$ 60,000 s$^{-1}$ (A and B) give noticeably poor agreement with the data for [ClO$_2^-$] $>$ 4 mM. An assumed $k_{\text{cat}}$ of 120,000 s$^{-1}$ (C) gives much better agreement between the simulated traces and the data, while a value of 200,000 s$^{-1}$ (D) is obviously too high. This analysis suggests that the true $k_{\text{cat}}$ under these conditions is $\sim$ 120,000 s$^{-1}$, in agreement with published results (2).
**Figure S3.** Mössbauer spectrum (4.2 K/53 mT parallel field) of a freeze-quench sample prepared by mixing a solution of 10 mM Fe^{II}-Mb (2.5 mM ^{57}\text{Fe-Mb} and 7.5 mM ^{56}\text{Fe-Mb}) with 0.25 equivalent volumes of a solution of 100 mM ClO$_2$ and freeze-quenching quenched after 15 ms. The spectrum has a 7 ± 3% contribution from oxy-Mb (shown in red).
REFERENCES


Appendix D:

A 2.8 Å Fe–Fe Separation in the Fe$_{\text{III/IV}}$ Intermediate (X) from *Escherichia coli* Ribonucleotide Reductase

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A 2.8 Å Fe–Fe Separation in the Fe$_{\text{III/IV}}$ Intermediate (X) from *Escherichia coli* Ribonucleotide Reductase

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ABSTRACT

A class Ia ribonucleotide reductase (RNR) employs a \( \mu\)-oxo-Fe\( _{2}^{III/IV} \)/tyrosyl radical cofactor in its \( \beta \) subunit to oxidize a cysteine residue \( \sim 35 \) Å away in its \( \alpha \) subunit; the resultant cysteine radical initiates substrate reduction. During self-assembly of the \textit{Escherichia coli} RNR-\( \beta \) cofactor, reaction of the protein's Fe\( _{2}^{II/II} \) complex with O\(_{2}\) results in accumulation of an Fe\( _{2}^{III/IV} \) cluster, termed X, which oxidizes the adjacent tyrosine (Y) 122 to the radical (Y\(_{122}\)) as the cluster is converted to the \( \mu\)-oxo-Fe\( _{2}^{III/III} \) product. As the first non-heme high-valent-iron enzyme complex to be identified and the key activating intermediate of class Ia RNRS, X has been the focus of intensive efforts to determine its structure. Initial characterization by extended X-ray absorption fine structure (EXAFS) spectroscopy yielded a \( 2.5 \) Å Fe–Fe separation (\( d_{Fe-Fe} \)), which was interpreted to imply the presence of three single-atom bridges (O\(^{2-}\), HO\(^{-}\), and/or \( \mu\)-1,1-carboxylates). This short \( d_{Fe-Fe} \) has been irreconcilable with computational models, which all have \( d_{Fe-Fe} \geq 2.7 \) Å. To resolve this conundrum, we applied our recently developed method of generating O\(_{2}\) \textit{in situ} from chlorite using the enzyme chlorite dismutase to prepare X at \( \sim 2.0 \) mM, > 2.5 times the concentration realized in the previous EXAFS study. The increased concentration of the intermediate afforded significantly improved signal-to-noise ratio of the EXAFS, which permitted acquisition of data at higher values of \( k \) (the wave number of the photoelectron). The measured value of \( 2.80 \pm 0.02 \) Å is fully consistent with computational models containing a \((\mu\)-O\(^{2-}\))\(_{2}\)-Fe\( _{2}^{III/IV} \) core. The correction of \( d_{Fe-Fe} \), combined with recent re-examination of X by \(^{17}\)O- and \(^{1}\)H electron-nuclear double resonance spectroscopy, brings the experimental data and computational models into full conformity and thus informs analysis of the mechanism by which X generates Y\(_{122}\).
INTRODUCTION

Ribonucleotide reductases (RNRs) catalyze the conversion of ribonucleotides to deoxyribonucleotides, thus providing all organisms with precursors for the \textit{de novo} synthesis and repair of DNA.\textsuperscript{1,2} All RNRs identified to date utilize a free-radical mechanism. A transient cysteine thiyl radical (C\textbullet{}), generated \textit{in situ} in the first step of the reaction, abstracts a hydrogen atom (H\textbullet{}) from the 3\textsuperscript{'}-position of the bound nucleotide. The mechanism by which the C\textbullet{} is generated in each turnover is the basis for the division of RNRs into classes I-III.\textsuperscript{1,2}

A class Ia RNR, such as the prototypical orthologue from aerobically-growing \textit{Escherichia coli} (Ec), functions as a 1:1 complex of homodimeric subunits, $\alpha_2$ and $\beta_2$. The $\alpha$ subunit binds substrates and allosteric effectors and contains the C residue (C\textsubscript{439} in Ec RNR) that is oxidized to the C\textbullet{}, whereas the $\beta$ subunit self-assembles a $\mu$-oxo-Fe\textsubscript{2}\textsuperscript{III/III}/tyrosyl radical cofactor that functions to generate the C\textbullet{} reversibly in each catalytic cycle.\textsuperscript{3-5} The functional cofactor is produced by reaction of the Fe\textsubscript{2}\textsuperscript{II/II} complex of $\beta$ with O\textsubscript{2}.\textsuperscript{6} Addition of O\textsubscript{2} yields a $\mu$-peroxo-Fe\textsubscript{2}\textsuperscript{III/III} (P) complex\textsuperscript{7-9} that is reduced upon cleavage of the O–O bond of the peroxo moiety. In the Ec $\beta$ reaction, this reduction step is mediated by one electron shuttled through the solvent accessible W\textsubscript{48} (the residue is transiently oxidized to its W\textsubscript{48}\textsuperscript{+•} form);\textsuperscript{10} the diiron cluster also donates an electron to generate a state containing an Fe\textsubscript{2}\textsuperscript{III/IV} complex (termed cluster X).\textsuperscript{11} The Ec W\textsubscript{48}\textsuperscript{+•} can be reduced \textit{in vitro} by small-molecule reductants including ascorbate and thiols,\textsuperscript{10,12} but it is possible that an accessory protein serves as the reductant \textit{in vivo}. The decay of the W\textsubscript{48}\textsuperscript{+•} leaves X to oxidize the nearby Y residue to the stable Y\textbullet{}.\textsuperscript{11,13} In the process, X is reduced to the $\mu$-oxo-Fe\textsubscript{2}\textsuperscript{III/III} cluster of the active $\beta$ subunit.\textsuperscript{6,13,14} The Y\textbullet{}, at position 122 in Ec $\beta$, is strictly conserved among all class Ia and Ib RNRs and is absolutely required for their activity.\textsuperscript{1,3,15}
The importance of X to the function of class Ia RNRs (which include the *Homo sapien* orthologue) has made it a prime target for structural characterization. For *Ec* RNR, the rapid rate at which X decays (~ 1 s\(^{-1}\) in the wild type β; 0.2 s\(^{-1}\) in the Y\(_{122}F\) variant at 5 °C\(^{13,14}\)) has thus far prevented characterization by X-ray crystallography. Instead, the freeze-quench technique has been used to trap the intermediate, and it has then been characterized by a variety of spectroscopic methods.\(^{14,16-22}\) Density functional theory (DFT) calculations have afforded models for its diiron core, and these models have been evaluated for consistency with the spectroscopic data.\(^{23-27}\) This approach, now commonplace in investigations of reactive metalloenzyme intermediates,\(^{28,29}\) has thus far failed to forge a consensus regarding the structure of X. The primary reason is that the short Fe-Fe separation (d\(_{Fe-Fe}\) ~ 2.5 Å)\(^{20}\) of the intermediate determined by extended X-ray absorption fine structure (EXAFS) spectroscopy seemingly requires a structure with three single-atom bridges provided by some combination of the protein carboxylate ligands and O\(_2\)/solvent-derived O\(^{2-}\)/HO\(^-\) ligands. Such a structure has been disfavored on energetic grounds in computational studies. Indeed, structures favored in these studies have values of d\(_{Fe-Fe}\) ≥ 2.7 Å and no more than two single-atom oxygen bridges.\(^{16,24-26}\) Furthermore, none of the available synthetic models for X has had such a short d\(_{Fe-Fe}\).\(^{30-32}\)

We sought to resolve the conundrum concerning the structure of X by revisiting the irreconcilably short d\(_{Fe-Fe}\) determined in the initial EXAFS study. The kinetics of the activation reaction preclude trapping of X in pure form, with a maximum fraction of ~ 0.7 having been achieved in published studies. In cases of such heterogeneity, resolution of the superimposed scattering interactions from the different species present in the sample is facilitated by acquisition of data up to high values of \(k\), the modulus of the photoelectron scattering vector. However, the intensity and signal-to-noise ratio of the EXAFS data decays with increasing \(k\) (the
signal is weighted by $k^3$ and thus is diminished by a factor of three for each unit increase in $k$). In the previous study, this trend conspired with the relatively low [X] ($< 0.76$ mM) achieved in the samples to limit useful data to $k < 12.6$ Å$^{-1}$. To permit acquisition of useful data at increased $k$, we trapped X at greater concentration by applying our recently developed method of in situ O$_2$-generation from ClO$_2^-$ by the heme enzyme, chlorite dismutase.$^{33}$

The 4.2-K/53-mT Mössbauer spectra of samples prepared by mixing one reactant solution containing a high concentration of the pre-formed Fe$_2^{II/II}$ complex of Ec RNR-β-Y122F and a catalytic concentration (12.5 µM) of chlorite dismutase with 0.25 equivalent volumes of a second reactant solution containing sodium chlorite and freeze-quenching after a reaction time of 0.3 s (at 5 °C) reveal the presence of ~ 65% X, comparable to the maximum fraction obtained in the previous EXAFS study, as well as ~ 18 % unreacted Fe$^{II}$ species and ~ 18 % of µ-oxo-Fe$_2^{III/III}$ product cluster (Figures S1, S2, and S3 of the Supporting Information). This fraction of X corresponds to 2.0 mM, more than 2.5 times the maximum concentration used in the previous EXAFS study.

The X-ray absorption near-edge structure (XANES) spectra (Figure S4) show a higher K-edge absorption energy (the energy at which the 1s-core electron is ejected) for samples containing X than for the samples of the unreacted Fe$_2^{II/II}$-β starting material. However, the edge of the X samples lies at a lower energy than for samples of the µ-oxo-Fe$_2^{III/III}$ product. This phenomenon, also observed by Riggs-Gelasco et al.,$^{20}$ may result from the contribution of the unreacted Fe$_2^{II/II}$ component in the freeze-quenched samples of X.

Fe K-edge EXAFS data over $k = 0.3 – 16$ Å$^{-1}$ are shown in Figure 1, along with fits to the raw data based on the parameters given in Table S1. For samples enriched in X, the Fourier transform (FT) of the EXAFS data over this range of $k$ (Figure 1A, right panel) reveals a
prominent, non-nearest-neighbor scattering interaction that can be modeled as an Fe-scatterer at \(R \sim 2.8\) Å. Consistent with this assignment, the feature is not observed in samples lacking X: the corresponding data for the reactant complex lack evidence of any Fe–Fe scatterer (Figure 1B), presumably because \(d_{\text{Fe-Fe}}\) is too large (~3.8 Å\(^3\)) in this form of the cluster, whereas the spectra of samples containing the \(\mu\)-oxo-Fe\(_{2}^{III/III}\) product cluster exhibit a prominent Fe-scatterer at \(R = 3.21\) Å (Figure 1C), consistent with the reported \(d_{\text{Fe-Fe}}\) of this form.\(^{35,36}\)

The fit to the unfiltered EXAFS data for the samples containing X (Figure 1A, left panel) corresponds to a total coordination number of 4.75: 3 O/N at 2.06 Å, 1 O/N at 1.98 Å, 0.75 O at 1.79 Å, 0.75 Fe at 2.79 Å, and 0.25 Fe at 3.21 Å. The occupancies of the latter two interactions account for the heterogeneity of the sample, specifically the fractions of X and \(\mu\)-oxo-Fe\(_{2}^{III/III}\) cluster determined from the Mössbauer data. The contribution to the EXAFS data from the ~18% Fe\(_{2}^{II/II}\) component in the samples is not obvious and is not accounted for in the analysis. However, consideration of this minor component would correct the total coordination number to ~5. A short Fe–O interaction at 1.78 Å is likely to arise from an oxo bridge, and the occupancy of 0.75 is consistent with the presence of two such \(\mu\)-oxo ligands (see Tables S4 and S5 for additional fitting results). Fits to the Fourier-filtered EXAFS data for the samples enriched in X, with the filter (\(R\) window = 0.5 – 2.5 Å) applied to investigate only the nearest-neighbor (first-shell) Fe interactions, agree well with the model used to fit the first-shell interactions of the unfiltered data (Figure S5; fit parameters provided in Table S6). However, the Fourier-filtered EXAFS data for the non-nearest-neighbor (second-shell) Fe interactions (\(R\) window = 2.5 – 3.5 Å\(^{-1}\)) are not fit well by the model used to fit the second-shell Fe interactions of the unfiltered EXAFS data (Figure S6; fit parameters provided in Table S7). The model includes only two Fe interactions at 2.8 and 3.2 Å; the discrepancy between the fit and the data suggests the need to
include a third metal-scattering interaction. Including this third interaction as an Fe scatterer at 3.0 Å significantly improves the agreement between the fit and the data (Figure S7; parameters provided in Table S8). The occupancy of this third interaction could not be unequivocally determined, as values ranging from 0.25 to 0.38 could give fits of equivalent quality (see Tables S1-S3 for additional fitting results). Table 1 reports the parameters for the fit to the unfiltered EXAFS spectrum with three Fe–Fe interactions (2.8, 3.0, and 3.2 Å) at the occupancies given. Irrespective of the origin of this apparent additional interaction at 3.0 Å, the predominant interaction that can be assigned to an Fe scatterer is at ~ 2.8 Å, and there is no evidence for an interaction at the previously reported d_{Fe-Fe} of ~ 2.5 Å.

To clarify the structure of the diiron core of X and rationalize the 2.8 Å d_{Fe-Fe}, we generated a series of structural models by broken-symmetry DFT methods, following previous work by Noodleman and coworkers (see Supporting Information for a more detailed description). The models were derived from the X-ray crystallographic data (1RIB) of Ec β by modifying the ligation. Two main candidates were examined in detail, a di-µ-oxo-µ-1,3-carboxylato core structure (Figure 2) and a variant with one of the oxo bridges protonated to µ-hydroxo. The di-µ-oxo variant has distance parameters that closely match the experimentally determined values, including, most notably, the d_{Fe-Fe} of 2.8 Å (Table 2).

It is noteworthy that the DFT calculations imply that protonation of one of the µ-oxo bridges should result in an elongation of the Fe-Fe separation to ~ 3.0 Å, which matches the second d_{Fe-Fe} included for the better fit to the EXAFS data (raw and Fourier-filtered) on the samples highly enriched in X. Conceivably, the two protonation states of the X core might both be present in the sample, perhaps in equilibrium.
The effect of including the tyrosine 122 residue that is oxidized by X in the DFT calculations was also evaluated. The presence of Y_{122} has a minor effect on the optimized geometries, resulting in only a slight elongation (~ 0.02 Å) of the Fe–Fe core (Table 2). Thus, it seems unlikely that the structure of X in the β-Y_{122}F variant could be significantly different from that formed in the wild-type protein.

We attempted to understand the basis for the discrepancy between the d_{Fe-Fe} of 2.8 Å determined here and the previous results that yielded a d_{Fe-Fe} of 2.5 Å. As noted above, resolution of the superimposed non-nearest scattering interactions in heterogeneous samples of reactive intermediates is challenging. Oscillations in scattering intensities that reveal the non-nearest neighbor distances, can, for individual components having similar but non-identical scattering distances, destructively interfere over a narrow range of k. As the range of k is expanded and the number of oscillations increased, additional regimes of constructive interference make this situation more apparent. Whereas the data analyzed in the previous study were limited to k = 2 - 12.6 Å\(^{-1}\), the elevated concentration of X in the samples prepared here and the consequent improvement in the signal-to-noise ratio of our data allowed acquisition of meaningful scattering intensities at an extended range of k (0.3 - 16 Å\(^{-1}\)). To evaluate whether this difference might be a plausible explanation for the discrepant results, we examined FTs of unfiltered EXAFS data with cutoffs at k = 11, 12, 13, 14, 15, and 16 Å\(^{-1}\) (Figure 3). The intensity of the 2.8-Å peak in the FT is indeed diminished with decreasing k (see cutoffs at 13 and 12 Å\(^{-1}\)). Upon truncation of the EXAFS data at k = 11 Å\(^{-1}\), the Fe–Fe scattering interaction is effectively obscured. This effect appears to be a consequence of the presence of more than one Fe scatterer in the sample, as similar treatment of the data obtained on the sample enriched in the μ-oxo-Fe\(_2^{III/III}\) form of the protein does not result in diminution of the FT peak (Figure S8). The
analysis suggests that sample heterogeneity and the narrow range of $k$ interrogated could have conspired to obscure the 2.8 Å Fe–Fe scattering interaction. However, in no case would truncation of our data have led to the erroneous assignment of a 2.5-Å scattering interaction, leaving this aspect of the discrepancy unexplained.

This re-examination of the structure of X and subsequent upward adjustment of its $d_{Fe-Fe}$ calls into question the short $d_{Fe-Fe}$ s reported for other O$_2$-derived diiron intermediate complexes. For example, the high-valent Fe$_2^{IV/IV}$ complex, Q, that accumulates during the conversion of methane to methanol by the soluble methane monoxygenase from *Methylosinus trichosporium* OB3b was characterized by EXAFS, and the measured $d_{Fe-Fe}$ of 2.46 Å led to the proposal of a di-$\mu$-oxo "diamond core" structure. Subsequently, EXAFS characterization of the $\mu$-peroxo-Fe$_2^{III/III}$ complexes that accumulate in the reactions of M ferritin from frog and the D$_{84}$E/W$_{48}$A variant of *Ec* RNR-β led to the proposal of similar values of $d_{Fe-Fe}$ (~ 2.5 Å) even in these mid-valent complexes. In general, the structures dictated by these surprisingly short Fe-Fe separations have been irreconcilable with synthetic and computational models. As we have shown here, the range of $k$ interrogated, which is limited by signal-to-noise ratio and thus by sample concentration, may be the critical factor that led to these potentially erroneous $d_{Fe-Fe}$ measurements. Re-examination of these other complexes and re-determination of their Fe-Fe separations would seem to be warranted.
ASSOCIATED CONTENT

Supporting Information. Method of sample preparation; Mössbauer spectra of the samples containing \( X \), the reactant complex, and the product of decay of \( X \); XANES spectra of the samples containing \( X \), the reactant complex, and the product complex; additional presentations of Fourier filtered EXAFS data and their fits; description of computational methodology and tables with results. This material is available free of charge via the Internet at http://pubs.acs.org

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ACKNOWLEDGEMENT

ABBREVIATIONS

RNR, ribonucleotide reductase; \( Ec \), Escherichia coli; DFT, Density Functional Theory; EXAFS, extended X-ray absorption fine structure; \( k \), photoelectron wave vector; XANES, X-ray absorption near structure; FT, Fourier transform; BS, broken symmetry; COSMO, conductor-like screening model; \( \varepsilon \), dielectric constant
REFERENCES


**FIGURES, TABLES, AND LEGENDS**

**Figure 1.** Fe K-edge EXAFS data (left panel) and their Fourier-transforms (right panel) for samples containing X (A), the Fe$^{II/III}$ reactant complex (B) and µ-oxo-Fe$^{III/III}$ product (C). Fit parameters are provided in Tables S1, S9, and S10.
Figure 2. Structural models for the Fe$^{III/IV}_2$ core of X derived from broken-symmetry DFT calculations. Left: $\mu-(O^2)_{2}$ core; Right: $\mu-[(O^2^-)(OH^-)]$ core.
Figure 3. FT of EXAFS data of samples containing X plotted with different cutoffs of the $k$-range. The red line is drawn at the middle of the $\sim 2.8$ Å peak.
Table 1. Fe K-edge EXAFS ($k = 0.3 - 16 \text{ Å}^{-1}$) fitting results of samples containing X for which the fit model includes three discernable Fe–Fe interactions.

<table>
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<th>Scatterer Type</th>
<th>N</th>
<th>R</th>
<th>$\sigma^2$</th>
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<tr>
<td>O</td>
<td>0.75</td>
<td>1.78</td>
<td>0.0033</td>
</tr>
<tr>
<td>O/N</td>
<td>3</td>
<td>2.06</td>
<td>0.0078</td>
</tr>
<tr>
<td>O/N</td>
<td>1</td>
<td>1.98</td>
<td>0.0027</td>
</tr>
<tr>
<td>Fe</td>
<td>0.4</td>
<td>2.79</td>
<td>0.0016</td>
</tr>
<tr>
<td>Fe</td>
<td>0.35</td>
<td>3.00</td>
<td>0.0035</td>
</tr>
<tr>
<td>Fe</td>
<td>0.25</td>
<td>3.21</td>
<td>0.0040</td>
</tr>
<tr>
<td>$F$</td>
<td>0.363</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_0$</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Resolution</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

N: occupancy; R: distance (Å); $\sigma^2$: Debye-Waller factor ($\times 10^{-3}$ Å$^2$); $E_0$: threshold energy shift (eV); $F$: fit error
Table 2. Calculated distances of Fe interactions for models of the Fe$_2^{III/IV}$ core of X determined by broken-symmetry DFT with COSMO $\varepsilon = 10$.

<table>
<thead>
<tr>
<th>Model</th>
<th>Distance (Å)</th>
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<tr>
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<td>Fe–Fe</td>
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<td>$\mu$-O$_2^-$:</td>
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<tr>
<td>($\mu$-O$_2^-$):-Y</td>
<td>2.83</td>
</tr>
<tr>
<td>$\mu$-[(O$_2^-$):(OH$^-$)]</td>
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<tr>
<td>$\mu$-[(O$_2^-$):(OH$^-$)]:Y</td>
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</tr>
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</table>
SUPPORTING INFORMATION

A 2.8 Å Fe–Fe Separation in the Fe$_2^{III/IV}$ Intermediate (X) from *Escherichia coli* Ribonucleotide Reductase

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EXPERIMENTAL PROCEDURES

Protein expression, purification and sample preparation. The methods for the overexpression and purification of Ec β-Y122F have been described. The methods for the overexpression and purification of Ec β-Y122F have been described. Two sets of samples containing the Fe_2^{III/IV} complex (X) were prepared using the in situ generation of O_2 from ClO_2, but with the substitution of iso-pentane with liquefied ethane (T ~ -150 °C) as the cryo-solvent. After evaporation of ethane at ~ -130 °C under reduced pressure, the powdered samples were packed into Mössbauer and EXAFS sample holders that have been described. The final concentrations of components for the first set of samples (batch 1) were 4.26 mM β, 6.19 mM ^{57}Fe, 10 mM ascorbate, 20 µM Cld, and 16 mM ClO_2. The second set of samples (batch 2) contained 4.26 mM β, 6.16 mM Fe, 20 µM Cld, and 16 mM ClO_2. All reactions to generate X were quenched after 0.3 s after mixing at 5 °C.

To prepare samples containing reactant Fe_2^{II/II} (diferrous) complex, a 3 mL solution of O_2-free β (4.26 mM), ^{57}Fe^{II} (6.16 mM), and Cld (20 µM) was sprayed into liquefied ethane in a rapid freeze-quench setup. The frozen powdered sample was packed into Mössbauer and EXAFS sample holders upon removal of the cryo-solvent, as previously described.

Samples containing the μ-oxo-Fe_2^{III/III} (diferric) product complex were initially prepared and quenched identically to samples containing X (batch 2). Upon removal of the liquefied ethane, the frozen powdered sample was allowed to thaw at ~ 5 °C for 1 hr before being re-loaded into the freeze-quench syringe and again sprayed into liquefied ethane. The cryo-solvent was removed and the powdered sample packed into Mössbauer and EXAFS samples holders, as previously described. The final concentrations of components were identical to those of X, batch 2 samples.

Mössbauer spectroscopy. The Mössbauer spectrometer has been described. Spectra were collected at 4.2 K with a 53-mT magnetic field externally applied either parallel or perpendicular to the γ-beam. The relative amount of intermediate X contained in the samples was estimated by analysis of the Mössbauer spectra using the program WMOSS (Seeco, Edina, MN). For the spectral deconvolution, we used experimental reference spectra of the reactant Fe_2^{II/II} and the μ-oxo-Fe_2^{III/III} product complexes (Figure S3), while the features of intermediate X were simulated using the commonly used spin Hamiltonian formalism with parameters reported previously. The relative amount of X can also be obtained from analysis of the difference spectrum (parallel – perpendicular), because only the spectral features of X depend on the orientation of the magnetic field.

XAS Data Collection and Analysis. All data were acquired in fluorescence mode at low temperature (~ 10 K) with a 30-element germanium detector (SSRL, BL7-3) as previously described. The background was removed using the program EXAFSPAK (k-weight = 3; spline range k = 0.3 – 16 Å⁻¹). EXAFS data were analyzed using EXAFSPAK as previously described.
The XAS data from the two batches of samples for X were analyzed individually before being combined to yield a greater signal-to-noise ratio. Only first scans were used for the analysis presented herein in order to minimize the effect of photoreduction of the species by the X-ray beam. A total of 83 first scans were averaged for the analysis of the data acquired for samples of X.

**Density Functional Theory Calculations.** All DFT calculations have been performed using Gaussian 03 package. Geometry optimizations were performed on the modified coordinates from X-ray crystal structure of *Escherichia coli* ribonucleotide reductase protein R2 (PDB: 1RIB). First, a rough optimization was carried out for the high spin (HS) state i.e. assuming ferromagnetic coupling between iron centers, using spin-unrestricted BP86\[^{10,11}\] DFT method *in vacuo* with Aldrichs SVP basis set on all atoms. During the optimization the linking H atoms on all the ligating amino acids were fixed. Using obtained geometries, a broken symmetry state (BS) determinant emulating the antiferromagnetic coupling with $S_{\text{tot}}=1/2$ have been formed by permutating the HS wavefunctions so that in the $\alpha$-spin domain only the d-orbitals of Fe\[^{III}\] ($S=5/2$) are populated and only the d-orbitals of Fe\[^{IV}\] ($S=2$) are populated in the $\beta$-spin domain. Permutations were defined using home written programs based on the largest contributions to the wavefunctions. The obtained solutions were projected to a larger basis set 6-31G*, re-converged with a single-point calculation on the unrestricted B3LYP\[^{14,15}\] level *in vacuo* and tested for stability.

A second step of the geometry optimization was carried out using a larger basis sets (Fe: 6-311G*, other atoms 6-31G) utilizing the obtained BS wavefunctions as initial guess. In this case a Gaussian implementation of the solvation model COSMO\[^{16}\] (C-PCM)\[^{17}\] has been utilized to account for the protein environment. Similar to the initial step the linking H atoms on all the ligating amino acids were fixed.
Figure S1. 4.2-K Mössbauer spectra of samples (X, batch 1) used for XAS. Spectra were collected with a 53-mT magnetic field applied parallel (top) or perpendicular (middle) to the γ-beam. The vertical bars depict the experimental data, whereas the solid black lines represent the sum of the individual components. The red and green lines are the experimental reference spectra of the Fe$_{2}^{II/II}$ reactant and μ-oxo-Fe$_{2}^{III/III}$ product complexes (Figure S3), scaled to 20% and 22%, respectively. The blue lines are simulations of X using the published parameters, scaled to 62% of the total area. The experimental difference spectrum (vertical bars, bottom) only depends on the features of X, because the features of the Fe$_{2}^{II/II}$ and μ-oxo-Fe$_{2}^{III/III}$ forms do not depend on the orientation of the magnetic field and therefore cancel out, and therefore provide an alternative way to determine the amount of X from the amplitude of the difference spectrum. The solid black line overlaid with the difference spectrum is the simulation of X, assuming 62% intensity.
Figure S2. 4.2-K/53-mT Mössbauer spectra of samples containing X (batch 2) used for XAS. The orientation of the 53-mT magnetic field is indicated at the left side of the spectra. Samples were analyzed as described in the legend of Figure S1. There is an 18% contribution to the spectra from the unreacted Fe$_{II/II}$ complex (red), and an 18% contribution from the µ-oxo-Fe$_{III/III}$ product complex. The contribution of X (blue) is scaled to 67% of the total intensity.
Figure S3. 4.2-K/53-mT Mössbauer spectra of samples containing the reactant Fe$_2^{III/II}$ complex (top) and the μ-oxo-Fe$_2^{III/III}$ product complex (bottom) of Ec β-Y$_{122}$F used for XAS. The samples were prepared as described (vide supra). The spectrum of the reactant complex is a broad quadrupole doublet with parameters typical of high-spin Fe$^{II}$. The spectrum of the product complex exhibits the prominent features of the μ-O-Fe$_2^{III/III}$ complex (solid black line), in addition to minor amounts of a magnetically split feature on the baseline (tentatively attributed to a high-spin Fe$^{III}$ contaminant; ~10%, see black arrows) and a weak absorption in the central portion of the spectrum (tentatively assigned to a non-μ-O-Fe$_2^{III/III}$ form; <5%, see red arrows).
Figure S4. X-ray Absorption Near Edge Structure (XANES) spectra of samples enriched in X (blue), the reactant complex (red), and the product complex (green). The edge energy of the sample containing X is between that observed in the spectra of the reactant and product control samples. This can be explained by the fact that unreacted diferrous component of the samples containing X skews the edge toward a lower energy.
Figure S5. Fourier filtered EXAFS data (left) and its Fourier-transform (FT, right) showing the nearest-neighbor Fe interactions for samples containing X. The filter was applied to cover $R$-window = 0.5 – 2.5 Å. The data are shown as the black spectra, and fits are shown as the red spectra. The results for the fits are provided in Table S6.
Figure S6. Fourier filtered EXAFS data (left) and its FT (right) showing the non-nearest neighbor Fe interactions for samples containing X. The filter was applied to cover $R$-window = 2.5 – 3.5 Å. The data (black traces) were fit to a model that included two discernable Fe–Fe interactions (one for X and one for the Fe$_{\text{III/III}}$ product). The red spectra represent the fits with results provided in Table S7.
Figure S7. Fourier filtered EXAFS data (left) and its FT (right) showing the non-nearest neighbor Fe interactions of samples containing X. The filter was applied to cover $R$-window = 2.5 – 3.5 Å. The data (black traces) were fit to a model that included three discernable Fe–Fe interactions (two forms of X at 2.8 Å and 3.0 Å, and the $\mu$-oxo-Fe$_2$$^{III/III}$ product). The red spectra represent the fits with results provided in Table S8.
Figure S8. FT of the EXAFS data evaluated at two extreme ranges of $k$ for samples containing the $\mu$-oxo-Fe$_{\text{III/III}}^\text{III}$ product. The black spectrum shows the FT for EXAFS data with $k = 0.3 – 16 \text{ Å}^{-1}$, whereas the blue spectrum shows the FT for EXAFS data with $k = 0.3 – 11 \text{ Å}^{-1}$. 
Table S1. Fe K-edge EXAFS (\(k = 0.3 - 16 \text{ Å}^{-1}\)) fitting results of the raw data acquired for samples containing X. The fit model included two discernable Fe–Fe interactions.

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<th>R</th>
<th>(\sigma^2)</th>
</tr>
</thead>
<tbody>
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<td>0.0056</td>
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<td>Resolution</td>
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N: coordination number; R: distance (Å); \(\sigma^2\): Debye-Waller factor (\(\times\) 10^{-3} Å^2); \(E_0\): threshold energy fit (eV); F: fit error

Table S2. Fe K-edge EXAFS (\(k = 0.3 - 16 \text{ Å}^{-1}\)) fitting results of the raw data acquired for samples containing X. The fit model included two discernable three Fe–Fe interactions. The occupancies for the first two Fe–Fe interactions were allowed to vary during the fit.

<table>
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<th>Scatterer Type</th>
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Table S3. Fe K-edge EXAFS (\(k = 0.3 - 16 \text{ Å}^{-1}\)) fitting results of the raw data acquired for samples containing X. The fit model included two discernable three Fe–Fe interactions. The occupancies and \(\sigma^2\) for the first two Fe–Fe interactions were allowed to vary during the fit, while all of the other parameters were fixed.

<table>
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<th>Scatterer Type</th>
<th>N</th>
<th>R</th>
<th>(\sigma^2)</th>
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Table S4. Fe K-edge EXAFS ($k = 0.3 – 16 \text{ Å}^{-1}$) fitting results of the raw data acquired for samples containing X. The fit model included two discernable three Fe–Fe interactions. The occupancy for the short Fe–O interaction was set to depict one of such interaction.

<table>
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Table S5. Fe K-edge EXAFS ($k = 0.3 – 16 \text{ Å}^{-1}$) fitting results of the raw data acquired for samples containing X. The fit model included two discernable three Fe–Fe interactions. The occupancy for the short Fe–O interaction was set to depict four of such interactions.

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Table S6. Fe K-edge EXAFS ($k = 0.3 – 16 \text{ Å}^{-1}$) fitting results of the Fourier filtered data for samples containing X. The filter was applied to cover $R$-window = 0.5 – 2.5 Å in order to probe the nearest-neighbor Fe interactions.

<table>
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Table S7. Fe K-edge EXAFS ($k = 0.3 – 16 \text{ Å}^{-1}$) fitting results of the Fourier filtered data for samples containing X. The filter was applied to cover $R$-window = 2.5 – 3.5 Å in order to probe the non-nearest-neighbor Fe interactions. The fit model included two Fe–Fe interactions.
Table S8. Fe K-edge EXAFS ($k = 0.3 - 16 \text{ Å}^{-1}$) fitting results for the Fourier filtered data for samples containing X. The filter was applied to cover $R$-window = 2.5 – 3.5 Å in order to probe the non-nearest-neighbor Fe interactions. The fit model included three Fe–Fe interactions.

<table>
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Table S9. Fe K-edge EXAFS ($k = 0.3 - 16 \text{ Å}^{-1}$) fitting results of the raw data acquired for samples containing the Fe$_2^{II/III}$ reactant complex.

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Table S10. Fe K-edge EXAFS ($k = 0.3 - 16 \text{ Å}^{-1}$) fitting results of the raw data for samples containing the Fe$_2^{III/III}$ product complex.

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<tr>
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200
Table S11. Distances and Mulliken Spin populations for the structural models of X discussed in the manuscript.

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Coordinates of the models from the table above:

(µ-O^2)_{2}

Fe  -0.057278  0.097612  -0.006475
Fe  2.743711  -0.110872  0.052159
O   1.461830  0.175920   1.222093
C   -3.283465 0.627591  -2.983248
C  -2.236373  1.078998  -1.987219
O  -1.504187  0.138841  -1.449507
O  -2.116111  2.323531  -1.715430
C   0.715516  -4.205052  0.047881
C   0.990561  -2.717573  0.057922
O  -0.051393  -1.938076  -0.021847
O   2.198977  -2.306073  0.135177
C  -3.150160  -1.740292  0.934227
C  -2.659211  -0.674129  1.854849
N  -1.513577  0.094192   1.587376
C  -3.193452  -0.245531  3.050834
C  -1.369598  0.959750   2.598288
N  -2.369866  0.783830   3.502164
C   4.037719  0.913516  -2.974026
C   4.634090  -0.318295  -2.328923
O   5.585371  -0.944198  -2.876456
O   4.142702  -0.697655  -1.160824
C   4.361488  4.059450  0.292765
C   3.231657  3.048032  0.180092
O   2.026553  3.485380  0.184210
O   3.589320  1.809175  0.066299
C   6.340567  0.175212  0.806620
C   5.429430  -0.432687  1.820811
N   4.041343  -0.573060  1.636794
C   5.740949  -0.930528  3.067820
C   3.545092  -1.144152  2.740058
N   4.545478  -1.374411  3.629122
O   1.519553  0.108435  -1.179231
O  -0.151849  2.124884  0.009470
H   0.013810  -4.459079  0.849443
H   4.338501  1.796695  -2.400843
H   5.039528  3.771081  1.102268
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\( \mu = \langle (O^2) - (OH) \rangle \)

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REFERENCES:


VITA

LAURA M. K. DASSAMA

EDUCATION

Graduate (2007-2013)  Ph.D., Biochemistry, Microbiology and Molecular Biology
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Advisor: Prof. Robert J. Stanley

PUBLICATIONS


ACADEMIC AWARDS

- Alfred P. Sloan Minority Ph.D. Scholar (2009-present)
- Carl Storm Underrepresented Minority Fellowship, Gordon Research Conference (2011)
- Travel Award, Department of Biochemistry, Microbiology, and Molecular Biology, The Pennsylvania State University (2011)
- Undergraduate Research Incentive Fund, Temple University (2006)