FORMATION AND FUNCTION OF A NOVEL HETEROBINUCLEAR
MN/FE REDOX COFACTOR IN RIBONUCLEOTIDE REDUCTASE
FROM CHLAMYDIA TRACHOMATIS

A Dissertation in
Biochemistry, Microbiology and Molecular Biology

by

Wei Jiang

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of
Doctor of Philosophy

December 2008
The dissertation of Wei Jiang was reviewed and approved* by the following:

Joseph Martin Bollinger, Jr.
Professor of Chemistry
Professor of Biochemistry and Molecular Biology
Dissertation Co-Adviser
Co-Chair of Committee

Carsten Krebs
Associate Professor of Chemistry
Associate Professor of Biochemistry and Molecular Biology
Dissertation Co-Adviser
Co-Chair of Committee

Squire Booker
Associate Professor of Chemistry
Associate Professor of Biochemistry and Molecular Biology

Ming Tien
Professor of Biochemistry and Molecular Biology

Michael T. Green
Associate Professor of Chemistry

Scott Showalter
Assistant Professor of Chemistry

Richard J. Frisque
Professor of Biochemistry and Molecular Biology
Head of the Department of Biochemistry and Molecular Biology.

*Signatures are on file with the Graduate School.
ABSTRACT

Ribonucleotide reductase (RNR) catalyzes the reduction of ribonucleotides to deoxyribonucleotides, the building blocks for DNA synthesis and repair. This remarkable reaction proceeds via a free-radical mechanism. A conventional class I RNR activates O₂ at a carboxylate-bridged Fe²⁺/II cluster to generate a stable tyrosyl radical (Y•) in its R2 subunit. The Y• is believed to oxidize a cysteine to cysteinyl radical (C•) in the R1 subunit transiently by a long-distance (~35 Å), inter-subunit, proton-coupled electron transfer (PCET) step, which is mediated by several redox-active amino acid residues. The C• then initiates the reduction of the substrate by abstracting the hydrogen atom from C3'.

RNR from Chlamydia trachomatis (Ct), a common human pathogen, is the prototype of a new subclass (class Ic RNR), in which the essential radical-harboring tyrosine residue is replaced by a phenylalanine. By a combination of activity assay, kinetic analysis and various spectroscopic methods, my research has demonstrated that Ct RNR employs a high-valent, heterobinuclear Mn⁴+/Fe³⁺ cluster for activity. During turnover, the Mn⁴+/Fe³⁺ cluster is transiently reduced to the Mn³+/Fe³⁺ state to generate the C• in the R1 subunit. So the Mn⁴+/Fe³⁺ cluster, specifically, the Mn⁴⁺ site, functionally replaces the Y• of a conventional class I RNR.

The Mn⁴+/Fe³⁺ cofactor is generated by reaction of O₂ with the reduced Mn²+/Fe²⁺ cluster. A Mn⁴+/Fe⁴⁺ intermediate accumulates and undegoes one-electron reduction of the Fe⁴⁺ site. The electron to reduce the Fe⁴⁺ site is shuttled by the near-surface residue, Y222, which has no functional cognate in the best-studied conventional class I RNRs. The Mn⁴+/Fe³⁺ cofactor can also be produced from the Mn³+/Fe³⁺ cluster by H₂O₂, an important physiological reactive oxygen species (ROS), suggesting that the class Ic RNR may confer to the pathogen greater fitness toward ROS produced as part of the host's immune response.

The active form of Ct R2 (Mn⁴+/Fe³⁺) is EPR-silent whereas the reduced form (Mn³+/Fe³⁺) is EPR-active. This is the first case that the reduced form of class I RNR can be monitored by EPR and other advanced paramagnetic resonance methods. Studies of reduction of Mn⁴+/Fe³⁺ cofactor by hydroxyurea and dithionite suggest that the outcome of the reduction is highly perturbed upon binding of substrate to the active site in the R1 subunit, although the binding site is presumably ~35Å away from the cofactor. Thus, Ct RNR provides new opportunities for dissection of the conformationally gated PCET step in a class I RNR and raises intriguing questions concerning the evolution of the elegant, intersubunit-PCET, radical initiation strategy.
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Acknowledgements

I deeply appreciate the unlimited support from both of my advisors, Prof. Marty Bollinger and Prof. Carsten Krebs, as well as all the patience (tolerance) and tremendous faith they gave me. Their enthusiasm for science and encouragement is the hidden motivation for all the achievements. Except for the direct guidance on the research, I also got abundant help in oral presenting, English writing, communications, etc. Trips to six conferences under their financial support are the most enjoyable experiences during my Ph.D. study. To join this lab is so far the most important and correct decision in my career.

We have a quite happy and productive cooperation with the Green lab at Penn State. The EXAFS analysis and DFT calculations done by Prof. Mike Green, Jarod Younker and Courtney Krest have expanded our research field a lot and led to a more comprehensive understanding for Ct RNR. I hope we can achieve more by using Mike’s other spectroscopic handles, e.g., rRaman, ENDOR, ESEEM, in near future.

I have never worked alone. Drs. Lana Saleh and Danny Yun, two previous graduate students, started this project from 2004. I am especially grateful to Lana, who gave me an awesome one-year training at the beginning. She has done most of the work on the O2 activation at Fe2I/II cluster in Ct R2 wt, Y338F, Y112F and part of the work in Y222F. In addition, she is also the first person who postulated that Y222 could play an important role in electron transfer during O2 activation. Eric Barr developed the protocol for the activity assay by mass spectrometry and offered technical helps for essentially everything I have ever touched in the lab. Dr. Gang Xing taught me how to operate with EPR spectrometer. Lee Hoffart first assigned the MnIV/FeIV and MnIV/FeIII species based on the Mössbauer analysis. Jiajia Xie constructed several variants, including Ct R1-C672S, Ct R1-Y990F, Ct R1-Y991F, and participated extensively in the research of interactions between R1 and R2 subunits. The studies on variants Ct R2-E89D and F127Y were mainly carried out by Monique Maslak Gardner, an undergraduate student at Penn State, and Hanne Nørgaard, an exchange Ph. D. student from the Technical University of Denmark, respectively. Denise Corner synthesized the 2’-azido-substrate analog for this project. I also want to thank Paul Varano, an undergraduate in our lab, who helped a lot with miscellaneous works in the last few months of my Ph.D. study so that I could focus on the thesis writing.

I owe my parents too much for their unconditional support across the Pacific Ocean. During the time in U.S., the trip back home is considered as my highest “reward” for working hard in the lab.

Last but not least, I thank my husband, Xin Li. He is my tutor in math and physics, a great listener for the scientific ideas and nagging complaint, a loyal personal driver, and an eligible shopping servant. He makes the spare time in State College, a village in the middle of nowhere, full of novelty and pleasure.
Chapter 1
Introduction to Ribonucleotide Reductases
Ribonucleotide Reductases: Function, General Mechanism and Classification

Ribonucleotide reductases (RNRs) are present in all cellular organisms reported so far. They catalyze the reduction of ribonucleotides to deoxyribonucleotides, which are the building blocks for DNA synthesis and repair. They provide the only de novo pathway for deoxyribonucleotide synthesis in vivo and are believed to be the link between the RNA and DNA world (1-3). Their central role in nucleotide metabolism made them an important target for antitumor and antiviral agents (4, 5).

This reaction involves replacement of the hydroxyl group on the 2’-carbon of the ribose moiety of nucleoside diphosphates (NDP) or triphosphates (NTP) by a hydrogen atom (6). This chemically very difficult reaction is made possible through the use of a free-radical mechanism. All RNRs share a common basic strategy for catalysis, in which a transient cysteine thiol radical (C•) in the active site initiates the chemical reduction by abstraction of the 3’-hydrogen atom of the ribose ring (7, 8). The C• is regenerated at the end of each cycle so that it can function catalytically.

Although the chemistry at the active site is very similar, different ways to generate the key C• have evolved. RNRs thus have been divided into three classes, Scheme 1-1: Classification of RNRs based on cofactors required to initiate the catalysis. Adapted from ref (10).
based on the metallocofactor required for the radical initiation process (Scheme 1-1) (1, 9, 10).

The enzymes from aerobically growing *Escherichia coli* (*Ec*), most eukaryotes including all mammals, and herpes simplex virus are termed class I RNRs, which are composed of a 1:1 complex of two homodimeric subunits, R1 (α2) and R2 (β2).1 R1 is the catalytic subunit, containing the active site for substrate reduction and the binding sites for allosteric effectors (1). R2 is the cofactor subunit, which harbors a carboxylate-bridged diiron(III/III) cluster in close proximity to a stable tyrosyl radical (Y•) (10, 12). During catalysis, the Y• in R2 oxidizes the key cysteine residue in R1 to C• by a long-distance, inter-subunit, proton-coupled electron transfer (PCET) reaction (10). The Fe_{2}^{III/III}-Y• cofactor is generated posttranslationally by activation of O_{2} at the reduced, Fe_{2}^{II/II} form of the cofactor (12, 13). Class I RNRs are further divided into three subclasses. Class Ia (e.g., *E. coli*, *Homo sapiens*) and Ib (e.g., *Salmonella typhimurium*) both contain the Fe_{2}^{III/III}-Y• cofactor, but they differ in their amino acid sequences, the physiological reductant, and some allosteric effectors (14, 15). The enzyme from human pathogen *Chlamydia trachomatis* (*Ct*) has most characters similar to class Ia RNRs, however, the crucial radical-harboring tyrosine residue is replaced by a phenylalanine (16). Several species that encode RNRs similar to *Ct* are grouped as class Ic (17). My Ph.D. thesis work focuses on mechanistic studies of *Ct* RNR as the prototype of class Ic, which will be discussed in details later.

The enzyme from *Lactobacillus leichmannii* serves as a prototype for class II RNRs, which are O_{2}-independent and exist widely in eubacteria and archaeabacteria (18). They require

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1 Some class I RNRs, e.g., the enzyme from *homo sapiens*, are believed to have α₆β₆ composition (11).
adenosylcobalamin (AdoCbl) as a cofactor to generate the C•. They consist of a single monomeric or homodimeric protein. The C-Co bond homolysis of AdoCbl produces cob(II)alamin and 5’-deoxyadenosylradical (5’-dA•), the latter of which generates C• in the active site. These two steps are believed to take place in a concerted fashion to prevent the accumulation of 5’-dA•, which partly overcomes the thermodynamically unfavorable C-Co homolysis reaction (19).

Class III RNRs only exist in anaerobic and facultative microorganisms, and only the latter case has been studied. They contain a stable glycyl radical (G•) in their active form as an initiator of catalysis (2). Like class I enzymes, they have an α2β2 structure. However, the G• is located on the large subunit (α), whereas the small subunit (β) is required for the posttranslational generation of the G•. The β subunit is a member of the “radical SAM” enzymes, which use a reduced [4Fe-4S] cluster to reductively cleave S-adenosylmethionine (AdoMet) to methionine and a 5’-deoxyadenos-5’-yl radical. The latter then generates the G• (20). The G• is sensitive towards oxygen, which results in cleavage of the α polypeptide chain of the α subunit (21). Class III RNRs use formate as the reducing equivalents, instead of thioredoxin or glutaredoxin in class I and II RNRs (22).

It was believed that there was the fourth class of RNRs (e.g., from Corynebacterium ammoniagenes), which was proposed to use a di-manganese cluster as the radical initiator (23). However, further research showed that it still required a diiron-Y• cofactor and was subsequently reassigned to class Ib (24). There was no conclusive evidence for a Mn-dependent RNRs until our recent report on the enzyme from Chlamydia trachomatis (see below).
Ec RNR as the Prototype of Conventional Class I RNRs

Structural Properties of Ec RNR

Our research is focused on class I RNR, with the enzyme from E. coli (Ec 1.17.4.1) as the prototype. Ec RNR has an $\alpha_2\beta_2$ quaternary composition. The crystal structures of R1 ($\alpha_\) (25) and R2 ($\beta_\) subunits (26) have been reported, but the structure of the $\alpha_2\beta_2$ holoenzyme has not yet been solved. A “docking model” based on surface complementarity in which R1 straddles on the top of the heart-shaped R2 was proposed (25), serving as the current model for interaction between R1 and R2 (Figure 1-1). The C-terminus of R2 is believed to be the “bridge” for the interaction of two subunits. Synthesized peptides corresponding to this region could compete with intact R2 for association to R1 and inhibit the enzyme activity (27). In the structure of R2,
the last detectable residue in Ec R2 is V340. The remaining 35 C-terminal amino acids are disordered and not observed. Co-crystallization of R1 with a 20-mer peptide corresponding to the C-terminus of R2 reveals that the last 15 residues (360-375) in R2 are inserted in a shallow groove between two helices in R1 (pink in Figure 1). Based on the docking model, the distance between diiron-Y• in R2 and active site in R1 is estimated to be ~35Å.

**Reductive Chemistry at the Active Site in R1 Subunit**

R1 ($\alpha_2$, Mr = 171,000) is the subunit that carries out catalysis. Perturbation of the system using site-directed variants of Ec R1 and mechanism-based inhibitors has provided the bulk of the insight into the reduction mechanism by inference. A model involving five essential cysteines (7, 28, 29) was proposed, and it was strongly supported by the structure reported later (25). C439 is thought to be transiently oxidized by one electron to the C• by the R2 subunit and to initiate the reaction by abstracting the 3'-hydrogen atom. C225 and C462 are oxidized to a disulfide concomitantly with substrate reduction. C754 and C759 in the C-terminus of R1 reduce the active-site disulfide and are subsequently reduced by an external protein reducing system such as thioredoxin or glutathione and glutaredoxin.

The current working model at the active site is shown in Scheme 1-2 (9). Once the thiyyl radical is generated, it is assumed to abstract the 3’ hydrogen of the substrate to generate a ribonucleotid-3’-yl radical intermediate to initiate the reaction. This intermediate is proposed to eliminate its 2’-hydroxyl group as water, and the 3’-hydroxyl group is deprotonated by glutamate E441 simultaneously, forming a 3’-keto-2’-deoxyribonucleotid-2’-yl radical, 1. Reduction of 1
proceeds via the oxidation of two cysteines to a disulfide. The 2′-deoxyribonucleotid-3′-yl radical, 2, is generated by protonation of glutamic acid E441. In the last step of the mechanism, the hydrogen atom that was originally removed from the substrate is re-abstracted by radical 2, regenerating the thyl radical on C439 and completing the reaction. The disulfide is reduced before the next turnover takes place.

Scheme 1-2: Proposed mechanism of substrate reduction by Ec R1 active site. Adapted from ref (9).

Because of the unfavorable kinetics in the enzyme, direct observation of C• is very difficult. So far, the only direct evidence for the existence of transient C• is from L. leichmannii (class II) RNR, in which a broad EPR (electron paramagnetic resonance) signal derived from an organic radical dipolar coupled to cob(II)alamin (8) is shown during catalysis. The chemical similarities
of substrate reduction, and the structure similarity in the region of the putative cysteine radicals among all three classes of enzymes, provide convincing evidence that a thiol radical, which initiates the nucleotide reduction by abstracting 3’-H from the substrate, is generated as a common strategy during catalysis (3, 15, 30).

**O₂ Activation at the Fe₂^{III/II} Cluster in R2 Subunit**

*Ec* R2 (β₂, Mr = 87,000) contains a carboxylate-bridged diiron center per β, which reacts in its Fe₂^{II/II} state with O₂ for the univalent oxidation of a buried tyrosine residue, Y122, which is 5.3Å away from the closest Fe atom (a process referred to as “O₂ activation”), thereby introducing the Fe₂^{III/III}-Y• cofactor that initiates catalysis by PCET (13, 31). The Y• exhibits a sharp absorption peak at 411 nm and an asymmetric doublet centered around \( g = 2.0 \) in the EPR spectrum (32, 33). Mössbauer spectroscopy demonstrated that the cofactor contains two nonidentical high spin Fe(III) ions in an antiferromagnetically coupled binuclear cluster with a diamagnetic (\( S_{\text{total}} = 0 \)) ground state (34). O₂

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**Figure 1-2**: Crystal structure of the diiron cluster in (A) Fe₂^{II/II} form of *Ec* R2 (PDB ID # 1PIY), (B) diferric form of *Ec* R2 (PDB ID # 1MXR) and (C) diferric form of MMOH from *M. capsulatus* (Bath) (PDB ID # 1MTY)
activation can be completely uncoupled to the subsequent PCET and nucleotide reduction. Thus, most of the studies on O₂ activation were carried out with R2 subunit only.

The crystal structures have shown the differences between Fe₂²⁺/²⁺ form, which is made by diffusing ferrous into crystals of apo protein anaerobically (35), and Fe₂³⁺/³⁺-Y, the met form lacking the radical (26, 36, 37) (Figure 1-2A and B). In the Fe₂²⁺/²⁺ cluster, Fe1 is 4-coordinate and Fe2 is 5-coordinate. E238 adopts a μ-(η¹,η²) coordination mode with one oxygen atom bridging the two iron ions and both oxygen atoms coordinated to Fe2. In the met form, one μ-oxo bridge derived from dioxygen (38) is generated and each Fe ion employs a terminal water/hydroxide ligand. E238 undergoes a carboxylate shift during the reaction and is a monodentate ligand to Fe2. Fe1 is thus 5-coordinate and Fe2 is 6-coordinate. The orientation of Y• in the active form was obtained by single-crystal high-field EPR (37). This study revealed a significant rotation of the tyrosyl side chain in the active radical state, away from the diiron center, compared to the orientation of Y122 in the structure of met-R2.

During O₂ activation, the four-electron reduction of dioxygen to water is balanced by oxidation of two Fe²⁺ ions to Fe³⁺, oxidation of Y122 by one-electron to the radical, and transfer of an “extra” electron from solution. The current proposed mechanism is shown in Figure 1-3.

The mechanism of cofactor assembly in wildtype Ec R2 was investigated using a combination of rapid kinetic methods in conjunction with various spectroscopic methods (39, 40). An intermediate, termed X, was first discovered to be kinetically competent to oxidize Y122 in the final and rate-limiting step in the presence of “extra” reductant, e.g., excess Fe²⁺ or ascorbate (31, 41). X exhibits a broad band with a detectable shoulder at 360 nm in the absorption
spectrum and a sharp, nearly isotropic $g = 2.0$ signal in X-band EPR. When the reaction is carried out with the isotope $^{57}$Fe, which has a nuclear spin $I = 1/2$, the signal is broadened, due to hyperfine coupling to two $^{57}$Fe nuclei. Mössbauer analysis indicated that X exhibits antiferromagnetic coupling between a high-spin Fe$^{III}$ ($S = 5/2$) and a high-spin Fe$^{IV}$ ($S = 2$) site that results in an $S_{\text{total}} = 1/2$ ground state ($39, 42$). The selective enrichment procedure of one Fe site with $^{57}$Fe in X showed that the site with greater affinity (Fe2) is oxidized to Fe$^{IV}$, while the site with lesser affinity (Fe1) is the high-spin Fe$^{III}$ of X ($43$). The structure of X is still debated. The distance between the two Fe atoms in X was suggested to be 2.5 Å by extended X-ray absorption fine structure (EXAFS) spectroscopy ($44$). Electron nuclear double resonance (ENDOR) spectroscopy revealed that X contains two oxygen atoms that are both initially derived from O$_2$, with one present as a μ-oxo bridge and one as a terminal water or hydroxide ligand ($45, 46$). Magnetic circular dichroism (MCD) spectroscopy and density functional theory (DFT) calculations reported by Solomon group suggested a bridged μ-oxo/μ-hydroxo [Fe$^{III}$(μ-O)(μ-OH)Fe$^{IV}$] structure ($47, 48$), although the calculations by Noodleman group

Figure 1-3: Current understanding on mechanism of Ec RNR. The red boxes indicate EPR-active cofactors. States encircled with a grey dotted line have not been directly detected.
preferred a bis-μ-oxo “diamond core” structure (49).

The intermediate X is three-electron oxidized relative to the Fe$_2^{II/II}$ form while the completed reduction of O$_2$ requires four electrons. The extra electron, which is required for the formation of X, is shuttled to the diiron cluster via the near surface residue W48 (50). W48 is connected to the buried diiron cluster by a network of hydrogen bonds involving D237 and one ligand of Fe1, H118 (Figure 1-2B) (36). It is transiently oxidized to a tryptophan cation radical (W48$^{+\cdot}$) before getting reduced in vitro by an exogenous facile one electron reductant (50). The reductant of W$^{+\cdot}$ in vivo remains unclear, but the [2Fe-2S]-cluster ferredoxin, YfaE, was suggested to be the possible electron donor in this process (51).

In the absence of obvious exogenous reductant, i.e., when W48$^{+\cdot}$ is not rapidly reduced, itself is also able to generate Y122•, leading to the formation of an intermediate containing both Y• and X, termed X-Y• (Figure 1-3, lower pathway) (50, 52). X then decays to Fe$_2^{III/III}$ form slowly. In general, X and W$^{+\cdot}$ each stores one of the two oxidizing equivalents. Depending on the availability of reductant, one of them generates Y122• while another one gets reduced (“wasted”). The existence of the precursor for both pathways, an X-W$^{+\cdot}$ diradical species, was supported by Mössbauer and EPR analysis (50). Kinetic studies showed that the formation of the X-W$^{+\cdot}$ is the first order in both O$_2$ and Fe$_2^{II/II}$-Ec R2 complex, even at the highest concentration that could be reached, implying that all the precursors to the diradical species are kinetically masked in wt Ec R2 reaction. In order to resolve this problem, O$_2$-activation reactions of several R2 variants were investigated.

There is a family of enzymes that use very similar carboxylate-bridged diiron clusters to
activate $O_2$ for oxidation reactions, some of which have been extensively studied, e.g., soluble methane monooxygenase hydroxylase (sMMOH) (53). sMMOH catalyzes the oxidation of the methane to methanol. Its $Fe^{II/II}$ form reacts with $O_2$ in $Fe^{II/II}$ form, carries out the 2-electron oxidation of the substrate and the diiron cluster is oxidized to $Fe^{III/III}$. Re-reduction of the $Fe^{III/III}$ cluster by the reductase completes the catalytic cycle. Two important intermediates were identified during the reaction of $Fe^{II/II}$ with $O_2$, named $P$ ($H_{peroxo}$) and $Q$, respectively. The first intermediate, $P$, is a $\mu$-1,2-peroxo-$Fe^{III/III}$ complex that exhibits spectroscopic properties $\lambda_{max} \sim 700$ nm and $\delta$ of 0.66 mm/s. $Q$, the successor of $P$, is the species that directly oxidizes the substrate, methane. $Q$ contains two antiferromagnetically coupled high-spin $Fe^{IV}$ ions that are linked by two single atom oxygen bridges to form a “diamond core” structure. The spectroscopic studies suggest that the O-O bond of $O_2$ is cleaved during the $P$ to $Q$ conversion (54).

Compared to other members of this enzyme family, R2 is unique in its exploitation of the two oxidizing equivalents asymmetrically to carry out a one electron oxidation reaction, and employing an aspartate (D84) instead of a glutamate (E) as the carboxylate ligand to Fe1 (Figure 1-2B and C). To test the correlation between the function and structure, the behavior of variant D84E was investigated. An intermediate that has a broad optical absorption band centered near 700nm accumulated during the reaction. Spectroscopic analysis and DFT calculations showed that it is a $\mu$-1,2-peroxo-$Fe^{III/III}$ species (55, 56), which is nearly identical to intermediate $P$ identified in MMOH (57, 58), suggesting that the symmetrical peroxide adduct is a common intermediate in the diverse oxidation reactions mediated by members of this class. It was worried that the substitution might cause this species to form in preference to a different intermediate in
wild-type Ec R2 reaction, rather than merely stabilizing the wild-type intermediate. Observations that the same species accumulates and decays to X during reaction of wild-type mouse R2 (59-61) removed the remaining doubt that this peroxo intermediate is “on pathway”.

The accumulation of the direct precursor of X-W⁺⁺ intermediate, a state assigned as [Fe₂O₂]⁴⁺, was trapped by double mixing experiments. In the W48A/Y122F variant employed in that study, the electron transfer to the diiron cluster is blocked from both outside (by substituting W48 with A) and inside (by substituting of Y122 with F). In the first mix, the [Fe₂O₂]⁴⁺ state is generated by reaction of the Fe₂⁰² form with O₂. In the second mix the solution is mixed with 3-methylindole (3-MI), the analogue of the truncated W48 sidechain. This reaction triggers rapid decay of the precursor to X. Following this chemical rescue methodology, the Mössbauer features were resolved by kinetic difference. It was proposed that there are at least two distinct antiferromagnetically coupled diiron (Fe₂<sup>III/III</sup>) clusters in quick equilibrium in this state (62, 63), which were proposed to be protonated successors to μ-1,2-peroxo-Fe₂<sup>III/III</sup> intermediate, P (56).

The complete understanding of the differences between R2 and other carboxylate-bridged diiron enzymes that carry
out two-electron oxidation led to the rational reprogramming of R2 into a self-hydroxylating monooxygenase. The key is to prevent the asymmetric exploitation of the two oxidizing equivalents in R2. The replacement of tryptophan 48 (W48) to phenylalanine (F) is required in order to block the transfer of the “extra” electron (Figure 1-4, blue circle). But W48F single variant gives a diradical intermediate X-Y•, suggesting that [Fe2O2]4+ species that generates W48•+ in wt Ec R2 oxidizes Y122 instead (64). Another substitution is thus applied, of which the aspartic acid 84 (D84) is replaced by a glutamic acid (E) to mimic the structure in MMOH (Figure 1-4, red circle). W48F/D84E variant carries out the ε-hydroxylation of F208 residue, implying that the oxidation outcome of diiron enzyme in this family is controlled by both “inner” sphere (D84) primary structure and “outer” spheres (W48) around the diiron cluster (65).

**PCET between R1 and R2 Subunits**

The question how does Y• on R2 generate the transient C• on R1 remains in mystery. Several features together make it one of the most complicated electron transfer system in biology. First, the process is highly reversible. Recent studies show that Y• is reduced and re-oxidized during each turnover but changes of the concentration of Y• during catalysis are not observed (10). Second, the distance between the two residues in Ec RNR is as long as ~35 Å, which is estimated by the “docking” model generated from the structure of individual subunits (25) and supported by pulsed electron-electron double resonance (PELDOR) spectroscopy and double quantum coherence (DQC) methods (66, 67). Using the Marcus-Levich equation, one can estimate the rate constant for electron transfer through a single-step tunneling over this long
distance to be $10^{-4}$ to $10^{-9}$ per second, \(^2\) which is far too slow to account for catalytic turnover number of 2 to 10 s\(^{-1}\) for \(Ec\) RNR (10). The electron transfer is thus believed to be mediated by a network of conserved, hydrogen-bonded, redox-active, aromatic amino acids (69-72), including W48 and Y356 in \(Ec\) R2 and Y731 and Y730 in \(Ec\) R1, by forming intermediate W• and Y• “pathway radicals” (Figure 1-5). These residues are conserved in all known class I RNRs and changing of any of them leads to the complete inactivation of the enzyme. This hypothesis is particularly supported by recent work from Gray and co-workers (73), which shows that a tryptophan can accelerate the rate of electron transfer by \(~300\)-fold than documented for single-step electron transfer when it is inserted between the electron donor, a photoexcited Re\(^{1}\)-diimine, and the acceptor, a Cu\(^{1}\) atom, which are 19 Å away in an engineered azurin protein.

Further, the electron transfer is believed to be coupled to proton transfer, referred as PCET (proton-coupled electron transfer). Without consideration of the possible effect by local protein environment, a Y• with the

\[ k_{ET} = k_{ET} (0) \exp (-\beta r) \], where \( \beta = 1.1-1.4 \text{ Å}^{-1} \), r = 35 Å, and \( k_{ET} (0) = 10^{13} \text{ s}^{-1} \) for an activationless process (68)

<table>
<thead>
<tr>
<th>reaction</th>
<th>( E^{\circ} ) (NHE)/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS• ( \rightarrow ) RSH</td>
<td>1.33</td>
</tr>
<tr>
<td>RS• ( \rightarrow ) RS(^{-})</td>
<td>0.77</td>
</tr>
<tr>
<td>WH•(^{+}) ( \rightarrow ) WH</td>
<td>1.15</td>
</tr>
<tr>
<td>W• ( \rightarrow ) WH</td>
<td>0.9-1.05</td>
</tr>
<tr>
<td>Y• ( \rightarrow ) YOH</td>
<td>0.83-0.94</td>
</tr>
<tr>
<td>Y• ( \rightarrow ) YO(^{-})</td>
<td>0.65</td>
</tr>
</tbody>
</table>

\(^2\) footnote: \( k_{ET} = k_{ET} (0) \exp (-\beta r) \), where \( \beta = 1.1-1.4 \text{ Å}^{-1} \), r = 35 Å, and \( k_{ET} (0) = 10^{13} \text{ s}^{-1} \) for an activationless process (68)
reduction potential of ~0.9 V vs NHE (normal hydrogen electrode) is not able to oxidize a cysteine to C• that has a reduction potential of 1.33V. However, the redox properties of amino acids can be modulated by their protonation states (Table 1-1). Deprotonation of the thiol group of the cysteine reduces the potential to 0.77 V, making the oxidation thermodynamically feasible (10).

Two main reasons prevent the direct observation of the PCET reaction. First, the reaction kinetics are unfavorable. Pre-steady-state kinetic experiments suggest that forward PCET to generate C• undergoes a rate-limiting physical step that is gated by the binding of a substrate and allosteric effector (PCET is thus considered to be “conformationally-gated”) before the subsequent fast events (74). In the steady state, the rate-determining step is either the same physical step or, alternatively, a step involved in re-reduction of R1 or a conformational change associated with this re-reduction, after dNDP formation and regeneration of the Y• (74). Intermediates are thus believed to be “kinetically masked”. Second, even if the potential pathway Y•' s accumulate, their UV/vis- and EPR-spectroscopic properties are expected to be indistinguishable from those of the PCET-initiating Y122•. Nevertheless, the radical communication among some of the residues on the proposed PCET pathway does have been observed during O2 activation. W•• can oxidize Y122 in the absence of obvious exogenous reductant as mentioned (52), and there is a rapid radical-transfer equilibrium between W48 and Y356, which is mediated by divalent cations, e.g., Mg2+, at concentrations similar to [Mg2+] employed in the standard RNR assay for optimum activity (~10 mM) (75).

Alternative methods have been applied. Stubbe, Nocera, and co-workers semi-synthesized
various R2 proteins with Y356 substituted by unnatural tyrosine derivatives (76), including 3-nitrotyrosine (77), various fluorinated tyrosine (78, 79), 4-aminophenylalanine (68), and 3,4-dihydroxyphenylalanine (80, 81). More recently, Stubbe, Schultz, and co-workers used the \textit{in vivo} suppressor tRNA/aminoacyl-tRNA synthetase method (82) to replace Y730 or Y731 with 3-aminotryosine (NH$_2$Y). These approaches result in a thermodynamic depression in the free-energy profile at the substituted position on the PCET pathway, causing the radical to reside on the unnatural residue, and thereby potentially solving the kinetic masking problem. In addition, the unnatural tyrosine analogs exhibit distinct new EPR features, which allows them to be detected more easily. In particular, the formation of NH$_2$Y• at 730 and 731 positions in R1 has been shown in a kinetically competent fashion during catalysis (82), probably may be the first example of authentic pathway radical intermediates in the PCET.

\textbf{RNR Inhibitors}

Because of the crucial role that RNR plays in metabolism, the inhibitors of RNRS are appealed to medical researches, as well as the biochemical studies. Inhibitors of class I RNRS are divided into three types.

1) Peptides derived from the C-terminal end of the R2 subunit. The interaction between R1 and R2 subunits are believed to be species-specific, making the peptides a type of potent specific inhibitor as an antiviral agent (83, 84). Peptides mimicking the C-termini of R2 have been shown to inactivate Ec RNR (27) and mouse RNR (85) competitively.

2) One electron reductant targeting the tyrosyl radical. The best characterized compound in
this category is hydroxyurea (HU). It can reduce the tyrosyl radical in the active form aerobically, yielding the intact Fe$_{III/II}$ cluster without radical (met form) \( (34) \). The possibility of HU as an inhibitor of human immunodeficiency virus-type 1 (HIV-1) replication was proposed \( (86) \). The enzyme is more sensitive to inhibition by HU during catalysis (i.e. in the presence of R1, allosteric effector and substrate) rather than with the R2 subunit only \( (87) \). The reason for this intriguing observation is unknown, but could be relevant to the conformational change upon holoenzyme assembly. A transient nitroxide-like radical have been detected in the reaction of HU with active R2 proteins from \textit{E. coli} and mouse, suggesting that the one-electron transfer from HU to the tyrosyl radical is the dominating mechanism under this condition \( (88) \).

3) Mechanism-based substrate analogs. Beginning with the study of 2’-deoxy-2’-chloro ribonucleotides \( (89) \), the effect of substrate analogs on RNRs, including 2’-halo-2’-deoxyribonucleotides, 2’-azido-2’-deoxyribonucleotides (Figure 1-6B) \( (89-92) \), 2’-deoxy-2’-methyleneccytidine 5’-diphosphate \( (93) \), 2’-fluoromethylene-2’deoxycytidine 5’-diphosphate \( (94, 95) \), 2’-deoxy-2’,2’-difluorocytidine (gemcitabine) 5’-diphosphate \( (93, 96) \) etc., have been extensively investigated. They all irreversibly inactivate the enzyme under turnover conditions, although by different mechanisms. Studies on these chemicals have offered precious information of reduction mechanism at the active site \( (4, 5) \), as well as their potential to be the antiviral and anticancer drugs \textit{in vivo} \( (97, 98) \). One of the most intriguing results is that incubation of 2’-azido-substituted substrate analogs results in the generation of a new stable radical species derived from R1 subunit concomitant with stoichiometric disappearance of Y$^\cdot$. Studies on \textit{Ec} RNR suggests that formation of the 3’-centered substrate radical leads to loss of
the azido moiety either as N$_3$- or N$_3$• (99), followed by the formation of a nitrogen-centered radical in which the cysteine sulfhydryl group of C225 is covalently attached to the ribose of the inactivator via an N$_3$-derived nitrogen and the 3’oxygen (Figure 1-6C), with the half life of 10 min at 25°C (92). As a result, the Y• is irreversibly reduced instead of being regenerated, as it is at the end of a normal turnover. The use of the 2’-azido-2’-deoxyribonucleotide with the anaerobic Ec RNR (class III RNR) results in irreversible loss of the G• (100). Thus, the 2’-azido-2’-deoxynucleotide analog is considered to be a good candidate for the identification of the C•-generating cofactor in RNRs.

**Current Understanding on Ct RNR as the Prototype of Class Ic RNRs**

*Discovery of Class Ic RNRs*

The R1 and R2 subunits of RNR from *Chlamydia trachomatis* (*Ct*) were first cloned, expressed and purified by McClarty group (16). *Ct* is a common obligate intracellular parasite and human pathogen that can lead to a variety of diseases, including trachoma (the leading cause of preventable blindness), sexually transmitted infections, infertility and ectopic pregnancy in women (101, 102). Comparison of the amino acid sequence of *Ct* RNR to those of other class I RNRs revealed that most of the residues, which are critical in catalysis, are conserved, with two
exceptions: a) the aspartate ligand to Fe1 (D84 in Ec R2) is substituted by glutamate (E89 in Ct R2), and the most striking change b) the radical-harboring tyrosine residue (Y122 in Ec R2) is replaced by a non-oxidizable residue, phenylalanine (F127 in Ct R2). These two changes were also observed in the other five chlamydial R2s, the sequences of which were accessible to the public data bases by that time. Nevertheless, the enzyme from Ct is catalytically active, despite the absence of the PCET-initiating Y• (I6).

The crystal structure of Ct R2 reported by Nordlund and Gräslund group confirms these features (Figure 1-7), in which F127 is located in the position where the radical harboring tyrosine is normally situated (I7). Moreover, these substitutions are also observed in other organisms including human pathogens Mycobacterium tuberculosis and Tropheryma whippelii. The Y•-less class I RNRs were named class Ic RNRs (I7). It was further suggested that the independence of the stable Y• might render the RNR of the pathogens more robust to reactive oxygen and nitrogen species [RO(N)S] produced in the host’s innate immune response (I7). A thorough understanding of the mechanism how the C• in the R1 subunit is generated could help in the design of new antibiotics and treatments for pathogens containing the class Ic RNR.

![Figure 1-7: Crystal structure of the Fe$_{\text{II}}$ cluster in (A) Ec R2 (PDB ID # 1MXR) and (B) Ct R2 (PDB ID # 1SYY). Adapted from ref (I7).](image-url)
Early Mechanistic Model of Ct RNR with $\text{Fe}_{2}^{\text{IV/III}}$ species as the PCET Initiator and the Intrinsic Problems

Subsequent work by Gräslund and co-workers focused on the discovery of the species that functionally replaces $Y\cdot$ in conventional class I RNRs to initiate the catalysis. They proposed that the $\text{Fe}_{2}^{\text{III/IV}}$ intermediate ($X_{Ct}$) is the PCET-initiating state. During $O_2$ activation at $\text{Fe}_{2}^{\text{II/II}}$-$Ct\ R2$, a singlet EPR signal with a $g$-value of 2.0 and a linewidth of $\sim18$ G was detected (17), while the final product, presumably the $\text{Fe}_{2}^{\text{III/III}}$ form, is EPR-silent. $^{57}\text{Fe}$- and $^1\text{H}$-ENDOR analysis of intermediate $X_{Ct}$ suggested the structure of two bridging oxo ligands and one terminal water ligand (103). $X_{Ct}$ decays reasonably fast (~0.2 s$^{-1}$ at 20°C) when R2 is alone, but is stabilized 400-fold under catalytic condition with the decay rate of $5\times10^{-4}$ s$^{-1}$ at 37°C (104). Moreover, the $X_{Ct}$ complex is induced when catalysis is initiated from $Ct\ R2$ in $\text{Fe}_{2}^{\text{III/III}}$ form. Based on these studies, the mechanism shown in Figure 1-8 was proposed for $Ct\ RNR$ (105).
This mechanism involves O$_2$ activation at Fe$_{\text{II/III}}$ form to generate X$_{Ct}$ (steps II and III), catalysis (steps IV to IX), and regeneration of X$_{Ct}$ after (occasional) reduction to the Fe$_{\text{III/III}}$ form (steps X and I).

We started our research by trying to reproduce what has been reported. The induction of X$_{Ct}$ species from Fe$_{\text{III/III}}$-Ct R2 under turnover conditions could not be reproduced, although the enzyme is active. Addition of a solution containing Fe$_{\text{II/II}}$-Ct R2 to an aerobic solution containing, R1, CDP and ATP results in generation and stabilization of X$_{Ct}$ complex. However, activity of these samples is identical to that of a sample of Fe$_{\text{III/III}}$-Ct R2 lacking detectable levels of X$_{Ct}$. In other words, we find that Ct RNR activity does not correlate with X$_{Ct}$ content. Moreover, replacement of the substrate, CDP, with N$_3$-ADP, the well-known RNR inhibitor, which should lead to the stoichiometric disappearance of the radical initiator, does not accelerate the decay of X$_{Ct}$.

Ct RNR is the first Example of a Mn-Dependent RNR

My Ph.D. work indicates that Ct RNR actually employs a heterobinuclear Mn/Fe cluster for catalysis. Evidences showing that a stable, high-valent Mn$_{\text{IV/III}}$ complex is the active form are present in Chapter 2. During catalysis, the Mn$_{\text{IV/III}}$ cofactor presumably generates the conserved C• in the R1 subunit by PCET and is reduced to the Mn$_{\text{III/III}}$ form. Thus, the Mn$_{\text{IV/III}}$ cofactor of Ct RNR, specifically the high-valent Mn$_{\text{IV}}$ site, functionally replaces the Y• cofactor of the conventional class I RNR. This is the first example for a redox-active Mn/Fe cofactor in biology and has subsequently allowed us to investigate various questions concerning
In Chapter 3, detailed mechanistic studies on assembly of Mn$^{IV}$/Fe$^{III}$ cofactor are present. As an analogy of O$_2$ activation at Fe$_2^{II/III}$ site in conventional class I RNRs, the fully reduced Mn$^{II}$/Fe$^{II}$ cofactor activates oxygen to form a high-valent Mn$^{IV}$/Fe$^{IV}$ intermediate. Reduction of this cluster, or, more specifically the Fe$^{IV}$ site, yields the active Mn$^{IV}$/Fe$^{III}$ state. The reduction during O$_2$ activation is mediated by the coordination of W51, the cognate of W48 in Ec R2, and a surface residue Y222, which does not have a redox-active cognate in Ec enzyme. Mn$^{IV}$/Fe$^{III}$ cofactor can also be generated by reaction between hydrogen peroxide (H$_2$O$_2$) and either Mn$^{II}$/Fe$^{II}$ or Mn$^{III}$/Fe$^{III}$ form. H$_2$O$_2$ as a two-electron oxidant can oxidize Mn$^{II}$/Fe$^{II}$ to Mn$^{IV}$/Fe$^{IV}$ through the formation of Mn$^{III}$/Fe$^{III}$, followed by the one-electron reduction to the formation of the active form. Chapter 2 and 3 are summarized in Figure 1-9.

The active form of Ct R2 (Mn$^{IV}$/Fe$^{III}$) is EPR-silent whereas the reduced form (Mn$^{III}$/Fe$^{III}$) is EPR-active (Figure 1-9). This is the first case that the reduced form of class I RNR can be monitored by EPR and other advanced paramagnetic resonance methods. Ct RNR thus offers
some unique opportunities to study the very challenging question: the details of the conformationally-gated long-range PCET between the R2 and R1 subunits. Preliminary studies of reduction of \( \text{Mn}^{IV}/\text{Fe}^{III} \) cofactor by hydroxyurea (HU) and dithionite are present in Chapter 4. It turns out that the outcome of the reduction is highly perturbed upon binding of substrate to the active site in the R1 subunit, although the binding site is presumably \( \sim 35\text{Å} \) away from the cofactor.

Compared to class I R2s, class Ic R2s have two conserved amino acid substitutions. The radical-harboring tyrosine (Y122 in \textit{Ec} R2) is replaced by phenylalanine (F127 in \textit{Ct} R2), and the conserved aspartate ligand to metal site 1 (D84 in \textit{Ec} R2) is replaced with glutamate (E89 in \textit{Ct} R2). We have termed these enzymes therefore also as “D/Y” and “E/F” R2s. The influence of these substitutions on the assembly and function in catalysis was investigated in the single variants \textit{Ct} R2-E89D and \textit{Ct} R2-F127Y. The E89D variant is much less efficient in incorporating Mn, but retains its activity, suggesting that E89 plays an important role for tuning the site for the Mn/Fe cofactor. The F127Y variant exhibits catalytic activity initially, but becomes completely inactive within one hour. Both residues are crucial for activity of \textit{Ct} RNR.

Chapter 6 is the outlook of this project. The absence of the essential, radical-harboring \( \text{Y}^\bullet \) in \textit{Ct} RNR offers the excellent possibility to detect small amounts of authentic \( \text{Y}^\bullet \) pathway radicals that could not be distinguished in the presence of the initiating \( \text{Y}^\bullet \) in conventional class I RNRs. The sensitivity of the EPR signal of the reduced \( \text{Mn}^{III}/\text{Fe}^{III} \)-state to binding of substrate to the holoenzyme, presumably \( \sim 35\text{Å} \) from the Mn/Fe center away, offers the unique possibility to monitor the conformational change required for PCET (“gating”). The possible chemical and
biological rationale for the replacement of D→E and Y→F will be explored. Bioinformatics searches identified 15 putative R2s, of which 10 are “D/Y” and 5 are “E/F”, suggesting that they use Fe$_{III/III}$-Y• and Mn$^{IV}/$Fe$_{III}$ cofactors, respectively. Because all 15 sequences are ≈70% identical and ≈83% similar, we will attempt to rationally redesign a “D/Y” enzyme into an “E/F” enzyme and vice versa. Finally, the existence of two distinct radical-generating cofactors in class I RNRs poses the question as to their evolution. It is speculated that they may have both evolved from a common, presumably Fe$_{2}$-containing ancestral protein (“Ur“-R2).
Chapter 2

*Ct* RNR Harbors A Stable Mn$^{IV}$/Fe$^{III}$ Cofactor that Initiates Catalysis
Discovery of the Heterobinuclear Mn/Fe cluster of Ct RNR

The identification of the cofactor required for the activity is the key step for any studies in enzymology. The first biochemical characterization of ribonucleotide reductase from *C. trachomatis* (*Ct* RNR) was reported in 2000 (16) and the crystal structure of the R2 subunit was revealed by 2004 (17). Its high similarity to conventional class I RNRs fooled all the people, including us at the beginning, to believe that it probably harbored a diiron cluster for catalysis, as all the class I RNRs do. Specifically, a high-valent Fe$^{II/IV}$ ($X_{ct}$) species was proposed to be the active form (104, 105).

Our study on *Ct* RNR started with a careful analysis on the correlation between iron content and catalytic activity (Figure 2-1). The activity [velocity (v)/[R2]] of the as-isolated R2 from cells grown in regular rich LB media with ambient iron concentration is 0.035 s$^{-1}$. The iron content of *Ct* R2 is ~0.75 equiv per R2 monomer, notably lower than 2 equiv in theory. This form can take up more iron (1.5 Fe, 2-fold increase), but the activity remains the same. Apo-*Ct* R2, generated by the removal of metal ions from the as-isolated form by reductive chelation, has almost no iron.

![Figure 2-1: Comparison of Fe content (top, on blue shaded part) and activity (s$^{-1}$) (bottom, on red shaded part) for *Ct* R2 prepared in different ways.]

\[ \text{LB rich media} \quad \text{LB rich media} \quad \text{minimal media} \]

\[
\begin{array}{c|c|c|c|c}
\hline
\text{Fe} & 0.75 & 0.05 & 0.05 & 0.75 \\
\text{activity (s}^{-1}) & 0.035 & 0.008 & 0.025 & 0.003 \\
\hline
\end{array}
\]

\[ \downarrow \text{Fe} \quad \downarrow \text{Fe} \quad \downarrow \text{Fe} \]

\[ \text{1.5} \quad \text{1.5} \quad \text{1.5} \]

\[ \text{0.035} \quad \text{0.010} \quad \text{0.035} \]

\[ [\text{Fe}/[\text{R2}]] \quad \nu/[\text{R2}] \text{ (s}^{-1}) \]

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\(^3\) All metal equivalencies and activities for the *Ct* R2 protein are reported on a per monomer basis in this thesis.
(<0.05), and as expected, has a reduced activity of 0.008 s\(^{-1}\). However, this protein can take up 1.5 Fe (30-fold increase), yet the activity increases only marginally (0.010 s\(^{-1}\)) and is significantly lower than that of the as-isolated form. We have also carried out the overexpression in cells grown in different media to compare activities of various as-isolated forms. Previous studies have shown that adding phenanthroline to LB rich media during cell growth can efficiently block iron entering overexpressed protein while other metal ions can still be incorporated \((106, 107)\). As-isolated \textit{Ct} R2 expressed under this condition had nearly no iron as expected (0.05 equiv), but is nearly as active (0.025 s\(^{-1}\)) as the protein obtained from cells grown in the absence of the chelator. Addition of Fe to this protein led to a 30-fold increase of Fe, but only a slight increase in activity that is comparable to the one from regular LB rich media. \textit{Ct} R2 obtained from cells grown on minimal media with iron supplement had an extremely low activity of 0.003 s\(^{-1}\), although it had a comparable Fe content to the one from LB rich media. Clearly, there is no correlation between iron content and enzyme activity of different preparations of R2, which is contrary to \textit{Ec} RNR \((34)\), suggesting that an unknown cofactor is crucial to the activity.

In parallel, the various \textit{Ct} R2 proteins prepared under different conditions, were examined by a combination of EPR and Mössbauer spectroscopies. EPR is sensitive to detect species with half-integer-spin ground states (e.g., \(S = 1/2, 2/3, 2/5\ldots\)). Mössbauer gives information of all Fe-containing species. In particular, species with integer-spin ground states (e.g., \(S = 0, 1, 2\ldots\)) usually give rise to a quadrupole doublet in Mössbauer spectra collected at zero or weak applied magnetic field, which is often sufficiently well resolved from other spectral components and make the detection and quantification much easier. Therefore, the combination of EPR and
Mössbauer spectroscopies is powerful to characterize all states of an Fe-containing enzyme. When the protein reconstituted by adding Fe$^{II}$ to the apo form aerobically was reduced by dithionite for a short time, a $g \sim 2$ EPR signal with multiple splitting lines was observed (Figure 2-2A). Substitution of $^{56}$Fe ($I = 0$) with $^{57}$Fe ($I = 1/2$) led to the broadening of lines, indicating that the electron spin is coupled to the $^{57}$Fe nuclear spin. Spin quantification showed that it accounted for $\sim 1\%$ of total iron concentration. We assumed that this “1%” species might be the real “active” form, and the multiple lines could arise from the hyperfine coupling to a transition metal with high nuclear spin number ($I$), e.g., $^{55}$Mn with $I = 5/2$. The EPR spectrum of an established Mn$^{III}$/Fe$^{III}$ complex with $S = 1/2$ ground state (Figure 2-2B) (108) is similar to this signal, suggesting that this minor, EPR-active form of Ct R2 may harbor a heterobinuclear Mn/Fe cluster.

Following the spectroscopic clue, we carried out the activity dependence on Mn and Fe. By reductive chelation of iron from the purified protein and subsequent dialysis against ethylenedinitrilotetraacetate (EDTA) (see Appendix B), R2 was isolated with less than 0.02 equiv iron (apo form) and very low catalytic activity ($\leq 0.01 \text{s}^{-1}$). The apo R2 so obtained is not
detectably active after anaerobic addition of 2 equiv of either Fe$^{II}$ or Mn$^{II}$ and exposition to O$_2$ for 10 min. By contrast, addition of both divalent metal ions to the apo protein increases the activity by more than 50-fold (Figure 2-3). A 1:1 ratio of Mn:Fe gives maximal activity (Figure 2-3A), and a total of two divalent metal ions per monomer was sufficient for ~85% of maximal activity (Figure 2-3B), suggesting the use of a Mn/Fe cofactor rather than Fe$_2$ cofactor by Ct RNR.

**Evidence for O$_2$ Activation**

Except the radical-harboring tyrosine, all the other residues on the proposed PCET pathway in Ct RNR are conserved (16), suggesting that the metallocofactor in R2 need to transiently oxidize the putative cysteine residue in R1 by one electron (12). Therefore, the fully reduced Mn$^{II}$/Fe$^{II}$-Ct R2 that should form upon addition of the divalent metal ions cannot be active. Rather, it is probably converted to an oxidized state by reaction with O$_2$, in analogy to the generation of the Fe$_2$ $^{III/III}$-Y• cofactor in conventional class I RNRs(12). This hypothesis was supported by activity assays on O$_2$ dependence. No turnover was detected after addition of an O$_2$-free R2 solution (containing R2, 0.75 equiv Mn$^{II}$ and 0.75 equiv Fe$^{II}$) to an O$_2$-free assay solution (containing R1, DTT, substrate CDP, and allosteric effector ATP), but the activity was observed when the same R2 solution was mixed with O$_2$-containing
assay solution. A comparable activity was also detected when the R2 solution was first exposed to O₂ before it was added to the O₂-free assay solution, suggesting that it did not require O₂ during the catalysis once R2 had been activated.

**Mn<sup>IV</sup>Fe<sup>III</sup> Complex as the Product of O₂ Activation**

The product of O₂ activation was prepared by mixing an equal volume of O₂-free solution containing Ct R2 with 0.5 equiv Fe<sup>II</sup> and 1.0 equiv Mn<sup>II</sup> (to disfavor the formation of diiron cluster) and O₂-saturated buffer at 5 °C for 10 min.<sup>4</sup> It exhibits no obvious EPR signal in X-band, perpendicular mode. Mössbauer analysis shows a quadrupole doublet at 4.2 K and zero field (Figure 2-4A). The parameters (isomer shift, δ, = 0.52 mm/s and quadrupole splitting parameter, ΔE<sub>Q</sub>, = 1.32 mm/s) establish that the iron site is converted to a high-spin Fe<sup>III</sup> by the reaction with O₂. The sharp (line-width, Γ, = 0.30 mm/s) doublet in zero magnetic field is markedly broadened when a weak (53 mT) external field is applied (Figure 2-4B).

![Figure 2-4: Mössbauer spectra of the final product of the reaction of Mn<sup>II</sup>Fe<sup>II</sup>-Ct R2 with O₂ and its reduction form by dithionite. (A and B) Mössbauer spectra of the product of O₂ activation acquired at 4.2 K in zero magnetic field (A) and with a 53-mT field oriented parallel to the γ beam (B). (C) Mössbauer spectrum at 190K and zero field of the reduction form of the final production after O₂ activation. The solid lines in (A) and (C) are theoretical quadrupole doublets with parameters described in the text.](image)

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<sup>4</sup>This method was used for preparation of the final product of O₂ activation in early studies. Mössbauer analysis showed that it gave ~75% Mn<sup>IV</sup>/Fe<sup>III</sup> and ~25% Fe<sup>II</sup>/Fe<sup>III</sup> complex. An improved methods (see Appendix B) was applied in later studies, which typically gave ~90% Mn<sup>IV</sup>/Fe<sup>III</sup> and ~10% Fe<sup>II</sup>/Fe<sup>III</sup> complex.
indicating that this complex has a paramagnetic ground state with an integer value of the total electron-spin quantum number \( S_{\text{total}} \). This characteristic distinguishes this product from the \( \text{Fe}^{\text{III/III}} \) clusters in conventional R2 proteins, which are diamagnetic \( (S_{\text{total}} = 0) \) due to antiferromagnetic coupling between the two high-spin \( \text{Fe}^{\text{III}} \) \( (S = 5/2) \) ions \( (34) \).

The presence of a Mn ion coupled to the \( \text{Fe}^{\text{III}} \) site is demonstrated by the characterization of the one-electron-reduced form of the complex produced by a brief \( (~ 2 \text{ min at } 22 \ ^{\circ}\text{C}) \) treatment with 20 mM dithionite. The reduced form is stable and does not react with \( \text{O}_2 \). The EPR spectra with multiple lines (Figure 2-5A) suggest hyperfine coupling to \( ^{55}\text{Mn} \ (I = 5/2) \). In addition, the lines are broadened by \( ^{57}\text{Fe} \) (Figure 2-5B). The \( g \)-value of \(~2 \) establishes that the reduced form has a coupled Mn/Fe cluster with \( S_{\text{total}} = 1/2 \).\(^5\) The Mössbauer spectrum of the reduced complex acquired at zero field and 190 K (Figure 2-4C) is a broad \( (\Gamma \sim 0.5 \text{ mm/s}) \) quadrupole doublet\(^6\) with parameters \( \delta = 0.43 \text{ mm/s} \) and \( \Delta E_Q = 0.81 \text{ mm/s} \), indicating that the iron site remains in the high-spin \( \text{Fe}^{\text{III}} \) state. Thus, the dithionite treatment reduces the Mn site but not the \( \text{Fe}^{\text{III}} \) site of the active state. The reduced Mn site must have a \( \text{Mn}^{\text{III}} \) \( (S_{\text{Mn(III)}} = 2) \), for coupling with the high-spin \( \text{Fe}^{\text{III}} \) site \( (S_{\text{Fe(III)}} = 5/2) \) to give \( S_{\text{total}} = 1/2 \). Therefore, the Mn in the product of \( \text{O}_2 \) activation is in \( \text{Mn}^{\text{IV}} \) configuration. Most likely, the cluster has a \( S_{\text{total}} = 1 \) ground state resulting from the antiferromagnetic coupling between the \( \text{Mn}^{\text{IV}} \) \( (S_{\text{Mn}} = 3/2) \) and high-spin \( \text{Fe}^{\text{III}} \) \( (S_{\text{Fe}} = 5/2) \) (will be presented in details later in this chapter).

\(^5\) Recall that these spectra are nearly identical to the ones we detected before the discovery of Mn/Fe cofactor as shown in Figure 2-2.

\(^6\) The \( S = 1/2 \) complex exhibits a magnetically split Mössbauer spectrum at low temperature, but the use of this higher temperature makes electronic relaxation fast compared with the \( ^{57}\text{Fe} \) nuclear precession frequency and collapses the spectrum into a quadrupole doublet.
**Mn^{IV}Fe^{III} Complex is the Active Form to Initiate PCET**

The most important question for *Ct* RNR was the identification of the “active form” that substitutes the Fe^{II/III}-Y• in conventional class I RNRs to initiate PCET. We speculated that if the Mn^{IV}/Fe^{III} cluster is the active form, it should be transiently reduced by one electron to the Mn^{III}/Fe^{III} form during catalysis (1).

The Mn^{III}/Fe^{III} form can be chemically generated by dithionite reduction as mentioned before. Interestingly, in the presence of R1, substrate (CDP), and allosteric effector (ATP) during reduction, the same redox state (Mn^{III}/Fe^{III}) is generated, but the features of the EPR spectrum are much sharper and more featured (Figure 2-5C and D). This observation suggests that the binding of R1 (in particular the R1-substrate-effector complex)\(^7\) to the R2 subunit probably causes a conformational change in R2 that is propagated to the cofactor and induces greater homogeneity, a phenomenon not previously observed in conventional class I RNRs. By simulation of the spectrum of the ^{56}Fe-containing sample (Figure 2-5C, dashed trace), the \(g\)-tensor of the \(S = 1/2\) ground state (2.030, 2.020, 2.015) and the manganese hyperfine coupling tensor, \(A_{Mn} [269, 392, 314] \text{ MHz}\), were determined. The significant anisotropy of \(A_{Mn}\) is consistent with the assignment as Mn^{III} (109, 110). Additional evidence for the Fe^{III} configuration is provided by the ^{57}Fe hyperfine coupling (Figure 2-5D, solid trace), which can be reproduced (Figure 2-5D, dashed trace) with an isotropic \(A_{Fe} [64.5, 64.5, 64.5] \text{ MHz}\) typical of high-spin Fe^{III} (111).

\(^7\) Detailed studies on this phenomenon will be present in Chapter 4.
The substrate analog, 2'-azido-2'-deoxyadenosine-5'-diphosphate (N3-ADP), was used to confirm the conclusion that the MnIV/FeIII cluster is the functional cofactor. In the previous work of Ec RNR, it has been shown that the treatment with 2'-azido-2'-deoxynucleotide results in the
stoichiometric scavenging of $Y^\bullet$ and the formation of a nitrogen-centered free radical \((89, 99)\), in which a cysteine sulfhydryl group of the enzyme is believed to be covalently linked to the ribose of the inactivator via an $N_3$-derived nitrogen \((92)\). For the case of \(Ct\) RNR, treatment with $N_3$-ADP should lead to the irreversible one-electron reduction of the “active form” as well, i.e., the conversion of the EPR-silent Mn\(^{IV}/Fe^{III}\) cluster to the EPR-active Mn\(^{III}/Fe^{III}\) state, together with the formation of nitrogen-centered radical. As expected, the same EPR signal emanating from the Mn\(^{III}/Fe^{III}\) cluster seen upon dithionite reduction under turnover condition was observed (Figure 2-5\(E\) and \(F\)), together with additional sharp features at $g \approx 2$ (marked by arrows; see also Figure 2-5\(G\)). These features are nearly identical to those observed for the N-centered radical reported in \(Ec\) RNR (Figure 2-5\(H\)) \((112)\). As a control, the signal did not develop under the same conditions when the inactivator was replaced by the natural substrate, CDP. Thus, the active form must be the EPR-silent species, Mn\(^{IV}/Fe^{III}\)-\(Ct\) R2.

**Reconsideration of the Previous \(Ct\) RNR Studies**

We attempted to reconcile our conclusion that Mn\(^{IV}/Fe^{III}\)-R2 is the active form with previous observations suggesting that Fe\(^{III/IV}\)-R2 is active \((17, 104, 105)\). Suspecting that the modest activity that we and others observe without adding Mn\(^{II}\) to R2 might result from a Mn "contaminant" (in R2 or another assay component), we tested for development of the very

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\(^8\) The spectrometer conditions for Figure 2-5\(G\) are: $T = 14$ K; \(\nu = 9.45\) GHz; \(P = 10\) $\mu$W; modulation frequency, 100 kHz; modulation amplitude, 2 G; time constant, 0.167 s, and 10 scans per spectrum, while the ones for Figure 2-5\(H\) are: $T = 101$ K; \(\nu = 9.43\) GHz; \(P = 1\) mW; modulation frequency, 100 kHz; modulation amplitude, 1.5 G; and time constant, 0.126 s. The calculation shows that the shift on the scale of field is due to the minor difference of the microwave frequency, which caused the $g = 2$ position shift from \(3380\) G (Figure 2-5\(G\)) to \(3372\) G (Figure 2-5\(H\)).
distinctive EPR signal of the Mn\textsuperscript{III}/Fe\textsuperscript{III}-R2•R1 complex after treatment with dithionite R1, CDP and ATP. The spectrum was readily detected (Figure 2-6A). Its intensity relative to that exhibited by the purposefully generated Mn\textsuperscript{III}/Fe\textsuperscript{III}-R2 sample (~ 4-5%; compare to Figure 2-6B) correlates with the ratio of enzymatic activity of the two R2 forms.

We suggest that the activity previously attributed to the Fe\textsuperscript{II/IV}-R2 complex resulted from a Mn\textsuperscript{IV}/Fe\textsuperscript{III}-R2 contaminant that escaped detection (104, 105). First, Figure 2-3 shows that only ~ 0.2 equiv contaminating Mn would have been required to give the maximum R2 activity previously reported (230 nmol dCDP•min\textsuperscript{-1}•mg\textsuperscript{-1}, ν/[R2] = 0.155 s\textsuperscript{-1}) (104). Whereas this quantity is five times the ~ 0.04 equiv detected in our preparations (vide supra), the different methods of subunit preparation and perhaps different enzyme concentrations employed in the activity determinations [not reported in (104) but expected to have been lower than in our assays due to their use of the more sensitive radiometric assay] could have resulted in a greater Mn/R2 stoichiometry in the previous study. Second, much lower activities of < 75 nmol•min\textsuperscript{-1}•mg\textsuperscript{-1} (ν/[R2] < 0.05 s\textsuperscript{-1}), similar to the activities of some of our preparations of R2 before activation with Mn\textsuperscript{II} and Fe\textsuperscript{II}, were reported in the subsequent study by the same group (105). This large variation in specific activity seems inconsistent with the authors' belief that

![Figure 2-6: (A) X-band EPR signal showing formation of Mn\textsuperscript{III}/Fe\textsuperscript{III} complex upon dithionite treatment of a sample prepared with R2 reconstituted only with 1.5 equiv Fe\textsuperscript{II}. Spectrometer conditions: T = 14 K; ν = 9.45 GHz, P = 1 mW; modulation amplitude, 4 G; microwave frequency, 9.45 GHz; time constant, 0.167 s; resolution of field axis, 1024 points, 25 scans. (B) Single scan of the sample as for Figure 2-5C with otherwise same spectrometer conditions for A.](image)
addition of only Fe\textsuperscript{II} should have fully activated R2 but could be explained by variation in the quantity of Mn in their preparations. With uncoupled Mn\textsuperscript{II} present, R2 would have been activated by addition of only Fe\textsuperscript{II}, but the extent of activation would have depended on the quantity of contaminating Mn. Third, in contrast to all other conventional class I RNRs, the active R2 state has no obvious EPR signal. Thus, if uncoupled Mn\textsuperscript{II} were initially present, addition of Fe\textsuperscript{II} in the presence of \( \text{O}_2 \) would have trapped the Mn in an EPR-silent state, allowing it to escape detection by the reported EPR experiments \((104, 105)\). We suggest that these considerations can reconcile all previous observations with our proposal that Mn\textsuperscript{IV}/Fe\textsuperscript{III}-R2 is the sole active form.

**Detailed Characterization of the Mn\textsuperscript{IV}/Fe\textsuperscript{III} Active Form**

Although heterobinuclear Mn/Fe complexes of various oxidation states \((108, 113, 114)\), including one example of an inorganic Mn\textsuperscript{IV}/Fe\textsuperscript{III} complex \((115)\), have been reported, \( \text{Ct} \) RNR is, to our knowledge, the first case in which an enzyme has been shown to use a Mn/Fe cluster as a redox cofactor. The analysis on Mn\textsuperscript{IV}/Fe\textsuperscript{III}-\( \text{Ct} \) R2, the active form, by variable-field Mössbauer spectroscopy has allowed us to study the electronic structure in detail.

The 4.2K/zero-field Mössbauer spectrum of a
sample identical to Figure 2-4. A comprises two quadrupole doublets with similar isomer shifts ($\delta$) but different quadrupole splittings ($\Delta E_Q$). The minor doublet (25% of the total intensity) matches the spectrum of the Fe$_{2}^{III/III}$ cluster, which is characterized by $\delta = 0.50$ mm/s and $\Delta E_Q = 0.79$ mm/s (Figure 2-7, solid line). The major doublet (75% intensity) arises from the functional Mn$^{IV}$/Fe$^{III}$ cofactor. To analyze the Mössbauer spectra collected in variable applied magnetic fields, it was necessary first to remove the 25% contribution attributed to the Fe$_{2}^{III/III}$ complex. The derived spectra of the Mn$^{IV}$/Fe$^{III}$ complex are shown in Figure 2-8.

Simulations are carried out with the assumption of slow relaxation and according to the spin Hamiltonian (Equation 2-1) with respect to the total electron spin ground state, $S_{total} = 1$.

$$H = \beta \mathbf{S} \cdot \mathbf{g} \cdot \mathbf{B} + D \left( S_z^2 - \frac{S(S+1)}{3} \right) + E \left( S_x^2 - S_y^2 \right) + \frac{eQV}{4} \left[ I_z^2 - \frac{I(I+1)}{3} + \frac{\eta}{3} (I_x^2 - I_y^2) \right] + \mathbf{S} \cdot \mathbf{A} \cdot \mathbf{I} - g_N \beta_N \mathbf{B} \cdot \mathbf{I} \quad (2-1)$$

In Equation 2-1, the first term represents the electron Zeeman effect, the second and third terms represent the axial and rhombic zero field splitting of the electronic ground state, the fourth term represents the interaction between the electric field gradient and the nuclear quadrupole moment of the Fe sites, the fifth term represents the magnetic hyperfine interaction of the electronic spin with the $^{57}$Fe nucleus, and the last term represents the nuclear Zeeman interaction. The $\mathbf{A}$-tensor is given with respect to the total spin. All tensors are assumed to be collinear.

The zero-field spectrum is a sharp quadrupole doublet with parameters ($\delta = 0.52$ mm/s and $\Delta E_Q = 1.32$ mm/s) typical of high-spin Fe$^{III}$, which were fixed in the following analysis. The other parameters in the simulations, according to Equation 1, are the asymmetry parameter, $\eta$, 
the axial and rhombic zero-field splitting (ZFS) parameters of the $S_{\text{total}} = 1$ ground state, $D_{S=1}$ and $(E/D)_{S=1}$, respectively, and the hyperfine tensor for the Fe$^{\text{III}}$ ion, $(\mathbf{A}/g_N \beta_N)_{\text{Fe}}$.

The fact that the overall splitting of the spectrum is larger at 4 T than at 8 T reveals that the electronic Zeeman term (first term in Equation 2-1) dominates the ZFS interaction at these magnetic field strengths. As a consequence, the spin expectation value of the ground state ($\langle S \rangle$) and the internal magnetic field [$B_{\text{int}} = -\langle S \rangle \cdot (\mathbf{A}/g_N \beta_N)_{\text{Fe}}$] approach their limiting values (corresponding to $\langle S \rangle = -1$) at these applied fields, and the hyperfine tensor can be determined accurately from the spectra. In a spin-coupled cluster, the $A$-tensor for the iron ion with respect to the $S_{\text{total}} = 1$ ground state ($A_{\text{Fe}}$) is given by Equation 2-2, in which $c_{\text{Fe}}$ and $a_{\text{Fe}}$ represent the vector coupling coefficient and the intrinsic hyperfine tensor, respectively, with the assumption that the total spin states are well separated in energy (i.e., $J \gg D$) (116).

$$A_{\text{Fe}} = c_{\text{Fe}} a_{\text{Fe}}$$ (2-2)

For high-spin Fe$^{\text{III}}$ sites, $a_{\text{Fe}}$ is dominated by the Fermi contact term and exhibits nearly
isotropic values of ~ -30 MHz. For the $S_{\text{total}} = 1$ state, $c_{\text{Fe}} = +7/4$. Thus, $A_{\text{Fe}}$ is expected to be nearly isotropic with values of ~ -53 MHz. Indeed, analysis of the spectra yielded a nearly isotropic hyperfine tensor, $A_{\text{Fe}} = (-55.3, -53.6, -52.3)$ MHz, similar to the expected value.

With the $A_{\text{Fe}}$ determined from the 4-T and 8-T spectra, the ZFS-parameters were then determined accurately from the spectra with lesser applied magnetic fields, under which conditions the ZFS is significant compared to the electronic Zeeman term. Simulation of the spectra provided for determination of the magnetic-field dependence of $\langle S \rangle$, which determines the magnitude of the internal magnetic field and the resultant splitting in each spectrum. From the field-dependent spectra, $D_{S=1} = -1.9$ cm$^{-1}$ and $(E/D)_{S=1} = 0.33$ were found. $D_{S=1}$ is related to the intrinsic $D$-values of the Mn$^{IV}$ and Fe$^{III}$ ions via Equation 2-3,$^{10}$ with the assumption that the total spin states are well separated in energy (i.e., $J \gg D$) ($\text{II6}$).

$$D_{S=1} = d_1 D_{\text{Fe}} + d_2 D_{\text{Mn}} = 14/5 D_{\text{Fe}} + 3/10 D_{\text{Mn}}$$

(2-3)

$D_{S=1}$ is dominated by the contribution from the Fe$^{III}$ site$^{11}$ and can be rationalized with a value typical for high-spin Fe$^{III}$ ($|D_{\text{Fe}}|$ of ~ 0.7 cm$^{-1}$) ($\text{III}$).

The Mn$^{IV}$ and Fe$^{III}$ ions could conceivably couple ferromagnetically to yield an $S_{\text{Total}} = 4$ ground state. However, the inherent anisotropy of $\langle S \rangle$ of the $S_{\text{total}} = 4$ system precludes simulation

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For a cluster composed of two coupled ions with local spin $S_1$ and $S_2$ to give a total spin $S$: $c_1 = [S(S+1) + S_1(S_1+1) - S_2(S_2+1)] / [2S(S+1)]$, $c_2 = [S(S+1) + S_2(S_2+1) - S_1(S_1+1)] / [2S(S+1)]$. In this case, the values of $S = 1$, $S_{1(\text{Fe})} = 5/2$ and $S_{2(\text{Mn})} = 3/2$ give $c_{1(\text{Fe})} = +7/4$ and $c_{2(\text{Mn})} = - 3/4$.

For a cluster composed of two coupled ions with local spin $S_1$ and $S_2$ to give a total spin $S$: $d_+ = [3(S_1(S_1+1)-S_2(S_2+1))^2 + S(S+1)[3S(S+1) - 3 - 2S_1(S_1+1) - 2S_2(S_2+2)] / (2S+3)(2S-1)]$, $d_- = [4S(S+1) - 3] [S_1(S_1+1) - S_2(S_2+1)] / (2S+3)(2S-1)$. In this case, the values of $S = 1$, $S_{1(\text{Fe})} = 5/2$ and $S_{2(\text{Mn})} = 3/2$ give $d_+ = +31/10$, $d_- = +5/2$, thus $d_{1(\text{Fe})} = +14/5$ and $d_{2(\text{Mn})} = + 3/10$.

High-spin Fe$^{III}$ and Mn$^{IV}$ have well-isolated, nondegenerate orbital ground states and typically exhibit small intrinsic $D$ values.
of all the experimental spectra with a single set of spin-Hamiltonian parameters. Specifically, comparison of the 4-T and 8-T spectra establishes that $\langle S \rangle$ along all three molecular axes nearly reaches saturation in an applied field of $\sim 4$ T. $|D_{S=4}|$ would therefore have to be small ($< 0.5 \text{ cm}^{-1}$). Different from the case of $S_{\text{total}} = 1$ (Figure 2-9B), with a $D_{S=4}$ of this small magnitude, $\langle S \rangle$ (for $D_{S=4} < 0$) saturates at $\sim 30$ mT (Figure 2-9C), resulting in much greater magnetic hyperfine splitting in the weak-field spectra than is observed (Figure 2-9A). We have also considered different $D$ and $E/D$ values. The $E/D$-dependence clearly shows that the magnitude of the $z$-component is large for all $E/D$ values (Figure 2-9D) in the weak field (150 mT). For $D > 0$, the component with the large expectation value is in the $y$-direction (Figure 2-9E). All combinations of ZFS parameters have at least one large $\langle S \rangle$ value. Thus, ferromagnetic coupling to yield $S_{\text{total}} =$.
4 can be ruled out.

We have also characterized the Mn$^{IV}$/Fe$^{III}$-Ct R2 cofactor by extended X-ray absorption fine structure (EXAFS) spectroscopy and DFT calculations, with the collaboration of Dr. Mike T. Green lab in Penn State University. The detailed work is done by Younker et. al (117) and only the conclusions are summarized here. Fe and Mn $K$-edge EXAFS consistently yield a distance of 2.9 Å between two metal ions (Figure 2-10A). This distance is ~0.4 Å longer than the Fe-Fe distance found for the Fe$^{III/IV}$ cluster X from Ec RNR (44), suggesting that the cofactor of Ct R2 has a fundamentally different geometry. The Mn data also reveal the presence of a short 1.74 Å Mn-O bond. Computational models were derived from the crystal structure of Ct R2 in the inactive Fe$^{III/III}$ form (17). Systematic variation of the protonation states of the three OH$_x$ ligands in the starting structure and the metal ion [Fe$^{III}$ or Mn$^{IV}$] occupying site 1 generated a series of 12 models for the Ct R2 cofactor (Figure 2-10B), which were compared to the EXAFS measurements.

**Figure 2-10:** (A) Mn (top) and Fe (bottom) $K$-edge EXAFS data (left) and Fourier transforms (right) for the Mn$^{IV}$/Fe$^{III}$-Ct R2. Arrows designate metal-metal vector at 2.91 and 2.92 Å from Mn and Fe EXAFS, respectively. (B) 12 models differentiated by the protonation states of their bridging and terminal OH$_x$ ligands as well as the location of the Mn$^{IV}$ and Fe$^{III}$ ions. Models that are consistent with EXAFS are in blue with bond distances that correspond to EXAFS measurements in bold italics. Adapted from ref (117).
measurements and the parameters extracted from Mössbauer analysis. The core structure with bis-μ-oxo requires much shorter Mn-Fe distance than the measurement while the bis-μ-hydroxo requires longer, thus both possibilities can be ruled out. The structure with one μ-oxo bridge and one μ-hydroxo bridge connecting two metals (blue in Figure 2-10B) matches the experimental result very well. The site-placement of the metal ions and the protonation state of the terminal water ligand cannot be discerned from the available data. The favored model for the active form is presented in Figure 2-11.

**Discussions**

Our work on *Chlamydia trachomatis* RNR strongly suggests the first characterized Mn- and Fe-dependent RNR, as well as the first Mn/Fe cluster as a redox cofactor in biology. It is possible that other metals might substitute for Mn (or Fe) *in vivo*, but the subtlety of the cofactor’s function (gated electron transfer by a stable, high-valent complex) makes this possibility remote. Actually, the effects of CuII, CoII, NiII, ZnII and the combination of FeII and each ion have been tested. Neither obvious O2 activation (monitored by stopped-flow absorption spectroscopy) nor enhanced activity has been detected.

The RNR from *Corynebacterium ammoniagenes* (formerly called *Brevibacterium*...
ammoniagenes (23, 118) has been repeatedly reported as a Mn-dependent enzyme (class IV) by Auling and co-workers. As noted, Sjöberg and co-workers subsequently reassigned this RNR to class Ib that utilizes the Fe\textsuperscript{III/IV}-Y• cofactor to initiate PCET (24, 119). This controversy is a clear example that for enzymes harboring complex metallo-redox cofactors the activity should be correlated to the relative amounts of the different forms of the cofactor determined by spectroscopic methods. It is the same argument here for the assignment of Mn\textsuperscript{IV/III} cluster to be the active form in Ct RNR, instead of Fe\textsuperscript{III/IV} cluster. Another interesting example is that the assembly of Mn/Fe cofactor has also been reported in Ec R2 (114). The reaction of H\textsubscript{2}O\textsubscript{2} at Mn\textsuperscript{II/II}-Ec R2 generates the Mn\textsuperscript{III/III} cluster, which exhibits a quite similar EPR signal as the sharp and featured spectrum in Mn\textsuperscript{III/III}-Ct R2 shown in Figure 2-5C. Nevertheless, Ec RNR undoubtedly employs the Fe\textsuperscript{III/III}-Y• cofactor as the active form.

The identification of the Mn\textsuperscript{IV/III} cofactor in Ct RNR is the start (and the “rate-limiting step”) of this project. Detailed studies/discussion on the assembly of the active form, the PCET process, and the possible chemical/biological rationale for this novel cofactor will be presented in following chapters.
Chapter 3
Assembly of the Mn$^{IV}$/Fe$^{III}$ Cofactor in Ct R2
After the discovery of Mn$^{IV}$/Fe$^{III}$$-Ct$ R2 as the catalytically active form, the mechanism of its assembly was investigated. Previous studies on the assembly of Fe$_2$$^{III/IV}$-$Y\bullet$, the cognate in Ec R2 (31, 39, 41, 52), have shown how to illustrate the transient-state kinetics and characterize the intermediate during a single-turnover reaction, by using a combination of rapid kinetic methods in conjunction with various spectroscopic methods. Stopped-flow (SF) absorption spectroscopy, which rapidly mixes two or more reactant solutions and collects the real-time optical spectra of the mixture, is often used at the first step to measure the kinetics of reactants, intermediates, and products, provided that they have absorption bands in the observed region (usually between 300 to 700 nm) and accumulate to detectable levels. Further characterization depends on various spectroscopic methods. However, in contrast to SF-absorption spectroscopy, these methods cannot be carried out in real time. It usually takes minutes or even days to collect a single spectrum. The rapid freeze-quench (FQ) technique is thus applied to overcome this obstacle, which quenches the reaction after a certain amount of time after mixing by spraying the mixed solution into a solution of a cryosolvent (e.g., isopentane or liquefied ethane at -150 °C).

A high-valent Mn$^{IV}$/Fe$^{IV}$ intermediate has been detected during the reaction of O$_2$ activation at the Mn$^{II}$/Fe$^{II}$ form of $Ct$ R2. To our knowledge, it is the first example of such complex in chemistry and biology. Its properties have been carefully characterized by EPR and Mössbauer spectroscopies. The one-electron reduction of Mn$^{IV}$/Fe$^{IV}$ species, specifically, the Fe$^{IV}$ site, leads to the formation of Mn$^{IV}$/Fe$^{III}$ complex as the stable product. The shuttling of the “extra” electron is mediated by a near-surface residue Y222, which has a redox-inactive leucine in Ec R2 (L233). However, Y222 is not a required component for catalysis, implying the existence of branched
electron transfer pathways for activation and catalysis.

The effects of H$_2$O$_2$, one of the important physiological reactive oxygen species (ROS), on various forms of Ct R2 have been studied. The Mn$^{IV}$/Fe$^{III}$ form is completely stable and active in the presence of H$_2$O$_2$. The reduced form, Mn$^{III}$/Fe$^{III}$, is rapidly and quantitatively (>90%) reactivated by H$_2$O$_2$. The reaction involves the two-electron oxidation of Mn$^{III}$/Fe$^{III}$ to Mn$^{IV}$/Fe$^{IV}$, which then decays back by transfer of an electron via Y222 to the active Mn$^{IV}$/Fe$^{III}$ form. The reaction of the Mn$^{II}$/Fe$^{II}$ form towards H$_2$O$_2$ has also been investigated. The reaction is triphasic, corresponding to two successive two-electron oxidations to yield the Mn$^{III}$/Fe$^{III}$ and Mn$^{IV}$/Fe$^{IV}$ states, respectively, followed by one-electron reduction to Mn$^{IV}$/Fe$^{III}$.

The discussion of this chapter focuses on comparison of these reactions at Mn/Fe (in Ct) and Fe/Fe clusters (in Ct and also other conventional class I RNRs), which gives many interesting implications for the further understanding of this process.

**Kinetics of O$_2$ Activation at Mn$^{II}$/Fe$^{II}$-Ct R2**

Previous Mössbauer characterization on the product of reaction between Mn$^{II}$/Fe$^{II}$-Ct R2 (1.0 equiv Mn, 0.5 equiv Fe) and O$_2$ reveals ~75% of Mn$^{IV}$/Fe$^{III}$ and ~25% of Fe$^{II}$/Fe$^{III}$ form based on Fe distribution, which implies ~87% of the enzyme employs the heterobinuclear cluster during the reaction.\(^{12}\) Since the Mn$_2^{II/II}$ cluster can not react with O$_2$ (unpublished data), a higher ratio of Mn:Fe, typically 3:1, is applied in studies of this chapter to further favor the formation of

\(^{12}\) The relative amounts of Fe$_2$-Ct R2 and Mn/Fe-Ct R2 are obtained by dividing the relative amounts obtained by Mössbauer spectroscopy by the number of Fe ions in the cluster. For example, (Mn/Fe)$\% = (75/1) / (75/1 + 25/2) = 7/8 = \sim 87\%$. 

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the Mn/Fe cluster over the Fe₂ form.

The kinetics of reaction of Mn^{II}/Fe^{II}-Ct R₂ (0.40 mM R₂ monomer, 1.5 equiv Mn^{II}, 0.5 equiv Fe^{II}) with O₂ was first studied by SF absorption spectroscopy. An intense absorption feature centered at ~390 nm (Figure 3-1A) develops rapidly and then partially decays over the course of ~1 min, indicating the transient formation of an unknown intermediate before the formation of Mn^{IV}/Fe^{III} product (refer to Mössbauer spectroscopic characterization in Chapter 2).\(^{13}\) The kinetics was thus defined by monitoring the time dependence of the absorption at 390 nm (A₃₉₀). The effects of variation of [O₂] were interrogated (Figure 3-1B). The A₃₉₀-vs-time

---

**Figure 3-1:** Kinetics of the activation of Ct R₂ by stopped-flow absorption spectroscopy. (A) Spectra acquired at the indicated reaction times after mixing at 5 °C of an O₂-free solution of Mn^{II}/Fe^{II}-Ct R₂ (0.40 mM R₂ monomer, 0.5 equiv Fe, 1.5 equiv Mn) with an equal volume of O₂-saturated buffer. (B) Dependence of the kinetics of the reaction on [O₂] obtained with the white light source and photodiode array detector. An equivalent Mn^{II}/Fe^{II}-R₂ solution was mixed with 100% (black), 50% (red), or 25% (blue) O₂-saturated buffer. The inset shows the apparent first-order rate constant for the formation phase of the reaction versus [O₂], which gives a second order rate constant (slope) of 12 (± 3) mM⁻¹s⁻¹. (C) Dependence of the kinetics on the concentration of ascorbate obtained with the monochromatic light source and photomultiplier detector. A Mn^{II}/Fe^{II}-R₂ solution (0.80 mM R₂ monomer, 0.4 equiv Fe, 1.2 equiv Mn), which contained ascorbate at a concentration sufficient to give the indicated [ascorbate] after mixing, was mixed with 100% O₂-saturated buffer. The inset shows the apparent first-order rate constant for the decay phase versus [ascorbate], which gives a limiting reduction rate constant (asymptote of hyperbolic fit) of 1.0 (± 0.2) s⁻¹.

\(^{13}\) The results of O₂ activation at Fe₂^{II/III}-Ct R₂ will be presented later in this chapter. The absence of the band centered at 390nm during diiron reaction clearly shows that the observations in Figure 3-1 mainly reflect the Mn/Fe reaction.
traces were analyzed by nonlinear regression according to Equation 3-1, which gives absorbance as a function of time ($A_t$) for a system of two parallel, irreversible, first-order reactions in terms of rate constants for the two steps ($k_1$ and $k_2$), their associated amplitudes ($\Delta A_1$ and $\Delta A_2$), and the absorbance at time zero ($A_0$).

$$A_t = A_0 + \Delta A_1[1-\exp(-k_1t)] + \Delta A_2[1-\exp(-k_2t)]$$  \hspace{1cm} (3-1)

Although the $Ct$ R2 reaction comprises two sequential irreversible steps, formation of the intermediate is so much faster than its decay ($k_1 > 300k_2$) that the equation describing the sequential case simplifies to that describing the parallel case. Formation of the intermediate is kinetically first-order in [O$_2$]. From the dependence of the apparent first-order rate constant on [O$_2$] the second-order rate constant (slope) of 12 (± 3) mM$^{-1}$s$^{-1}$ (Figure 3-1B, inset) can be obtained.$^{14}$

Formation of the Fe$_2^{III/III}$-Y• cluster of a conventional class I RNR requires transfer of an "extra" electron to the buried cofactor during its reaction with O$_2$. It has been shown that ascorbate can donate this electron and accelerate the decay of the intermediate ($50$). Similarly, the Mn$^{IV}$/Fe$^{III}$ cofactor of active $Ct$ R2 is three oxidizing equivalents above the Mn$^{II}$/Fe$^{II}$-R2 complex, which reacts with the four-electron oxidant, O$_2$, to produce the active state. Thus, an extra electron is also required in activation of $Ct$ R2. We speculated the decay of the intermediate

$^{14}$ It is generally considered appropriate to invoke the pseudo-first-order approximation implicit in this fitting analysis only when one reactant is in at least 10-20-fold excess over the other. In these experiments, O$_2$ is in excess over the theoretical concentration of the reactive Mn$^{II}$/Fe$^{II}$ complex by a minimum of 2.3-fold and a maximum of 9.0-fold. However, within this range, the apparent first-order rate constant still behaves as a nearly linear function of the concentration of the excess reactant, and the error introduced into the second-order rate constant by the approximation is small (∼10%) in comparison with other sources (∼25% in the values of [O$_2$]).
is a reduction process and the effect of ascorbate was evaluated. Indeed, ascorbate accelerates decay of the intermediate in concentration-dependent fashion without affecting the kinetics of its formation (Figure 3-1C). A plot of the observed first-order rate constant for decay versus [ascorbate] is hyperbolic (Figure 3-1C, inset), suggesting that a unimolecular step is rate-limiting for reduction of the intermediate at high [ascorbate]. The nature of this step is discussed below.

**Spectroscopic Analysis of the Intermediate Reveals a Mn\textsuperscript{IV}/Fe\textsuperscript{IV} Complex**

The electronic structure of the intermediate that exhibits the intense band centered at 390 nm was probed by FQ-EPR and Mössbauer spectroscopies. Analysis of the data demonstrates that the intermediate has an $S_{\text{total}} = 1/2$ ground state as a consequence of antiferromagnetic coupling between the Mn\textsuperscript{IV} ($S_{\text{Mn}} = 3/2$) and high-spin Fe\textsuperscript{IV} ($S_{\text{Fe}} = 2$) ions.

**Characterization of the Intermediate by EPR Spectroscopy**

The difference between the EPR spectra of the reactant solution, which contains free Mn\textsuperscript{II} and the Mn\textsuperscript{II}/Fe\textsuperscript{II} complex, and of the same solution after exposure to O\textsubscript{2} for 10 min to yield the EPR-silent Mn\textsuperscript{IV}/Fe\textsuperscript{III} complex, resolves the EPR spectrum of the Mn\textsuperscript{II}/Fe\textsuperscript{II}-R2 complex, (Figure 3-2). It exhibits a broad resonance centered at $g \sim 2$ that shows hyperfine coupling characteristic of Mn\textsuperscript{IV} and Fe\textsuperscript{II}.

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\textsuperscript{15} It was observed later that this intermediate is photosensitive: its decay is accelerated by the intense white light source used with the photodiode array detector. Thus, the effect of ascorbate on the decay of the intermediate was analyzed based on data acquired with monochromatic 390 nm light and a photomultiplier detector. The dependence of formation on [O\textsubscript{2}] has not been repeated because it should not affect the formation rate constant. This phenomenon is similar to previously observed for the Fe\textsuperscript{II/IV} complex, Q, in the reaction of soluble methane monooxygenase, which is the redox cognate of this intermediate, a Mn\textsuperscript{IV}/Fe\textsuperscript{IV} cluster (vide infra).
of an $I = \frac{5}{2}$ $^{55}$Mn nucleus. Optimal detection of this signal requires relatively low temperature and high power (e.g., 4.2 K and 20 mW, as in Figure 3-2). Spectra of samples, which were freeze-quenched after mixing of an O$_2$-free solution of Mn$^{II}$/Fe$^{II}$-R2 (0.80 mM R2 monomer, 1.2 equiv Mn, 0.4 equiv Fe) with an equal volume of O$_2$-saturated buffer at 5 °C, were recorded under less stringent conditions (14 K and 20 µW) to reduce the contribution from the reactant complex. By subtracting the spectrum of the reactant solution under these conditions from the spectra of the freeze-quenched samples, the contribution of free Mn$^{II}$ (resulting from the use of excess Mn$^{II}$ in preparation of the reactant complex) was removed.

The time-dependent spectra (Figure 3-3A) illustrate that an intermediate with a sharp six-line EPR signal develops rapidly upon reaction with O$_2$ and then decays slowly.

An overlay of the scaled intensities of the EPR spectra with the trace calculated by using the rate constants extracted from the SF data (Figure 3-1C, black line) illustrates that the EPR signal and 390-nm absorption features arise from the same intermediate. The predicted kinetics of the intermediate were calculated according to Equation 3-2, which is derived from the scheme Mn$^{II}$/Fe$^{II}$ $\rightarrow$ intermediate $\rightarrow$ Mn$^{IV}$/Fe$^{III}$ with $k_1$ and $k_2$ as the irreversible first-order rate constants for two steps, respectively (for the derivation see Appendix B).
Figure 3-3: X-band EPR spectra at 14.0 (± 0.2) K of the Mn$^{IV}$/Fe$^{IV}$ intermediate in activation of Ct R2. (A) Spectra of samples freeze-quenched at various reaction times (indicated on the figure) after mixing at 5 °C of an O$_2$-free solution of Mn$^{II}$/Fe$^{II}$-R2 (0.80 mM R2 monomer, 0.4 equiv Fe, 1.2 equiv Mn) with an equal volume of O$_2$-saturated buffer. The spectrum of the recovered product (completion) sample has been scaled to account for the fact that it was manually frozen rather than being freeze-quenched (× 0.6, the "packing factor" typical of freeze-quenched samples). In addition, the appropriately scaled spectrum of the reactant sample (× 0.6 because it was manually frozen) was subtracted from the experimental spectrum of each sample to generate the spectra shown. (B) Kinetics of the Mn$^{IV}$/Fe$^{IV}$ intermediate from the intensity of its EPR spectra shown in A. The solid lines are kinetics calculated according to rate constants extracted from the stopped-flow (Figure 3-2C, black line) under the identical condition: $k_{\text{form}} = 10.8 \text{ s}^{-1}$, $k_{\text{decay}} = 0.021 \text{ s}^{-1}$. (C) Spectra of samples prepared by manual mixing of an O$_2$-free solution of Mn$^{II}$/Fe$^{II}$-R2 (3.0 mM R2 monomer, 0.75 equiv of each metal ion) at ambient temperature (22 ± 2 °C) with 9 equivalent volumes of O$_2$-saturated buffer and freezing after 20 ± 2 s. The first and third traces are the experimental spectra of the samples prepared with $^{56}$Fe and $^{57}$Fe, respectively. Spectrometer conditions were: microwave frequency, 9.45 GHz; microwave power, 20 µW; modulation frequency, 100 kHz; modulation amplitude, 10 G; scan time, 167 s; time constant, 167 ms. The second and fourth traces are simulations of the experimental spectra with the $g$, $A_{\text{Mn}}$, and $A_{\text{Fe}}$ tensors given in Table 1.
[intermediate]_i = [Mn^{II}/Fe^{II}]_0 \cdot [k_i/(k_i - k_1)] \cdot [\exp(-k_1 t) - \exp(-k_2 t)] 

(3-2)

The $g$-value of ~2 in the EPR spectrum indicates that the cluster has a $S_{\text{total}} = 1/2$, and the six lines separated by ~ 80 G reflects the hyperfine coupling to a single $^{55}$Mn nucleus. The second group of transitions (at ~ 3210 G) is nearly isotropic (i.e., all transitions are observed at the same magnetic field). The other five groups either are somewhat broadened or exhibit resolved features due to anisotropy of the $g$- and $A_{\text{Mn}}$-tensors. The resolution of the features within the groups, especially for the fifth and sixth groups, permits determination of the $g$- and $A_{\text{Mn}}$-tensors directly from spectral simulation (Figure 3-3C, second spectrum). $A_{\text{Mn}}$ is nearly isotropic [(247, 216, 243) MHz], similar to $A_{\text{Mn}}$ for the Mn$^{IV}$ site of Mn$_2^{III/IV}$ catalase [(235, 224, 252) MHz] (109)$^{16}$ and as expected for a Mn$^{IV}$ site (109, 110). For the simulation of the EPR spectrum of the intermediate prepared with $^{57}$Fe, the $g$- and $A_{\text{Mn}}$-tensors determined from the spectrum of the intermediate containing $^{56}$Fe were assumed and hyperfine coupling to the $^{57}$Fe was then imposed (Figure 3-3C, bottom spectrum). An isotropic $A_{\text{Fe}}$-tensor was assumed first, but it became obvious that the quality of the simulation could be improved considerably with an anisotropic $A_{\text{Fe}}$. By considering the EPR spectra together with the field-dependent Mössbauer spectra (vide infra), $A_{\text{Fe}}$ was determined.

**Characterization of the Intermediate by Mössbauer Spectroscopy**

$^{16}$ The Mn$^{IV}$ sites of the Mn$_2^{III/IV}$ cluster of catalase and the Mn$^{IV}$/Fe$^{IV}$ intermediate in Ct R2 have the same spin projection factors, and therefore the magnitudes of the $A_{\text{Mn}}$ tensors with respect to the total spin of the $S = 1/2$ ground states can be directly compared.
The 4.2-K/53-mT Mössbauer spectra (Figure 3-4A) of the Mn$^{II}$/Fe$^{II}$-R2 complex (top spectrum) and samples prepared by reacting this complex at 5 °C with O$_2$ for varying reaction times illustrate the accumulation of the intermediate to a high level and its subsequent decay to the previously characterized Mn$^{IV}$/Fe$^{III}$-R2 product (bottom spectrum, see Chapter 2). Importantly, comparison of the spectra of the 90-ms and 2-s samples shows that the dominant features in the latter spectrum (the broad lines indicated by the arrows) develop with the same kinetics as for the $g$ ~ 2 EPR signal and 390-nm absorption feature and are therefore associated with the same intermediate. Analysis of the spectra suggests that the intermediate accumulates to ~40% and ~70% of the total intensity at 90ms and 2s, respectively.

The Mössbauer spectra of the 2-s sample were thus collected at variable external magnetic field for detailed characterization (Figure 3-4B). Ideally, the spectral contributions of the minor species would be removed by subtraction of appropriate reference spectra in order to resolve the spectrum of the intermediate. However, the multiplicity and unknown identities of the minor species in this case make removal of their contributions impossible: (a) A high-spin Fe$^{II}$ species of unknown identity$^{17}$ as the major "contaminant". Its presence is most clearly revealed in the weak-field (B < 53 mT) spectra by peaks at -0.2 mm/s and +2.8 mm/s (~17% intensity; Figure 3-4B, middle spectrum, blue line). The magnetic field dependence of the Fe$^{II}$-associated spectral component is unknown. Fortunately, it is clear that, as expected for high-spin ($S = 2$) Fe$^{II}$, the Mössbauer features become broader and contribute little to the overall line-shape of the

\[17\] Candidates for the Fe$^{II}$ complex(es) are aqueous Fe$^{II}$, complexes in which the divalent metal is bound to R2 either in mononuclear fashion or in a homodinuclear or heterodinuclear cluster, or some combination of these. The Fe$^{II}$-associated features remaining in the spectrum of the 2-s sample are different from the spectrum of the reactant complex and thus cannot simply be removed by subtraction of this spectrum.
Figure 3-4: (A) Mössbauer spectra of an O₂-free sample containing Mn^{II}/Fe^{II} reactant complex (3.0 mM R2 monomer, 0.5 equiv Fe and 1.0 equiv Mn) (top), a sample in which this complex was reacted with an equal volume of O₂-saturated buffer at 5 °C and freeze-quenched at 90 ms (second spectrum) and 2 s (third spectrum), and a sample of the Mn^{IV}/Fe^{III} product of this reaction (bottom spectrum). Spectra were collected at 4.2 K in external magnetic fields of 53 mT oriented parallel to the γ-beam. The solid lines in the bottom spectrum represent the 25% contribution from the homodinuclear Fe₂^{III/III} complex. (B) 4.2-K Mössbauer spectra in varying magnetic fields (as indicated on the spectra) of the sample freeze-quenched at 2 s. Unless otherwise noted, the field was parallel to the γ-beam. The solid black lines plotted over the spectra are simulations of the spectra of the Mn^{IV}/Fe^{IV}-R2 intermediate with the parameters given in Table 1. They are scaled to account for 70% of the total intensity. The red lines are a simulation of the spectrum of the Fe₂^{III/IV} complex (11% of total intensity) with the published parameters, and the blue line is a quadrupole doublet with δ = 1.3 mm/s and ΔE_Q = 3.0 mm/s to illustrate the contribution from the Fe^{II} component of the sample (17% of total intensity).

The experimental spectrum of the 2-s sample with increasing field strengths. (b) The products of the reaction as previously characterized, which contains ~75% Mn^{IV}/Fe^{III}-R2 and ~25% of the
homodinuclear $\text{Fe}_2^{\text{III/III}}$ product (see Chapter 2). Given their slow formation, only a small fraction is in the form of the products at a reaction time of 2 s ($\leq 9\%$ total intensity). (e) The $\text{Fe}_2^{\text{III/IV}}$ precursor ($\text{X}_{Ct}$) to the $\text{Fe}_2^{\text{III/III}}$ product (see later this chapter about the analysis of $\text{O}_2$ activation at $\text{Fe}_2^{\text{II/II}}$-$\text{Ct}$ R2) ($120$). In particular, two features of $\text{X}_{Ct}$ are nearly fully resolved in the 13-mT spectrum and can be used to estimate the contribution from the complex ($\sim 11\%$; Figure 3-4B, bottom spectrum, red line). In addition, the highest-energy lines of the sub-spectra of the Fe$^{\text{IV}}$ and Fe$^{\text{III}}$ sites are coincident at 3.7 mm/s in the 8-T spectrum, resulting in a more intense line that reveals the presence of the complex (Figure 3-4B, top spectrum, red line).

Despite this heterogeneity, the predominance of the intermediate makes the positions and shapes of its features sufficiently clear for simulations (black lines in Figure 3-4B) with the spin Hamiltonian in Equation 3-3 ($111$).

$$\hat{H} = \frac{eQV}{12} \left[ 3I_{\text{Fe,z}}^2 - I_{\text{Fe}} (I_{\text{Fe}} + 1) + \eta \left( I_{\text{Fe,x}}^2 - I_{\text{Fe,y}}^2 \right) \right] + \langle S \rangle \cdot \mathbf{A}_\text{Fe} \cdot \mathbf{I}_\text{Fe} - g_{\text{n,Fe}} \beta_n \mathbf{B} \cdot \mathbf{I}_\text{Fe} \quad (3-3)$$

In particular, the contributions of species with integer-electron-spin ground states (Fe$^{\text{II}}$ species and the Mn$^{\text{IV}}$/Fe$^{\text{III}}$ and Fe$^{\text{III}}$/Fe$^{\text{III}}$ products) are canceled in field orientation dependent spectra (53 mT $\parallel 53$ mT $\perp$) and the contributions of the species with half-integer-spin (the Mn$^{\text{IV}}$/Fe$^{\text{IV}}$ intermediate and $\text{X}_{c,t}$) are resolved. The contribution from $\text{X}_{c,t}$ (red line) is small compared to that of the intermediate (black line). This difference spectrum provides constraints on the parameters of the Mn/Fe intermediate, in particular the isomer shift ($\delta$). Because $\delta$ must be determined from magnetically split spectra, the uncertainty in this crucial parameter is fairly large (0.06 mm/s).

---

$18$ The standard tactic of raising the temperature to make the electronic fluctuation rapid with respect to the nuclear precession frequency and thereby collapse the magnetic spectrum into a quadrupole doublet for more...
Nevertheless, even with the large uncertainty, the value of $\delta (0.17 \pm 0.06 \text{ mm/s})$ indicates that the intermediate has an Fe$^{IV}$ site. Indeed, the center of the range is essentially identical with $\delta$ of the Fe$_2^{IV/III}$ complex, Q, in the reaction of soluble methane monooxygenase (57, 121). Therefore, the iron site is reduced by one electron O$_2$ activation, from the Fe$^{IV}$ in the intermediate to Fe$^{III}$ in the product.

The hyperfine tensor for the Fe$^{IV}$ site with respect to the total spin of the ground state ($S_{\text{total}} = 1/2$), $A_{Fe}$, determines the splitting in the spectra and is given by the product of the intrinsic hyperfine tensor, $a_{Fe}$, and the spin projection factor, $c_{Fe}$ (Equation 3-4) (116).

$$A_{Fe} = c_{Fe} \cdot a_{Fe}$$ (3-4)

The components of $A_{Fe}$ are negative, as revealed by the decrease of the overall splitting with increasing applied magnetic field (e.g., compare the 53-mT, 4-T, and 8-T spectra in Figure 3) (122). The components of the intrinsic hyperfine tensor for iron, $a_{Fe}$, are dominated by the Fermi contact term and are negative. Thus, $c_{Fe}$ must be positive. A positive value of $c_{Fe}$ requires that $S_{Fe} > S_{Mn} (= 3/2)$. The Fe$^{IV}$ site must therefore be in the high-spin configuration ($S_{Fe} = 2$), rather than the intermediate spin ($S = 1$) configuration. For this spin system, $c_{Fe} = 2$ for the $S = 1/2$ ground state, giving $a_{Fe} = (-28.0, -29.7, -20.3)$ MHz. These values are almost identical to those of the high-spin Fe$^{IV}$ site of cluster X in E. coli R2 [$a_{Fe} = (-27.6, -27.6, -20.6)$ MHz] (42), consistent with the assignment of the Fe site of the Ct R2 intermediate as high-spin Fe$^{IV}$.

accurate determination of $\delta$ and $\Delta E_O$ failed. The 120 K/zero-field spectrum is very broad and featureless, implying that the fluctuation rate of the electronic states is comparable to the $^{57}$Fe Larmor frequency (the intermediate relaxation regime) at this temperature.
Comparison of the low-field spectra clearly illustrates the perturbation associated with the hyperfine coupling to the $I = 5/2$ $^{55}$Mn nucleus. Coupling between the electron spin of the ground state, $S = 1/2$, and the $^{55}$Mn nuclear spin, $I = 5/2$, leads to two states with spins $F = 2$ and $F = 3$ (with definition $F = S + I$). Therefore, for the simulation of the Mössbauer spectra, the $S \cdot A_{Mn} \cdot I_{Mn}$ is taken into account of the spin expectation value, $\langle S \rangle$, according to the solutions of Equation 3-5. The energies and $\langle S \rangle$ values are plotted in Figure 3-5 as a function of external magnetic field.

$$\hat{H} = \beta S \cdot g \cdot B + S \cdot A_{Mn} \cdot I_{Mn}$$ (3-5)

**Figure 3-5:** Field-dependence of the energies (A-C) and spin expectation values (D-F) for a spin system with $S = 1/2$ and $I = 5/2$, which couple via the hyperfine interaction to total spin states with $F = 2$ (red) and $F = 3$ (black). $A_{Mn}$ as determined from EPR was used for the calculation. The magnetic field is oriented along the $x$ (A and D), $y$ (B and E), and $z$ (C and F) direction. Dashed lines in D-F mark the field of 13mT and 53 mT.

For $B > 0.1$ T, the electron Zeeman effect (first term in Equation 3-5) dominates the $S \cdot A_{Mn} \cdot I$ term, the states are "pure" and characterized by the $M_S \ [= \pm 1/2]$ and $M_I \ [= \pm 1/2, \pm 3/2, \pm 5/2]$
quantum numbers and the spin expectation values are at their maxima, \( |\langle S \rangle| = 0.5 \). With weaker applied fields, the electron Zeeman interaction and \( A_{\text{Mn}} \) are comparable, leading to mixing of the states. For example, at a field of 13 mT (Figure 3-4B, bottom spectrum), the absolute magnitude of the internal magnetic field [given by \( -\langle S \rangle \times (A/g_N\beta_N)_\text{Fe} \)] is smaller for some of the states as a consequence of sub-saturating values of \( \langle S \rangle \) (Figure 3-5), resulting in reduced splitting and greater intensity in the center of the spectrum. As the external field is increased in the 0 – 0.1 T regime, \( \langle S \rangle \) and the magnetic splitting increase (compare to the 53-mT \( \parallel \) spectrum; Figure 3-4B, middle). For external fields \( B > 0.1 \) T, the internal field reaches its limiting value (\( |\langle S \rangle| = 0.5 \)) and splitting decreases again (as described above).

The spectroscopic parameters for Mn\(^{IV}/Fe^{IV} \) intermediate extracted from EPR and Mössbauer analysis are summarized in Table 3-1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fe(^{IV} ) site</th>
<th>Mn(^{IV} ) site</th>
</tr>
</thead>
<tbody>
<tr>
<td>( g )</td>
<td>2.017, 2.030, 2.027</td>
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</tr>
<tr>
<td>( A ) (MHz)</td>
<td>-55.9, -59.3, -40.5(^a)</td>
<td>247, 216, 243</td>
</tr>
<tr>
<td>( \delta ) (mm/s)</td>
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<td></td>
</tr>
<tr>
<td>( \Delta E_Q ) (mm/s)</td>
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<td></td>
</tr>
<tr>
<td>( \eta )</td>
<td>-10</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Sign determined from Mössbauer spectroscopy

**The Reduction of Mn\(^{IV}/Fe^{IV} \) Is Mediated by Y222**

It is a quite intriguing question how the 4-electron oxidized intermediates ([Fe\(_2\)O\(_2\)]\(^{4+} \) state in Ec R2 and Mn\(^{IV}/Fe^{IV} \) in Ct R2) are reduced by one electron during O\(_2 \) activation while the products, which is in the 3-electron oxidized state and also a strong oxidant (Fe\(_{2\text{III/III}}\)Y• in Ec R2
and Mn\textsuperscript{IV}/Fe\textsuperscript{III} in \textit{Ct} R2), is stable. Previous work on \textit{Ec} R2 has shown that W48, a near-surface, PCET pathway residue, shuttles one electron to the diiron center and is transiently oxidized to the cation radical W48\textsuperscript{+•} (50, 52). W48\textsuperscript{+•} then engages in a rapid redox equilibrium with Y356 only in the presence of > 10 mM Mg\textsuperscript{2+} (75). We therefore also intended to study how the extra electron is transferred to the Mn\textsuperscript{IV}/Fe\textsuperscript{IV} intermediate.

Prior to the discovery that the active form of \textit{Ct} R2 contains a heterobinuclear Mn/Fe cofactor, the initial experiments on the catalytically inactive homobinuclear Fe\textsubscript{2} form of the protein revealed the participation of Y222 in electron transfer to the cofactor during O\textsubscript{2} activation (see later in this chapter). Y222 is close to W51 (the cognate of W48 in \textit{Ec} R2) and has its phenolic hydroxyl group projecting outward from the surface of the protein into solution (Figure 3-6). The cognate of Y222 in \textit{Ec} R2 is a redox-inactive leucine residue (L233). To test whether this novel electron-transfer element also functions during formation of the active Mn\textsuperscript{IV}/Fe\textsuperscript{III} form of \textit{Ct} R2, the behavior of the variant Y222F were studied. The roles of two other residues that play roles in O\textsubscript{2} activation in \textit{Ec} R2 were investigated as well for parallel comparison: W51, the cognate of W48 in \textit{Ec} R2; and Y338, the cognate of Y356 in \textit{Ec} R2.

\textbf{Evidence for Electron Transfer by Y222 during O\textsubscript{2} Activation by the Mn\textsuperscript{II}/Fe\textsuperscript{II} Form}

The kinetics of the 390-nm absorption (Figure 3-7A) suggest that the substitutions have
either no effect (Y222F and Y338F) or a minor effect (W51F) on the development of Mn^{IV}/Fe^{IV} intermediate, and the Y338F substitution has no effect on decay of this feature. By contrast, both the W51F and the Y222F substitutions retard decay of this feature by ~10-fold. Freeze-quench EPR experiments on the reactions of the wt, W51F and Y222F variants were conducted to confirm that the absorbance-versus-time traces accurately reflect the kinetics of the intermediate (Figure 3-7, C-E). The kinetics (black squares, blue circles, and green triangles) extracted from the intensities of the sharp six-line EPR feature of the Mn^{IV}/Fe^{IV} complex agree well with traces calculated from Equation 3-2 by using the rate-constants

![Figure 3-7](image-url):

**Figure 3-7**: Kinetics of the Mn^{IV}/Fe^{IV} intermediate during reaction of the Mn^{II}/Fe^{II} complexes of wt (black), Y338F (red), Y222F (blue) and W51F (green) Ctr R2 proteins at 5 °C with O₂. (A) A390-versus-time traces during the reactions initiated by mixing of an O₂-free solution of Mn^{II}/Fe^{II}-Ctr R2 (0.80 mM monomer, 0.4 equiv Fe^{II}, and 1.2 equiv Mn^{II}) with an equal volume of O₂-saturated buffer at 5 °C. (B) Kinetics of the Mn^{IV}/Fe^{IV} intermediate from the intensity of the six-line EPR spectrum in wt (C), Y222F (D) and W51F (E). The reactions were initiated under reaction conditions identical to those in A and freeze-quenched at the indicated time. The solid lines in B are kinetics of the intermediate for the wt (black) and Y222F (blue) reactions calculated from Equation 3-2 according to rate constants extracted from the stopped-flow results: $k_{\text{form, wt}} = 10.8 \text{ s}^{-1}$, $k_{\text{decay, wt}} = 0.021 \text{ s}^{-1}$, $k_{\text{form, Y222F}} = 10.1 \text{ s}^{-1}$, and $k_{\text{decay, Y222F}} = 0.0021 \text{ s}^{-1}$. Spectrometer conditions: $T = 14.0 \pm 0.2 \text{ K}$; $\nu = 9.45 \text{ GHz}$; $P = 20 \mu\text{W}$; modulation frequency, 100 kHz; modulation amplitude, 10G; scan time, 167 s; time constant, 167 ms.
extracted from the stopped-flow data (Figure 3-7B), confirming that both W51F and Y222F stabilize the intermediate by retarding its reduction.

It was previously shown that ascorbate can accelerate the reduction of the Mn IV/Fe IV intermediate in the reaction of wt Ct R2. Additional stopped-flow absorption experiments were conducted to test whether reduction by ascorbate is mediated by Y222.19 As previously reported, decay of the intermediate is accelerated with a hyperbolic dependence on [ascorbate] in the reaction of the wt protein (Figure 3-8A, black in Figure 3-9D). The Y338F substitution has no significant effect on this behavior (Figure 3-8B, red in Figure 3-8D), consistent with its failure to retard the intrinsic (i.e., in the absence of ascorbate) decay of the intermediate. By contrast, the Y222F substitution (Figure 3-8C, blue in Figure 3-8D) drastically diminishes (by ~65-fold) the efficiency of ascorbate reduction (blue circles and fit line) from an apparent

Figure 3-8: (A-C) Dependence of the kinetics of the Mn IV/Fe IV intermediate on the concentration of ascorbate in the reactions of the Mn II/Fe II forms of (A) wt, (B) Y338F, and (C) Y222F Ct R2 proteins with O2. The stopped-flow experiments were carried out under conditions identical to those in Figure 3-7, with the exception of the presence of ascorbate (sufficient to give the indicated concentrations after mixing) in the protein solution. The solid lines were generated by fitting the Equation 3-1 to the 390-nm kinetic traces. The rate of formation of the Mn IV/Fe IV intermediate does not depend on [ascorbate]. (D) Dependence of the apparent first-order rate constant for the decay of the Mn IV/Fe IV intermediate (extracted from A-C) on the concentration of ascorbate.

19 The long time required for these experiments (~3 h) allowed for significant scavenging of O2 from the atmosphere of the anaerobic chamber by the Mn II/Fe II-R2 stock, resulting in a minor (~20%) diminution in the amplitude of the transient between the first (0 mM ascorbate) and last (15 mM ascorbate) experiments. The traces have been re-scaled to a common amplitude for purposes of comparison.
second-order rate constant \( (k) \) of \((1.3 \pm 0.3) \times 10^{-1} \text{ mM}^{-1}\text{s}^{-1}\) for the wt and Y338F proteins to \(k = (2.0 \pm 0.5) \times 10^{-3} \text{ mM}^{-1}\text{s}^{-1}\) for the Y222F variant. The results imply that Y222 mediates reduction of the intermediate both in the absence and presence of ascorbate.

**Branched Activation- and Catalysis-Specific Electron-Transfer Pathways in Ct RNR**

To verify that, despite the altered kinetics, decay of the MnIV/FeIV intermediate in the W51F and Y222F variants still yields the MnIV/FeIII product previously described for the wt protein, Mössbauer spectra of the products were acquired (Figure 3-9). As described in Chapter 2, the MnIV/FeIII cofactor has a triplet \( (S_{\text{total}} = 1) \) ground state resulting from antiferromagnetic coupling of its MnIV \((S_{\text{Mn}} = 3/2)\) and high-spin FeIII \((S_{\text{Fe}} = 5/2)\) ions. It gives rise to a sharp quadrupole doublet at 4.2 K in zero-field and a diagnostic broadening (due to the hyperfine interaction between the \(S = 1\) electron-spin ground state and the \(^{57}\text{Fe}\) nuclear spin) in the presence of a weak external magnetic field. Products of the Y222F, Y338F, and W51F reactions have spectra essentially identical to those of the wt protein in both zero and 53-mT field, implying that all reactions form the

![Figure 3-9: Mössbauer spectra of the final products of the O₂ reactions of the MnII/FeII cluster in wt, Y222F, Y338F, and W51F Ct R2. Spectra were collected at 4.2 K in external magnetic fields of 0 mT (A) and 53 mT (B) oriented parallel to the γ-beam. The sample preparation is described in Appendix B.](image-url)
Mn$^{IV}$/Fe$^{III}$ cofactor.

Activity measurement of the wt and variant R2 products was carried out to examine the roles of the three aromatic residues in catalysis. The wt and Y222F Ct R2 samples supported indistinguishable activities of $(0.60 \pm 0.06)$ s$^{-1}$ and $(0.58 \pm 0.02)$ s$^{-1}$ (mean $\pm$ standard deviation of 4 trials), respectively, implying that Y222 has no important role in the inter-subunit PCET step that initiates turnover. By contrast, the activities of the Y338F and W51F variants were less than the detection limit of the assay (0.001s$^{-1}$), suggesting that Y338 and W51, like their cognates in the mouse and Ec R2 proteins (71, 72, 123), are both essential for the inter-subunit PCET step. Thus, Ct R2 uses branched electron transfer pathways for activation (W51-Y222) and catalysis (W51-Y338), of which the former one is absent in Ec R2 (Figure 3-10).

**Figure 3-10:** Branched electron transfer pathways in Ct RNR. The catalysis-specific intersubunit pathway between the Mn/Fe cluster in R2 and the conserved cysteine residue in R1, C672, is indicated by the red dotted lines. The conserved residues proposed to participate in electron transfer are indicated. The activation-specific pathway is indicated by green dotted lines. This figure was adapted from the docking model for Ec RNR (25).

**Activation of the Mn$^{II}$/Fe$^{II}$ and Mn$^{III}$/Fe$^{III}$ Forms of Ct R2 by H$_2$O$_2$**

Based on the absence of the PCET-initiating Y• in Ct RNR, Nordlund, Gräslund and co-workers speculated that the use of a novel cofactor might render the RNR and therefore bacterium more robust to reactive oxygen and nitrogen species [RO(N)S] produced in the host's innate immune response (17). Indeed, several earlier studies had shown that the Y• in the
conventional class I system can be targeted by RO(N)S (124-129). Our discovery of the Mn/Fe cluster does not rule out this hypothesis. In light of the several precedents for the involvement of Mn in bacterial oxidative stress responses (130-132), e.g., the induction by *E. coli* in response to oxidative stress of a Mn-dependent paralogue to the constitutively expressed, Fe-dependent superoxide dismutase (130), its presence in the *Ct* RNR cofactor would seem to make this hypothesis even more attractive. Among the biologically important RO(N)S, H₂O₂ is the most stable and its chemistry the most simple. Moreover, its production is known to be an important component of innate immunity to bacteria (133). Previous work in *Ec* R2 showed that H₂O₂ can efficiently oxidize the Fe²⁺/²⁺ cluster to the met (Fe²⁺/²⁺) form (134, 135) but is then only very inefficiently converted from the met to the active (Fe³⁺/³⁺-Y•) form (25-30% Y• after 1.5-hour incubation) (136). We started to test the Nordlund/Gräslund hypothesis by examining the reactivity of the Mn/Fe cofactor toward H₂O₂.

**H₂O₂ does not inactivate Mn⁴⁺/Fe³⁺-Ct R2**

Incubation of active Mn⁴⁺/Fe³⁺-R2 (10 μM) with as much as 5 mM H₂O₂ for as long as 60 min before the protein is used to initiate the activity assay does not result in detectable loss of activity.

**H₂O₂ activates Mn³⁺/Fe³⁺- and Mn²⁺/Fe²⁺-Ct R2**

Mn³⁺/Fe³⁺-Ct R2 was prepared by treatment of Mn⁴⁺/Fe³⁺-Ct R2 with 7.5 molar equiv dithionite per R2 monomer (Appendix B), which diminished the activity to a very low level
(v/[R2] < 0.02 s⁻¹). Subsequent treatment with H₂O₂ for 10 min restores activity (Table 3-2). Under these conditions, ten molar equiv. H₂O₂ are required to maximally reactivate Ct R2. The activity is then not significantly different from the activity of the Mn⁴⁺/Fe³⁺-R2 before its reduction with dithionite.

Mössbauer spectroscopy was used to verify that this reactivation reflects conversion of the inactive Mn³⁺/Fe³⁺-Ct R2 to the active Mn⁴⁺/Fe³⁺ form. The Mn⁴⁺/Fe³⁺-Ct R2 sample exhibits a sharp quadrupole doublet (δ = 0.52 mm/s and ΔEQ = 1.38 mm/s) at 4.2 K and zero field, showing that ~90% of the iron is in the form of the Mn⁴⁺/Fe³⁺ cluster (solid line in Figure 3-11A).²⁰ The sample also contains a minor (~10%) contaminant of the homobinuclear Fe²⁺/Fe³⁺ complex (red line). At 190 K and zero-field (Figure 3-11B), the isomer shift of the Fe³⁺ site is slightly diminished (δ = 0.47 mm/s) by the second-order Doppler effect (II). The quadrupole splitting is temperature-independent (ΔEQ = 1.37 mm/s) and identical within the experimental uncertainty (0.03 mm/s) to the value at 4.2 K.

Treatment of this sample with 1.2 equiv of dithionite alters the 190-K/zero-field spectrum (Figure 3-11C) to a much broader quadrupole doublet with parameters (δ = 0.45 mm/s, ΔEQ =

<table>
<thead>
<tr>
<th>equiv. of H₂O₂</th>
<th>activity (%)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>&lt;5</td>
</tr>
<tr>
<td>2.5</td>
<td>19 ± 9</td>
</tr>
<tr>
<td>5</td>
<td>48 ± 29</td>
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<tr>
<td>10</td>
<td>83 ± 28</td>
</tr>
<tr>
<td>20</td>
<td>93 ± 21</td>
</tr>
<tr>
<td>50</td>
<td>90 ± 23</td>
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Mn³⁺/Fe³⁺ -- 100⁰

²⁰ ΔEQ is slightly larger than the value reported previously, ΔEQ = 1.32 mm/s. We attribute this to the presence of a greater concentration of glycerol in this sample (45% v/v) than in the previous sample (10% v/v).
0.83 mm/s) still characteristic of high-spin Fe$^{III}$ ($I$), implying (also see Chapter 2) that the Fe site of the cofactor is not reduced.

The Mössbauer spectra of this sample after its subsequent treatment with excess H$_2$O$_2$ (300 mM or 100 equiv for 15 min) confirm the essentially quantitative conversion of the dithionite-inactivated (Mn$^{III}$/Fe$^{III}$) R2 back to the active Mn$^{IV}$/Fe$^{III}$ state (Figure 3-11D and E). The spectra are identical within the experimental uncertainty to those of the Mn$^{IV}$/Fe$^{III}$-R2 sample prior to its reduction with dithionite (Figure 3-11A and B). As the solid lines in D and E indicate, ~90% of the intensity is again attributable to the quadrupole doublet of Mn$^{IV}$/Fe$^{III}$-R2 [$\delta = 0.47$ mm/s and $\Delta E_Q = 1.34$ mm/s at 190 K (D) and $\delta = 0.52$ mm/s and $\Delta E_Q = 1.35$ mm/s at 4.2 K (E)].

*Figure 3-11.* Mössbauer spectra showing the reduction of Mn$^{IV}$/Fe$^{III}$-R2 to Mn$^{III}$/Fe$^{III}$-R2 by dithionite and re-oxidation to Mn$^{IV}$/Fe$^{III}$-R2 by H$_2$O$_2$. The temperature and magnetic field are indicated on the spectra. A and B: a sample of Mn$^{IV}$/Fe$^{III}$-R2 prepared as described in Appendix B. C: a sample after treatment of Mn$^{IV}$/Fe$^{III}$-R2 with 1.2 equiv dithionite for 1 h. D and E: the dithionite-reduced sample after subsequent treatment with 300 mM H$_2$O$_2$ for 15 min. The solid black lines plotted over the spectra in A - E are simulations with parameters with parameters quoted in the text. The red line is the experimental spectrum of Fe$^{III}$/Fe$^{III}$-R2, scaled to 10% of the total intensity of spectrum A.

The Mössbauer spectra of this sample after its subsequent treatment with excess H$_2$O$_2$ (300 mM or 100 equiv for 15 min) confirm the essentially quantitative conversion of the dithionite-inactivated (Mn$^{III}$/Fe$^{III}$) R2 back to the active Mn$^{IV}$/Fe$^{III}$ state (Figure 3-11D and E). The spectra are identical within the experimental uncertainty to those of the Mn$^{IV}$/Fe$^{III}$-R2 sample prior to its reduction with dithionite (Figure 3-11A and B). As the solid lines in D and E indicate, ~90% of the intensity is again attributable to the quadrupole doublet of Mn$^{IV}$/Fe$^{III}$-R2 [$\delta = 0.47$ mm/s and $\Delta E_Q = 1.34$ mm/s at 190 K (D) and $\delta = 0.52$ mm/s and $\Delta E_Q = 1.35$ mm/s at 4.2 K (E)].

*Kinetics and mechanism of the reaction of Mn$^{III}$/Fe$^{III}$-R2 with H$_2$O$_2$*
Stopped-flow absorption and freeze-quench EPR experiments were used to define the kinetics and mechanism of H₂O₂-mediated reactivation of the dithionite-generated Mn^{III}/Fe^{III}-R2. An intense ~ 390-nm absorption band, similar to the feature associated with the Mn^{IV}/Fe^{IV}-R2 intermediate in the reaction of the fully reduced (Mn^{II}/Fe^{II}) protein with O₂, develops rapidly upon mixing of Mn^{III}/Fe^{III}-R2 with H₂O₂ and then decays slowly (data not shown). Analysis of A₃₉₀-versus-time traces for the reaction (Figure 3-12A) according to Equation 3-1 reveals that the formation phase has the expected first-order dependence on [H₂O₂], whereas the decay phase is relatively insensitive to [H₂O₂] (inset to Figure 3-12A). The slope of the plot of the apparent first-order rate constant for the formation phase (k_{obs}) versus [H₂O₂] gives a second order rate constant of 8 ± 1 M⁻¹s⁻¹ for reaction of Mn^{III}/Fe^{III}-R2 with H₂O₂. The kinetics of the decay phase (limiting k_{obs} = 0.06 ± 0.01 s⁻¹) are consistent with those previously observed for decay of the Mn^{IV}/Fe^{IV} complex to Mn^{IV}/Fe^{III}-R2.²¹ Thus, both the character of the spectral changes and the kinetics suggest the accumulation of the Mn^{IV}/Fe^{IV}-R2 intermediate.

The accumulation of this complex was directly demonstrated by freeze-quench EPR experiments (Figure 3-12B). The dithionite-reduced R2 reactant exhibits a broad, poorly defined g ~ 2 EPR spectrum (top spectrum) from the antiferromagnetically coupled (S = 1/2) Mn^{III}/Fe^{III} cluster.²² Upon mixing of this reactant with H₂O₂, the well-defined, sharp, six-line signal

²¹ The study of H₂O₂ effects was carried out before we noticed that the Mn^{IV}/Fe^{IV} intermediate is photosensitive. Since the formation phase should not be interfered, the analysis is still based on the data obtained with the white light source. The decay rate constant here is consistent with the one extracted from the O₂-activation at Mn^{II}/Fe^{II}-Ct R2 observed under the same condition.

²² We have seen marked variability in the shape of this signal. In particular, it varies considerably with the Mn/R2 stoichiometry and the details of how the protein has been reconstituted prior to reduction.
characteristic of the Mn\textsuperscript{IV}/Fe\textsuperscript{IV}-R2 intermediate (red spectrum in Figure 3-12) develops rapidly and then slowly decays. The kinetics of the intermediate obtained from the intensity of the EPR signal at different reaction times (green circles in Figure 3-12) agree well with the A\textsubscript{390}-versus-time trace from the stopped-flow experiment with the same [H\textsubscript{2}O\textsubscript{2}].

Figure 3-12: Reaction of the Mn\textsuperscript{III}/Fe\textsuperscript{III}-Ct R2 with excess H\textsubscript{2}O\textsubscript{2} observed by SF absorption and EPR spectroscopies. (A) Kinetics obtained by SF spectroscopy of the reaction initiated by mixing of the Mn\textsuperscript{III}/Fe\textsuperscript{III}-R2 (0.40 mM R2 monomer, 0.75 equiv of Fe, 0.75 equiv of Mn) at 5 °C with an equal volume of the same buffer, which contains H\textsubscript{2}O\textsubscript{2} at a concentration sufficient to give the [H\textsubscript{2}O\textsubscript{2}] of 5 mM (red), 15 mM (blue), 50 mM (green), and 150 mM (black) after mixing. The green circles are the EPR signal intensities from (B) scaled for direct comparison to the absorbance changes. The solid lines are fits according to Equation 3-1. The inset shows the apparent first-order rate constants (k\textsubscript{obs,1} and k\textsubscript{obs,2}) for the formation and decay phases (blue circles and red squares, respectively) versus [H\textsubscript{2}O\textsubscript{2}], which gives a second-order rate constant (slope) of 8 ± 1 M\textsuperscript{-1}s\textsuperscript{-1} for formation and a limiting rate constant of 0.06 ± 0.01 s\textsuperscript{-1} for decay. (B) X-band EPR spectra at 14.0 (± 0.2) K of freeze-quenched samples from the reaction between Mn\textsuperscript{III}/Fe\textsuperscript{III}-R2 and 50 mM H\textsubscript{2}O\textsubscript{2}. The reaction was initiated by mixing Mn\textsuperscript{III}/Fe\textsuperscript{III}-R2 (0.90 mM R2 monomer, 0.75 equiv of each metal) with 0.5 equivalent volumes of 150 mM H\textsubscript{2}O\textsubscript{2}, and samples were rapidly frozen at the indicated reaction times. For the spectra of the three freeze-quenched samples that are shown (indicated by reaction time), the spectrum of the reactant sample was scaled appropriately and then subtracted from the experimental spectrum of each sample to resolve the contribution of the Mn\textsuperscript{IV}/Fe\textsuperscript{IV}-R2 intermediate. The red spectrum is of the Mn\textsuperscript{IV}/Fe\textsuperscript{IV}-R2 intermediate in the reaction of Mn\textsuperscript{II}/Fe\textsuperscript{II}-R2 with O\textsubscript{2}. Spectrometer conditions were: \( \mu = 9.45 \) GHz; P = 200 \( \mu \)W; modulation frequency, 100 kHz; modulation amplitude, 10 G; scan time, 167 s; time constant, 167 ms.
Kinetics and mechanism of the reaction of Mn\textsuperscript{II}/Fe\textsuperscript{II}-Ct R2 with H\textsubscript{2}O\textsubscript{2}

Previous studies have shown that the fully reduced (Fe\textsubscript{2}\textsuperscript{II/II}) form of Ec R2 reacts with H\textsubscript{2}O\textsubscript{2} to produce met-R2 (134, 135). The cognate reaction in Ct R2 would convert the Mn\textsuperscript{II}/Fe\textsuperscript{II} complex to Mn\textsuperscript{III}/Fe\textsuperscript{III}-Ct R2, which should, as demonstrated above, then react with a second equiv of H\textsubscript{2}O\textsubscript{2} to yield the active state. Indeed, treatment of the Mn\textsuperscript{II}/Fe\textsuperscript{II}-R2 complex with excess H\textsubscript{2}O\textsubscript{2} results in activity equivalent to that produced by treatment with O\textsubscript{2}. In addition, the 4.2-K/zero-field Mössbauer spectra (Figure 3-13) of the products of the H\textsubscript{2}O\textsubscript{2} (hash marks) and O\textsubscript{2} (solid line) reactions are nearly identical. In both cases, application of the 53-mT magnetic field elicits the same diagnostic broadening of the quadrupole doublet (data not shown), confirming the formation of Mn\textsuperscript{IV}/Fe\textsuperscript{III}-Ct R2 as the ultimate product also in the reaction of the fully reduced protein with H\textsubscript{2}O\textsubscript{2}.

The expectation that this conversion proceeds by two sequential reactions with H\textsubscript{2}O\textsubscript{2} via Mn\textsuperscript{III}/Fe\textsuperscript{III}- and Mn\textsuperscript{IV}/Fe\textsuperscript{IV}-Ct R2 intermediates was confirmed by SF absorption and freeze-quench EPR experiments. A\textsubscript{390} versus-time traces from the reaction exhibit two resolved development phases followed by a slower decay phase (Figure 3-14A). The data were analyzed by nonlinear regression according to Equation 3-6, in which \( k_1 \) - \( k_3 \) are apparent first-order rate constants, \( \Delta A_1 - \Delta A_3 \) are amplitudes for the exponential phases, and \( A_0 \) is the absorbance at time.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure3-13}
\caption{The 4.2K/zero-field Mössbauer spectra of the products of reactions between Mn\textsuperscript{II}/Fe\textsuperscript{II}-Ct R2 cluster and H\textsubscript{2}O\textsubscript{2} (hash marks) or O\textsubscript{2} (solid lines). Samples were prepared by exposure of Mn\textsuperscript{II}/Fe\textsuperscript{II}-Ct R2 (1.5 mM R2 monomer, 1 equiv Mn\textsuperscript{II}, 0.5 equiv Fe\textsuperscript{II}) to 300 mM H\textsubscript{2}O\textsubscript{2} or 1 mM O\textsubscript{2}.}
\end{figure}
zero. The assumption of a pseudo-first-order excess of H$_2$O$_2$ inherent in these equations is met by the experimental conditions. The assumption of irreversibility is reasonable because cleavage of H$_2$O$_2$ to water is highly exergonic.

\[ A_t = A_0 + \Delta A_1\left[1 - \exp(-k_1t)\right] + \Delta A_2\left[1 - \exp(-k_2t)\right] + \Delta A_3\left[1 - \exp(-k_3t)\right] \]  \hfill (3-5)

Both development phases exhibit an approximately first-order dependence on [H$_2$O$_2$] (inset to Figure 3-14A). The plot of $k_{obs}$ for the slower of the two phases versus [H$_2$O$_2$] (blue circles) gives second-order rate constants of $1.7 \pm 0.3$ mM$^{-1}$s$^{-1}$ for the first phase (purple diamonds) and $8 \pm 1$ M$^{-1}$s$^{-1}$ for the second phase (blue circles). (B) X-band EPR spectra at 14.0 ($\pm$ 0.2) K of freeze-quenched samples from the reaction of Mn$^{II}$/Fe$^{II}$-R2 and H$_2$O$_2$. The reaction was initiated by mixing Mn$^{II}$/Fe$^{II}$-R2 (0.90 mM R2 monomer, 0.5 equiv Fe$^{II}$, 1.0 equiv Mn$^{II}$) with 0.5 equivalent volumes of 45 mM H$_2$O$_2$, and samples were rapidly frozen at the indicated reaction times. For the spectra of the three freeze-quenched samples that are shown (indicated by reaction time), the spectrum of the reactant sample was scaled appropriately and then subtracted from the experimental spectra to resolve the contributions of Mn$^{III}$/Fe$^{III}$-R2 and Mn$^{IV}$/Fe$^{IV}$-R2. The arrows in the spectra of 0.09-s and 1.5-s samples indicate features of Mn$^{III}$/Fe$^{III}$-R2. Spectrometer conditions were: $\mu = 9.45$ GHz; P = 200 $\mu$W; modulation frequency, 100 kHz; modulation amplitude, 10G; scan time, 167s; time constant, 167ms.
a second-order rate constant (8 ± 1 M⁻¹s⁻¹) for combination with H₂O₂ that is indistinguishable from the value determined for the reaction of Mn^{III}/Fe^{III}-Ct R2 with H₂O₂. Values of k_{obs} for the decay phase are also indistinguishable from those for the decay phase in the reaction of Mn^{III}/Fe^{III}-R2 at equivalent concentrations of H₂O₂. Thus, the slower development phase and the decay phase reflect conversion of Mn^{III}/Fe^{III}-R2 to Mn^{IV}/Fe^{IV}-R2 (development) and then to Mn^{IV}/Fe^{III}-R2 (the decay). The more rapid development phase corresponds to conversion of Mn^{II}/Fe^{II}-R2 to Mn^{III}/Fe^{III}-R2 by the first reaction with H₂O₂. The second-order rate constant for this step, 1.7 ± 0.3 mM⁻¹s⁻¹ (from the inset to Figure 3-14A, grey diamonds), is ~ 200 times that for the second H₂O₂-mediated cluster oxidation and similar to the rate-constant for conversion of fully reduced (Fe^{II}/II) Ec R2 to met-R2 by H₂O₂ [6 ± 1 mM⁻¹s⁻¹ (unpublished data)].

EPR spectra of samples freeze-quenched during the reaction provide additional evidence for sequential accumulation of Mn^{III}/Fe^{III}-R2 and Mn^{IV}/Fe^{IV}-R2 intermediates (Figure 3-14B). The spectral features of the first intermediate are broad and thus neither as intense nor as easily quantifiable as the features of Mn^{IV}/Fe^{IV}-R2. Nevertheless, these features can readily be discerned (arrows in Figure 3-14B) at shorter reaction times (e.g., 0.090 and 1.5 s) in the regions outside the sharp six-line spectrum of Mn^{IV}/Fe^{IV}-R2 and can be seen to decay at longer times (13 s). The much sharper and more well-defined features of Mn^{IV}/Fe^{IV}-R2 become apparent even early in the reaction but increase and become predominant at longer reaction times (13 s). Thus, the kinetics reflected in the EPR spectra are qualitatively consistent with the sequential formation of Mn^{III}/Fe^{III}-R2 and Mn^{IV}/Fe^{IV}-R2 intermediates on the pathway to the active Mn^{IV}/Fe^{III}-R2 product.
O₂ and H₂O₂ reactions at Fe₂²⁺⁻²⁻ Ct R2

The O₂ activation on the catalytically inactive homobinuclear (Fe₂) form of Ct R2 has been studied.²³ Consistent to the previous observation by Nordlund, Gräslund and co-authors (17, 103-105), our work also shows the transient accumulation of Xct in the reaction of Fe₂²⁺⁻²⁻-R2 with O₂ by SF-absorption, FQ-EPR and Mössbauer spectroscopies (120). In addition, the SF data also shows a rapid development of the sharp ~ 410-nm absorption signature of a tyrosyl radical (Figure 3-15A, marked by arrow), which was confirmed by FQ-EPR (120). This peak decays within 50 seconds to yield the featureless spectrum of the Fe₂³⁺⁻³⁻ product (blue spectrum).²⁴

During activation of Ec R2, a transient W₄₈ cation radical (W₄₈⁺) accumulates under these conditions (50, 52), and the presence

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²³ This study was initially carried out by Lana Saleh, the previous graduate student in our lab. She has done most of the work on the O₂ activation at Fe₂³⁺⁻³⁻ cluster in Ct R2 wt, Y338F, Y112F and part of the work in Y222F. In addition, she is the first person who postulated that Y222 could play a role in electron transfer pathway during O₂ activation.

²⁴ The lack of intense absorption features for the Fe₂³⁺⁻³⁻ cluster in the 360-600 nm regime, which contrasts with the spectrum of the Fe₂³⁺⁻³⁻ cluster in Ec R2, is consistent with the conclusion from X-ray crystallography that the Ct R2 product has two bridging hydroxo or water ligands (17) rather than the single μ-oxo of the Ec cluster (26).
of a high concentration (> 10 mM) of Mg$^{2+}$ engages a rapid redox equilibrium between W48 and the next residue in the PCET pathway, Y356, resulting in co-accumulation of Y356• and W48$^{\text{III}•}$ (75). To test whether the transient Y• detected in the Ct R2 reaction resides on Y338, the cognate of Ec R2 residue Y356, Y338 was replaced by F and O$_2$ activation by the Y338F variant examined. The absorption signature of the transient Y• is not diminished in the variant (Figure 3-15B, arrow), and is even somewhat enhanced, implying that Y338 is not the primary site of the transient Y• in the Ct R2 reaction. Similarly, substitution (by F) of Y112, a residue near the cofactor but with no cognate in the Ec protein and no known or suspected role in PCET, does not diminish the absorption signature of the transient Y• (not shown).

Prospecting for other candidates for the site of the transient Y•, we noted that Y222 is close to W51 (Figure 3-6), and hypothesized that it could be the site of the transient Y•. Indeed, the signature of the transient Y• is not observed (or is much less prominent) in the reaction of the Y222F variant of Ct R2 (Figure 3-15C). Rather, a new, broad, transient absorption centered at ~550 nm develops instead (marked by arrow). This feature is reminiscent of the signature of the W48$^{\text{III}•}$ in the Ec R2 reaction (50, 52). These observations suggest that Y222 is the site of the transient Y• in the reaction of the wild-type Ct R2 protein and that its substitution by F causes accumulation of a W$^{\text{III}•}$, presumably residing on W51. The latter assignment is supported by experiments on the W51F variant (Figure 3-15D). Reaction of the Fe$_2^{\text{III/II}}$ complex of W51F Ct R2 with O$_2$ does not result in accumulation of the transient absorption band at ~550 nm. Rather, yet another transient absorption feature, a broad intense band at ~700 nm (arrow) that our previous work on D84E variants of Ec R2 implies is attributable to a μ-(1,2-peroxo)-Fe$_2^{\text{III/II}}$
complex (P) (56, 137, 138), is observed. Thus, an oxidized diiron intermediate accumulates in place of the Y222• (or W51•+) in the W51F variant. The simplest interpretation of these results is that W51 and Y222 cooperate to transfer an electron to the diiron cluster during O₂ activation, with the Y222• being the more stable of the pathway radicals.

The assignment of P in the reaction of W51F is supported by Mössbauer analysis (Figure 3-16). The spectra of samples freeze-quenched early in the reaction exhibit two quadrupole doublets that are transient and therefore associated with intermediates. One doublet has \( \delta = 0.63\text{mm/s} \) and \( \Delta E_Q = 1.55\text{mm/s} \) (red), which are very similar to those of P in Ec-R2 (\( \delta = 0.63\text{mm/s}, \Delta E_Q = 1.58\text{mm/s} \)) (137). The other one has \( \delta = 0.49\text{mm/s}, \Delta E_Q = 0.93\text{mm/s} \) (blue). These parameters and the fact that it gives rise to quadrupole doublet feature suggest that it also emanates from a diiron(III/III) cluster. One of the possibilities is that it arises from a cognate of the [Fe₂O₂]⁴⁺ state identified in the aforementioned studies on Ec-R2 variants (62, 63), which is believed to be the protonated successors of P (56).

The demonstration that Mn^{III}/Fe^{III} form of Ct R2 readily reacts with H₂O₂ contrasts with previous reports of very slow and inefficient conversion of the cognate (Fe₂^{III/III} or met) form of Ec R2 to the active, Fe₂^{III/III}/Y• state (136). To assess whether (i) the substitution of the metal ion, (ii) additional, more subtle structural differences between the active sites of the Ct and Ec R2
proteins, or (iii) some combination of these factors is primarily responsible for the greater reactivity of the Ct protein, the reaction of the homobinuclear Fe$_2^{III/III}$ form of Ct R2 with H$_2$O$_2$ was examined by SF absorption spectroscopy. Development of absorption is much (~10-fold) slower in this reaction than in the reaction of the Mn$_{III}$/Fe$_{III}$ protein (black in Figure 3-17). The H$_2$O$_2$ reaction at Fe$_{II/II}$-Ct R2 gives a quick formation phase (red in Figure 3-17), which reflects the formation of Fe$_{III/III}$ complex, followed by the similar slow development as in the reaction initiated with Fe$_2^{III/III}$. Mössbauer analysis shows that the final product of reaction of excess H$_2$O$_2$ at Fe$_{II/II}$-Ct R2 is primarily a Fe$_{II/III}$ complex (data not shown). These results establish that substitution of the Fe$_{III}$ by Mn$_{III}$ is important, if not primarily determinant, in the greater H$_2$O$_2$ reactivity of the III/III oxidation state of the Ct R2 protein compared to Ec R2.

**Discussion**

![Figure 3-17: Kinetics of the reactions of Fe$_{II/II}$-R2 (red) and Fe$_{III/III}$-R2 (black) with excess H$_2$O$_2$. An O$_2$-free solution of Fe$_{II/II}$-R2 (0.40 mM R2 monomer, 1.5 equiv Fe$_{II}$) was mixed at 5 °C with an equal volume of 100 mM H$_2$O$_2$ solution (red). Fe$_{III/III}$-R2 was prepared by adding 1.5 equiv of Fe$_{II}$ to an air-saturated solution of apo R2 (0.1 mM). The solution was dialysed against EDTA for four hours to remove excess iron and then against buffer to remove the EDTA. A solution of 0.40 mM Fe$_{III/III}$-R2 monomer was mixed at 5 °C with an equal volume of 300 mM H$_2$O$_2$ solution (black). The absorbance at 370 nm versus time traces (the Fe$_{II/III}$ cluster X has strong shoulder at this wavelength) for both reactions were analyzed by non-linear regression according to Equation 3-1.](image-url)
To our knowledge, the Mn$^{IV}$/Fe$^{IV}$ intermediate formed during O$_2$ activation in Ct R2 is the first example of such a complex ever reported. In view of the X-ray structure of the (presumptively) Fe$_2$$^{III/III}$ form of the Ct R2 protein by Högbom, et al. (17), which suggested a bis-(µ-hydroxo)-dimetal core, and previous studies suggesting formation of a (µ-O)$_2$-Fe$_2$$^{IV/IV}$ complex, Q, in the catalytic cycle of soluble methane monoxygenase (139), we consider it very likely that the Mn$^{IV}$/Fe$^{IV}$ intermediate also has this [M$_2$O$_2$(H)$_n$]$^{(4+n)+}$ "diamond core" structure. Its half-integer ($S$ = 1/2) electron-spin ground state, which contrasts with the $S$ = 0 ground state of Q, and heterobinuclear rather than homobinuclear nature should afford unique opportunities to test this hypothesis and probe details of the core structure by ENDOR and X-ray absorption experiments.

Previous spectroscopic studies on intermediates during O$_2$ activation are usually hampered by two technical obstacles for sample preparation: a) the purity of the intermediate due to its fast decay, and b) the concentration of the intermediate limited by the O$_2$ solubility. The Y222F variant, which slows down the decay of Mn$^{IV}$/Fe$^{IV}$ intermediate dramatically to give $t_{1/2}$ = ~ 300 s, leads to the application of a new strategy. Instead of the quick mixing of the protein solution and an O$_2$-saturated buffer solution, the concentrated Mn$^{II}$/Fe$^{II}$-Y222F is directly exposed to O$_2$ gas (~1.5 atms) with vigorous stirring of the solution and the reaction is hand-quenched in liquid ethane (~ - 180 °C) within ~2 min. Samples with [Mn$^{IV}$/Fe$^{IV}$] of ~ 1.4 mM and purity > 75%

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25 The solubility of O$_2$ in H$_2$O at 5 °C under 1 atm of O$_2$ is 1.9 mM. The maximum concentration of O$_2$ after mixing is usually ~1.2 mM (when $V_{protein}$: $V_{O2}$ = 1:2). In order to keep O$_2$ in excess, the concentration of the reactive site (e.g., Fe$_2$$^{III/III}$ for Ec R2) should not be higher than 1.2 mM. In addition, the purity of the intermediate species is usually no higher than ~80%. So the typical concentration of the intermediate in the final sample is lower than 1 mM.
have been prepared for EXAFS spectroscopy. Again, we collaborated with the Green group at Penn State University for this study. The preliminary data suggests that the distance between the two metals is between 2.7-2.8 Å, remarkably longer than the reported number in complex Q (2.46 Å). Further investigation on the Mn^{IV}/Fe^{IV} intermediate is undergoing.

The proposed mechanisms of \( \text{O}_2 \) activation at \( \text{Fe}^{\text{II/III}}-\text{Ec R2} \) and \( \text{Fe}^{\text{II/III}}-\text{Ct R2} \) are shown in Scheme 3-1 (top and middle) and they are believed to be quite similar. A tryptophan residue (W48 in \( \text{Ec R2} \) and W51 in \( \text{Ct R2} \)) shuttles one electron to the diiron cluster through a network of hydrogen bonds for the asymmetric exploiting of the two oxidizing equivalents. Y222 locates next to the W in \( \text{Ct R2} \) is the new element on the electron transfer pathway. The difference in the

\[
\text{Scheme 3-1: Proposed mechanisms of } \text{O}_2 \text{ activation at } \text{Fe}^{\text{II/III}}-\text{Ec R2 (top), Fe}^{\text{II/III}}-\text{Ct R2 (middle), and Mn}^{\text{II/III}}-\text{Ct R2 (bottom) clusters. The intermediates marked with red frames are directly observed in reactions of corresponding wt proteins.}
\]
location of the transient radical in the diiron reactions, i.e., the accumulation of \( X-Y^* \) di-radical state in \( Ct \) R2 instead of \( X-W^+ \) state in \( Ec \) R2 (50), can be explained by the lesser reduction potential of (neutral) \( Y^* \) compared to \( W^+ \) (10). The phenolic hydroxyl of Y222 projects outward from the surface of \( Ct \) R2 into solution and should readily lose its proton to solvent or buffer upon phenol oxidation, localizing the "hole" at this site in preference to the more solvent-protected W51, which hydrogen bonds via its indole N-H to Asp226 and may lose its proton less readily upon oxidation to the cation radical.

The comparison between the \( O_2 \) activation at the Mn/Fe cluster and the Fe/Fe cluster, in particular, in the same enzyme \( Ct \) R2 (Scheme 3-1, middle and bottom), reveals significant differences in their kinetics, implying that the substitution of the metal ion is more important than active-site tuning in causing these differences. It has been shown that diiron-\( O_2 \) complexes that are two-electron oxidized relative to the product (\( Fe_{2}^{III/III} \)) cluster do not accumulate during \( O_2 \) activation. \( P \), the \( \mu-(1,2\text{-peroxo})-Fe_{2}^{III/III} \) complex, does not accumulate (or only to a very low level) in the reaction of wildtype \( Ec \) and \( Ct \) R2 but has been identified in their variant proteins [\( Ec-R2-D84E \) (137)and \( Ct \) R2-W51F, see above]. \( Q \), the \( Fe_{2}^{IV/IV} \) complex, has never been detected in any wt or variants R2 protein. It is at least in part because transfer of the extra electron from the cofactor-proximal tryptophan residue is so rapid (\( > 400 \text{ s}^{-1} \) at 5 °C in the \( Ec \) R2 reaction) (50). Further evidence suggests that this electron transfer step occurs concomitantly (perhaps even concertedely) with cleavage of the O-O bond of the peroxo-\( Fe_{2}^{III/III} \) complex (63). The coupling of the oxidation of the tryptophan to O-O bond cleavage should make this step thermodynamically favorable, resulting in the observed accumulation of the amino acid radical.
By contrast, the Mn\textsuperscript{IV}/Fe\textsuperscript{IV} complex accumulates nearly to the stoichiometric level during O\textsubscript{2} activation in wt Ct R2. Based on the “diamond core” structure, the O-O bond of O\textsubscript{2} has presumably already been cleaved (without net reduction) in the Mn\textsuperscript{IV}/Fe\textsuperscript{IV} intermediate. The failure of a state containing the Mn\textsuperscript{IV}/Fe\textsuperscript{III} cofactor and an amino acid radical (the cognate of the X-W48\textsuperscript{**} di-radical state (50) in Ec R2 and X-Y222\textsuperscript{•} di-radical state in Ct R2) to accumulate could thus be explained by the lack of coupling of amino acid oxidation to O-O cleavage, as well as a relatively modest reduction potential of the Mn\textsuperscript{IV}/Fe\textsuperscript{IV} intermediate. The "saturation" of the observed rate constant for the decay of the Mn\textsuperscript{IV}/Fe\textsuperscript{IV} intermediate by ascorbate is consistent with this scheme (Scheme 3-1, bottom). A value of 1.0 ± 0.3 s\textsuperscript{-1}, the asymptotic value of \(k_{\text{obs}}\) for decay of the intermediate (Figure 3-8D), could represent the forward rate constant of the step in which the Mn\textsuperscript{IV}/Fe\textsuperscript{IV} complex is reduced by W51.

It remains to be seen whether the Mn\textsuperscript{IV}/Fe\textsuperscript{IV} intermediate, like Q (54) and X (31, 41, 52), forms from a (μ-peroxo)-M\textsubscript{2}\textsuperscript{III/III} intermediate. The stopped-flow and freeze-quench EPR data provide no evidence for the accumulation of such a complex. Thus, as in E. coli R2, it might prove necessary to perturb the reaction kinetics (e.g., by replacement of a ligand, as in the D84E substitution in E. coli R2 that was shown to stabilize the peroxide intermediate (137)) to permit accumulation of a peroxide precursor to the Mn\textsuperscript{IV}/Fe\textsuperscript{IV} intermediate, if it indeed exists.

The discussion about the reactivity of Mn\textsuperscript{III}/Fe\textsuperscript{III} cluster towards H\textsubscript{2}O\textsubscript{2}, and the possible biological implication of the branched electron transfer pathways in Ct RNR, will be presented in Chapter 5.
Chapter 4
Reduction of the Mn$^{IV}$/Fe$^{III}$ Cluster as a Probe for Dissecting PCET
As reported in Chapter 2, the products of dithionite reduction on Mn$^{IV}$/Fe$^{III}$-Ct R2 exhibit distinct EPR signals, depending on the absence or presence of R1-effector-substrate complex (turnover conditions). Similar observations have been obtained in hydroxyurea (HU) reaction. HU is a well-known radical scavenger for conventional class I RNR (34). The studies on Ec RNR suggest that HU can reduce the Fe$_2^{III/III}$-Y• cofactor by one-electron to the met form (Fe$_2^{III/III}$-Y) either in the free R2 or under the turnover conditions, but with different kinetics (87). HU can also inhibit the activity of Ct RNR and thus the growth of Chlamydia (140). Detailed studies on HU reduction in Ct RNR presented in this chapter have shown that, different from Ec RNR, the outcomes are completely different under these two conditions. The differences almost certainly reflect structural changes at the cofactor site that are part of the PCET “gate” and HU can be used as a probe for PCET in future studies.

**Requirement for the Different Outcome of Reduction at the Mn$^{IV}$/Fe$^{III}$ Cluster by Hydroxyurea and Dithionite**

Previous studies have shown that the Mn$^{IV}$/Fe$^{III}$ cluster in the R2 subunit can be reduced to the Mn$^{III}$/Fe$^{III}$ form by dithionite (DT) (see Chapter 2). The reduction can occur in the free R2 protein, or under turnover conditions, i.e. in the presence of R1, allosteric effector ATP, and substrate CDP. Although the products have the same redox state, the latter one exhibits a much sharper and more featured EPR signal. To understand the requirement for this change, each component has been left out in turn and the EPR spectra are shown in Figure 4-1A. As a control, the spectrum of the Mn$^{IV}$/Fe$^{III}$ cluster under the regular turnover condition (A2) was collected. It
does not exhibit an obvious EPR signal, indicating that DTT, the reductant used to regenerate R1 subunit after each turnover, is not strong enough to reduce the Mn\textsuperscript{IV}/Fe\textsuperscript{III} cluster. When dithionite was applied, the conversion from Mn\textsuperscript{IV}/Fe\textsuperscript{III} to Mn\textsuperscript{III}/Fe\textsuperscript{III} occurs. The reduction with free R2 (A3), R2:R1 (A4), and R2:R1:ATP complex (A5) are essentially the same, indicating that the binding of the substrate, the missing component in all three samples, is the key for the generation of well-resolved spectrum (A7). When R1, DTT, and CDP are added in the absence of the allosteric effector, ATP, the spectrum (A6) is the superposition of the broad and sharp spectra, suggesting that ATP also has some effects during this process, but only when the substrate CDP has already bound to the enzyme.

**Figure 4-1**: X-band EPR spectra showing the requirement for different outcomes of reduction at Mn\textsuperscript{IV}/Fe\textsuperscript{III}-Ct R2 by dithionite (DT) (A) or hydroxyurea (HU) (B). The presence (+) or absence (-) of each component in samples are listed on the right side of the corresponding spectrum. 300 μM of R2 monomer (0.75 equiv Mn\textsuperscript{IV}/Fe\textsuperscript{III}) were applied in all samples. [R1] = 900 μM, [DTT] = 10 mM, [CDP] = 4 mM, [ATP] = 1 mM, [DT] = 20 mM, [HU] = 20 mM if present. All the components except DT or HU were quickly pre-mixed and the reductant was added and incubated for 5 min at ambient temperature anaerobically before freezing. Spectrum B5 has been expanded by 5-fold. Spectrometer conditions: T = 14.0 ± 0.2 K; ν = 9.45 GHz; P = 200 μW; modulation frequency, 100 kHz; modulation amplitude, 10G; scan time, 167 s; time constant, 167 ms.
Gräslund and co-authors have reported that the reduction by HU under turnover conditions can also generate the well-resolved spectrum of the Mn\textsuperscript{III}/Fe\textsuperscript{III} cluster (141). Thus, the same strategy was applied to study the HU reduction (Figure 4-1B). In contrast to incubation with dithionite, the incubation of HU and Mn\textsuperscript{IV}/Fe\textsuperscript{III}-Ct R2 does not generate any species that exhibit X-band EPR signals (B3), neither does the R2:R1 complex (B4). When ATP is added, a small amount of the well-resolved signal is observed (B5), which could be explained by the low level of ADP (substrate) contamination in ATP supply. Again, the binding of substrate, CDP, turns out to be the key for the development of the sharp EPR signal (B7). In this case, the ATP can even be left out without any perturbation (B6).

This is the first time that substrate binding in a class I RNR can be directly observed by a spectroscopic method, although the binding site is very far away (~ 35Å in Ec RNR) from the metal cluster. We postulate that the binding of the substrate probably causes conformational changes in the holoenzyme that are propagated to the cofactor and induce greater homogeneity, which are almost certainly parts of the PCET “gate”.

The EPR spectra presented above only represent one time point in the reaction with dithionite or HU. We have therefore studied the kinetics of the reduction of the turnover Mn\textsuperscript{IV}/Fe\textsuperscript{III}-Ct R2:R1:CDP:ATP complex in more detail. We chose HU for these studies because the EPR spectra of the HU-reduced Mn\textsuperscript{III}/Fe\textsuperscript{III} complex are better resolved and do not show any evidence for the broad, heterogeneous Mn\textsuperscript{III}/Fe\textsuperscript{III} complex. In addition, HU as a well-defined RNR inhibitor has been used as a clinic drug and its effects on Ct RNR should be of more general interest.
**Time-Dependence of the One-Electron Reduction of Mn$^{IV}$/Fe$^{III}$-Ct R2:R1:ATP:CDP by HU**

The kinetics of the reduction of Mn$^{IV}$/Fe$^{III}$-Ct R2:R1:ATP:CDP with HU was first assessed by SF absorption spectroscopy. Mn$^{IV}$/Fe$^{III}$-Ct R2 and the reduction products have broad absorption bands between 300-500 nm without prominent peaks. $A_{390}$ was picked up to represent the kinetics of the reaction (Figure 4-2A), which was analyzed by nonlinear regression according to Equation 4-1. It gives absorbance as a function of time ($A_t$) for a system of two

![Graph](attachment:image.png)

**Figure 4-2:** Kinetics of HU reduction at Mn$^{IV}$/Fe$^{III}$-Ct R2 under turnover conditions. (A) A solution containing 400 $\mu$M R2 monomer (0.75 equiv Mn$^{IV}$/Fe$^{III}$), 800 $\mu$M R1 monomer, 20 mM DTT, 2mM ATP was mixed with an equal volume of buffer containing 8 mM CDP and 40 mM HU at ambient temperature and $A_{390}$ was measured by SF absorption spectroscopy (open circles). The solid line is the fitting according to Equation 4-1, which gives a rate constant of 0.12 ± 0.03 s$^{-1}$ for the faster decay phase. (B) X-band EPR spectra of the timecourse of Mn$^{III}$/Fe$^{III}$-Ct R2$_R$R$_1$ cluster. Samples are freeze-quenched at the indicated time during the same reaction as (A). The spectrum of a sample quenched at 20 ms was subtracted from the experimental spectrum of each sample to generate the spectra shown. Spectrometer conditions: $T = 14.0 \pm 0.2$ K; $\nu = 9.45$ GHz; $P = 200$ $\mu$W; modulation frequency, 100 kHz; modulation amplitude, 10G; scan time, 167 s; time constant, 167 ms. (C) The EPR signal intensities in (B) are extracted. The solid line is the fitting according to Equation 4-2, which gives a rate constant of 0.1 ± 0.03 s$^{-1}$.

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26 This experiment was carried out by Jiajia Xie, a graduate student in our lab.
parallel, irreversible, first-order reactions in terms of rate constants for the two steps (\(k_1\) and \(k_2\)), their associated amplitudes (\(\Delta A_1\) and \(\Delta A_2\)), and the absorbance at time zero (\(A_0\)).

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

The fitting gives the first-order rate constant of \(0.12 \pm 0.03\) s\(^{-1}\) for the faster decay phase, which we speculated reflects the formation of \(\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}-\text{Ct R2:R1:ATP:CDP}\) complex. This hypothesis is supported by FQ-EPR data (Figure 4-2B). The intensity of the well-resolved \(\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}-\text{Ct R2:R1:CDP:ATP}\) complex was analyzed by nonlinear regression according to Equation 4-2, which is derived from the scheme \(\text{Mn}^{\text{IV}}/\text{Fe}^{\text{III}} \rightarrow \text{Mn}^{\text{IV}}/\text{Fe}^{\text{III}}\) with \(k_1\) as the irreversible first-order rate constants.

\[
[Mn^{III}/Fe^{III}]_t = [Mn^{IV}/Fe^{III}]_0 \cdot [1-\exp(-k_1t)]
\]

The first-order rate constant of \(0.10 \pm 0.03\) s\(^{-1}\) extracted from the fitting (Figure 4-2C) is consistent to the faster phase in SF. Interestingly, spin quantification shows that the maximum amount of \(\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}\) cluster generated by treatment with HU represents \(\sim 50\%\) of the total concentration of the Mn/Fe cofactor, providing one of several lines of evidence that the R2:R1 complex displays half-of-sites reactivity.

**Mössbauer Characterization of the Two Distinct Products upon HU Treatment**

The reasons of the slower decay phase in HU reduction during turnover conditions remain unclear. In addition, although the incubation of free \(\text{Mn}^{\text{IV}}/\text{Fe}^{\text{III}}-\text{Ct R2}\) with HU does not generate any EPR-active species, the marked loss of intensity of absorbance between 300-500 nm (Figure
4-3) and the decrease of the activity (data not shown) after incubation strongly suggest that a reaction occurs. Therefore, we used Mössbauer spectroscopy, which gives information of all Fe-containing species, to monitor this conversion.

The 4.2-K/53-mT Mössbauer spectra of samples, in which the Mn\textsuperscript{IV}/Fe\textsuperscript{III}-R2:R1:CDP:ATP complex was reacted with HU for 1 min (black) and 10 min (red) are shown in Figure 4-4A. These two reaction times correspond to completion of the first, fast phase (1 min) and approximately midpoint of the second conversion. Both spectra display two broad bands at -5 mm/s and +5 mm/s, which emanate from the Mn\textsuperscript{III}/Fe\textsuperscript{III}-R2:R1:CDP:ATP complex. Analysis of magnetic-field-dependent spectra of this component, which is described below, reveals that it accounts for \(~50\%\) of total intensity, consistent to the results from spin quantification by EPR. The remaining portion of the 1-min spectrum is (within the experimental uncertainty of the method) identical to the features of the Mn\textsuperscript{IV}/Fe\textsuperscript{III}-R2 complex described in Chapter 2. We therefore assign this component to the Mn\textsuperscript{IV}/Fe\textsuperscript{III}-R2:R1:CDP:ATP complex and conclude that formation of the R1:R2 complex does not perturb the Mössbauer-spectroscopic properties of the Mn\textsuperscript{IV}/Fe\textsuperscript{III} cluster. Comparison of the 1-min spectrum to the 10-min spectrum reveals that prolonged incubation with HU yields a new quadrupole doublet (indicated by arrows). This new quadrupole doublet only accounts for \(~10\%\) of the total intensity of the spectrum.
The development of this quadrupole doublet is also observed when free Mn$^{IV}$/Fe$^{III}$-R2 is reacted with HU. Incubation of 20 mM HU for 30 min led to 36% conversion of Mn$^{IV}$/Fe$^{III}$ to the new species (Figure 4-4B, from black hashed lines to green line). Further addition of HU and longer incubation times (blue and red lines) leads to progressive conversion of this new species. Its isomer shift ($\delta \sim 0.50$ mm/s) suggest that it is also a high-spin Fe(III)-containing complex.

Thus, the Mössbauer characterization shows two distinct products upon HU reduction at the Mn$^{IV}$/Fe$^{III}$-R2 cluster. Under the turnover condition, ~50% of the Mn$^{IV}$/Fe$^{III}$-cluster is quickly reduced by one electron to Mn$^{III}$/Fe$^{III}$ form while the rest is slowly converted to an EPR-silent, unknown species. When free Mn$^{IV}$/Fe$^{III}$-Ct R2 is incubated with HU, the only product is this unknown species. Detailed characterization of these two products is presented below.
Mössbauer-Spectroscopic Characterization of the Mn$^{III}$/Fe$^{III}$-Ct R2:R1 complex

The presence of three different species in HU-treated sample under turnover conditions complicates the detailed spectroscopic characterization of the Mn$^{III}$/Fe$^{III}$–R2:R1:CDP:ATP complex. Therefore, we sought for an alternative way of generating such complex for its spectroscopic characterization. The obvious choice is a sample generated by reaction of Mn$^{IV}$/Fe$^{III}$-R2:R1:ATP with the mechanism-based inhibitor 2’-azido-2’-deoxyribonucleotide, because it yields the well-resolved EPR signal of the Mn$^{III}$/Fe$^{III}$ cluster that is indistinguishable from that of the HU-generated Mn$^{III}$/Fe$^{III}$-R2:R1:CDP:ATP complex. (see Chapter 2)

The 4.2-K/variable-field Mössbauer spectra of a sample, in which the Mn$^{IV}$/Fe$^{III}$-R2:R1:ATP complex was exposed to N$_3$-ADP for 10 min at the ambient temperature (800 μM R2 with 0.75 equiv Mn$^{IV}$/Fe$^{III}$ cluster, 1.2 mM R1, 10 mM DTT, 1 mM ATP, 1 mM N$_3$-ADP). The strength and the orientation of the field are as indicated. The blue lines are the reference spectra of Mn$^{IV}$/Fe$^{III}$ cluster (50% intensity). The red lines are the simulation of Mn$^{III}$/Fe$^{III}$ cluster as described in the text (45%).

Figure 4-5: The 4.2-K/variable field Mössbauer spectrum of the sample in which the Mn$^{IV}$/Fe$^{III}$-Ct R2:R1:ATP complex was exposed to N$_3$-ADP for 10 min at the ambient temperature (800 μM R2 with 0.75 equiv Mn$^{IV}$/Fe$^{III}$ cluster, 1.2 mM R1, 10 mM DTT, 1 mM ATP, 1 mM N$_3$-ADP). The strength and the orientation of the field are as indicated. The blue lines are the reference spectra of Mn$^{IV}$/Fe$^{III}$ cluster (50% intensity). The red lines are the simulation of Mn$^{III}$/Fe$^{III}$ cluster as described in the text (45%).
marks.\textsuperscript{27} The spectra reveal that this sample contains approximately equimolar amounts of the Mn\textsuperscript{IV}/Fe\textsuperscript{III} and Mn\textsuperscript{III}/Fe\textsuperscript{III}-forms. Their features are reasonably well resolved in most of the spectra. The blue lines represent the previously reported experimental spectra of the Mn\textsuperscript{IV}/Fe\textsuperscript{III}–R2 form (50% of intensity).\textsuperscript{28} The Mössbauer-spectroscopic properties of the Mn\textsuperscript{IV}/Fe\textsuperscript{III}-R2 and Mn\textsuperscript{IV}/Fe\textsuperscript{III}-R2:R1:”N•-ADP”:ATP complexes are indistinguishable. The red lines are simulations of the Mn\textsuperscript{III}/Fe\textsuperscript{III}-R2:R1:”N•-ADP”:ATP complex with respect to the total spin, $S = 1/2$, using the following parameters: $\delta = 0.50$ mm/s, $\Delta E_Q = 1.0$ mm/s, eta = -3, $A_{Fe}/g_S\beta_N = (-52, -52, -52)$ T. Using the spin projection factor of $+7/3$, the $A_{Fe}$-tensor with respect to the total spin corresponds to an intrinsic $a_{Fe}$-tensor of $(-22.3, -22.3, -22.3)$ T, which is typical for a high-spin Fe\textsuperscript{III} complex ($II$). The simulations of the Mn\textsuperscript{III}/Fe\textsuperscript{III} component correspond to 45% of the total intensity, providing additional evidence for half-of-sites reactivity.

As described previously for the Mn\textsuperscript{IV}/Fe\textsuperscript{IV} intermediate (see Chapter 3), in small externally applied fields (B < 0.1 T) the electron Zeeman interaction and the Mn-hyperfine interaction are comparable in magnitude. In zero applied field, the $S = 1/2$ and $I = 5/2$ spins couple to $F = 2$ and $F = 3$ states ($F = S + I$). The strength of the magnetic field required to dominate $A_{Mn}$ depends strongly on the anisotropy of $A_{Mn}$. Thus, the greater anisotropy of $A_{Mn(III)}$ [(269, 392, 314)MHz] compared to that of $A_{Mn(IV)}$ [(247, 216, 243)MHz] makes this effect more pronounced in Mn\textsuperscript{III}/Fe\textsuperscript{III}-R2\textsubscript{R1} (Figure 4-6).

\textsuperscript{27} We recently encountered a problem from the old $^{57}$Co radioactive source, which gives a broad background noise signal from some paramagnetic species. It has been removed from the 4-T and 8-T spectra to generate the ones shown in Figure 4-5. These spectra will be re-collected by using the new source for future publication. \textsuperscript{28} The Mn\textsuperscript{IV}/Fe\textsuperscript{III} cluster has an integer spin ground state and the spectrum should not exhibit the dependence on the orientation of the external field. Thus, its 53-mT spectrum collected in parallel mode is also considered to be the reference spectrum in perpendicular mode.
**Reaction of MnIV/FeIII-R2 with HU Yields a Diamagnetic FeIII-containing Cluster**

The samples for further characterization of the unknown, EPR-silent species was prepared by incubation of the free MnIV/FeIII-R2 with HU. The 4.2-K/zero-field Mössbauer spectra shown that incubation with 20 mM HU for 30 min reduces the features associated with MnIV/FeIII-R2 by 36 % and a new quadrupole doublet develops (Figure 4-7A, top). When a weak external magnetic field (53 mT) is applied, the spectrum emanating from MnIV/FeIII-R2 cluster is broadened due to hyperfine coupling with the \( S = 1 \) ground state (see Chapter 2), but the new quadrupole doublet remains the same as in zero-field (Figure 4-7A, middle). The reference spectra for the new species at 0mT and 53 mT, which are generated by subtracting 54% of the

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**Figure 4-6**: Field-dependence of the spin expectation values in MnIII/FeIII (A-C) and MnIV/FeIV (D-F) clusters. Both of them have a spin system with \( S = 1/2 \) and \( I = 5/2 \), which couple via the hyperfine interaction to total spin states with \( F = 2 \) (red) and \( F = 3 \) (black). \( A_{\text{Mn}} \) as determined from EPR was used for the calculation. The magnetic field is oriented along the x (A and D), y (B and E), and z (C and F) direction. Dashed lines mark the field of 13mT and 53 mT.
reference spectra of the Mn$^{IV}$/Fe$^{III}$-R2 cluster from the spectra of the HU-treated sample, are essentially the same (Figure 4-7A, bottom). They can be analyzed either with one asymmetric quadrupole doublet ($\delta = 0.49$ mm/s, $\Delta E_Q = 0.88$ mm/s) or two, symmetric quadrupole doublets with identical line width ($\delta = 0.55$ mm/s, $\Delta E_Q = 0.76$ mm/s and $\delta = 0.46$ mm/s, $\Delta E_Q = 0.94$ mm/s). The latter model is justified because the width of the low-energy line ($\Gamma = 0.38$ mm/s) is much greater than that of the high-energy line ($\Gamma = 0.24$ mm/s). The isomer shift values are typical of high-spin Fe$^{III}$, suggesting that the oxidation state of the iron does not change during the reaction. Moreover, the lack of magnetic broadening may indicate that it has a diamagnetic ($S = 0$) ground state.

For further characterization we have collected additional spectra of the sample of Mn$^{IV}$/Fe$^{III}$-R2 treated with 100 mM HU for two hours, because the zero-field spectrum indicated that it contains a large amount of the new species (Figure 4-4B, red line). However, Mössbauer spectra recorded over a wider range of Doppler velocities also reveal the presence of ~30% of a mononuclear high-spin Fe$^{III}$ complex (blue lines in Figure 4-7B).\textsuperscript{29} 50% of the intensity of the 53-mT spectrum can be attributed to the novel quadrupole doublet (red line in Figure 4-7B, top). In addition, there are broadened features indicated by arrows that are similar to those of the spectrum of Mn$^{IV}$/Fe$^{III}$-R2 (green line) and may indicate the presence of ~20% of that component. However, the 8-T spectrum clearly rules out the presence of more than 5% of the Mn$^{IV}$/Fe$^{III}$ form, because the well-resolved lines at -5 mm/s and +6 mm/s are not observed (green line in Figure

\textsuperscript{29} The mononuclear Fe$^{III}$ could represent Fe$^{III}$ in the di-metal site of Ct R2 after removal of the Mn, or it may represent Fe$^{III}$ in solution after it was removed from the cluster, or a combination thereof.
The central region of the 8-T spectrum is dominated by poorly resolved features that represent the novel cluster. They can be simulated with the values of $\delta$ and $\Delta E_Q$ determined from the zero-field spectrum and assuming diamagnetism. We note that simulated spectrum (red line) does not match the observed spectrum accurately. The slightly greater splitting suggests the presence of a weak internal magnetic field of $\sim$0.7 T that augments the externally applied field.

Similar behavior has been observed for in the high-field spectra of the non-heme Fe$_2^{III/II}$ cluster of soluble methane monooxygenase and models thereof (142). Münck and co-workers have

**Figure 4-7:** The 4.2-K Mossbauer spectra at the indicated external field with the orientation parallel to the $\gamma$-beam to characterize the product of reaction of free Mn$^{IV}$/Fe$^{III}$-Ct R2 and HU. (A) A sample containing Mn$^{IV}$/Fe$^{III}$-Ct R2 (3.3 mM R2 monomer, 0.75 equiv Mn$^{IV}$/Fe$^{III}$) (solid lines) was incubated with 20 mM HU at the ambient temperature for 30 min (hashed lines). The spectra with solid lines have been scale to 64% to show what is left after HU reduction. The bottom spectra are reference spectra of the new species at 0 mT (hashed lines) and 53 mT (solid line), which are obtained by subtracting the 64% of the spectra of the starting material from the spectra of the sample after incubation with HU. (B) The same Mn$^{IV}$/Fe$^{III}$-Ct R2 sample as (A) was incubated with 100 mM HU at the ambient temperature for 2 hours. The blue lines are the theoretical spectra of a high-spin Fe$^{III}$ (30% of total intensity). The red lines are simulations of a diamagnetic Fe$^{III}$-containing species (50%). The green lines are the reference spectra of Mn$^{IV}$/Fe$^{III}$-Ct R2 cluster (20%).
conclusively shown that this phenomenon can be explained by the antisymmetric exchange interaction, which causes admixture of excited paramagnetic states into the wave function of the diamagnetic ground state, resulting in an internal magnetic field oriented perpendicular to the applied magnetic field. It is likely that antisymmetric exchange causes the small internal field in the HU-generated, diamagnetic, Fe(III)-containing complex.

**Discussion**

Two distinct forms of the Mn$^{III}$/Fe$^{III}$ cluster have been identified with EPR-spectroscopy. A rather poorly resolved and heterogeneous spectrum is observed when Mn$^{IV}$/Fe$^{III}$-R2 is reduced by dithionite. On the other hand, a well-resolved signal is observed when the Mn$^{IV}$/Fe$^{III}$-R2:R1:CDP:ATP complex is treated either with dithionite or HU. The same, well-resolved signal is observed when the mechanism-based inhibitor N$_3$-ADP is used. The generation of the well-resolved EPR spectrum strictly depends on the presence of the R1 subunit and the substrate, despite the fact that the substrate binding site is estimated to be ~35Å away from the Mn/Fe cluster. The allosteric effector (ATP) also has some effects during dithionite reduction, but only when CDP is present. One of the possibilities is that the binding of CDP to the holoenzyme during reduction is the “switch” to turn on the generation of the Mn$^{III}$/Fe$^{III}$ cluster with well-resolved EPR features, while the binding of ATP enhances the affinity between the R1 and R2 subunits (Kasrayan, 2004, JBC) thus favor its formation over the Mn$^{III}$/Fe$^{III}$-R2 cluster.

The time-dependence of the reaction of Mn$^{IV}$/Fe$^{III}$-R2:R1:CDP:ATP with HU was studied
by a combination of spectroscopies. SF-absorption reveals that the reduction is biphasic. The first, fast phase ($k_1 = 0.1 \text{ s}^{-1}$) corresponds to the one-electron reduction to generate the well-resolved Mn$^{III}$/Fe$^{III}$-R2:R1:CDP:ATP, as was demonstrated by FQ-EPR. During the second, slow phase ($k_2 = 0.002 \text{ s}^{-1}$), a new species was detected by Mössbauer spectroscopy. The same product is generated, when Mn$^{IV}$/Fe$^{III}$-R2 is treated with HU in the absence of R1, substrate, and effector. In the following, the pathway of the one-electron reduction of the Mn$^{IV}$/Fe$^{III}$-R2:R1:CDP:ATP complex and the nature of the diamagnetic, Fe(III)-containing complex are discussed.

Pathways of one-electron reduction of Mn$^{IV}$/Fe$^{III}$-R2:R1:CDP:ATP by HU

More insight into the mechanism, by which HU reduces the Mn$^{IV}$/Fe$^{III}$-R2:R1:CDP:ATP complex was obtained from studies of site-directed variants of Ct RNR. These studies have been carried out together with Jiajia Xie, a graduate student from our group. In these experiments, each of the proposed PCET pathway residues and of the novel, branched ET pathway was changed (C672S, Y990F, and Y991F in Ct-R1 and Y338F, W51F, and Y222F Ct-R2). In addition to these single variants, the Y222F/Y338F Ct-R2 double variant was studied. The results are summarized in Figure 4-8. The well-resolved EPR signal of the Mn$^{III}$/Fe$^{III}$ cluster is observed upon treatment of the Mn$^{IV}$/Fe$^{III}$-R2:R1:CDP:ATP complexes with HU, except for the W51F and Y222F/Y338F Ct-R2 variants. These observations can be explained by the following hypothesis. Binding of CDP to the R1 subunit induces the conformational change, which open the gate for PCET (will be discussed in details in Chapter 6) and allow the excursion of the radical from its resting position on Mn$^{IV}$ onto W51, toward R1. The radical can then be quenched via either the
the catalysis pathway involving Y338, or, the activation pathway involving Y222. Only when both pathways are blocked (i.e. in W51F-R2 or Y222F/Y338F-R2) the ET to the Mn$^{IV}$/Fe$^{III}$ cluster is blocked.

*The nature of the diamagnetic, Fe(III)-containing complex*

The isomer shift 0.49 mm/s suggests that it contains a high-spin Fe$^{III}$ site. Further, it has a diamagnetic ground state. The following two candidates for this complex can be envisioned. First, it could be the two-electron-reduced form of the cofactor, Mn$^{II}$/Fe$^{III}$, which would be expected to have a diamagnetic ground state due to antiferromagnetic coupling of the Mn$^{II}$ ($S_{Mn} = 5/2$) and Fe$^{III}$ ($S_{Fe} = 5/2$) ions. The second possibility is that the species is a NO$^\bullet$ adduct of the Mn$^{III}$/Fe$^{III}$ cluster, generated by one-electron reduction of Mn$^{IV}$/Fe$^{III}$. The NO$^\bullet$ could be generated in solution by breakdown of HU (143). The likely electronic structure could entail binding of NO$^\bullet$...
to the Fe$^{III}$ site, yielding a $\{\text{FeNO}\}_6$ species. Intramolecular electron transfer from the Mn$^{III}$ site to the $\{\text{FeNO}\}_6$ complex could then yield a Mn$^{IV}$ site ($S_{\text{Mn}} = 3/2$) and a $\{\text{FeNO}\}_7$ fragment. $\{\text{FeNO}\}_7$ species that contain a high-spin Fe$^{III}$ site have been reported for numerous mononuclear non-heme enzymes and models thereof. They generally have an $S = 3/2$ ground state due to antiferromagnetic coupling of the Fe$^{III}$ site ($S_{\text{Fe}} = 5/2$) and the coordinated NO$^-$ ligand ($S_{\text{NO}} = 1$). The $S_{\text{total}} = 0$ ground state of the cluster, which is a $\{\text{MnFeNO}\}_10$ species, can then be rationalized by antiferromagnetic coupling of the Mn$^{IV}$ to the $\{\text{FeNO}\}_7$ fragment.$^{30}$

The most striking difference between the two possibilities for the diamagnetic Fe$^{III}$-containing species is the Mn oxidation state. X-ray absorption spectroscopy could be a useful tool to address this question, because the energy of the Mn-$K$-edge is sensitive to the Mn oxidation state. These experiments have thus far not been carried out yet, because the samples are heterogeneous. Mössbauer analysis reveals the formation of a significant amount of mononuclear Fe$^{III}$ ($\sim 30\%$), in addition to the $\sim 50\%$ of the diamagnetic Fe(III)-containing species. This suggests that the Mn/Fe-cluster is degraded and that there is also free Mn in the sample (or mononuclear Mn in the dimetal site of Ct R2, after removal of the Fe). There is no spectroscopic method that could be used to quantify the various forms of Mn in the sample. Mn-XAS experiments may provide useful information if it is possible to prepare samples, which contain only a small amount of mononuclear high-spin Fe$^{III}$, i.e. degraded clusters. These studies are ongoing, but have not yet been completed.

$^{30}$ Coordination of NO to the Mn ion is less likely. The high-spin Fe$^{III}$ site, which is indicated by the Mössbauer parameters, has $S_{\text{Fe}} = 5/2$, requiring that the $\{\text{MnNO}\}_5$ fragment has $S_{\text{MnNO}} = 5/2$ and is antiferromagnetically coupled to the Fe$^{II}$ site.
In order to discern between these two hypotheses, we carried out the following additional experiments, which collectively provide circumstantial evidence for the assignment of this species as the \(\{\text{MnFeNO}\}^{10}\) species. First, when a sample of \(\text{Mn}^{IV}/\text{Fe}^{III}-\text{R2}\) is treated with hydroxylamine (\(\text{NH}_2\text{OH}\) or HA), the quadrupole doublet characteristic of the diamagnetic Fe\(^{III}\)-containing complex is also observed. However, when the two-electron reductant hydrazine, \(\text{N}_2\text{H}_4\), is used, the quadrupole doublet is not detected in the Mössbauer spectra. Second, the diamagnetic Fe\(^{III}\)-containing species does not react with \(\text{H}_2\text{O}_2\). Given that the \(\text{Mn}^{II}/\text{Fe}^{II}\) and \(\text{Mn}^{III}/\text{Fe}^{III}\) forms of \(\text{Ct R2}\) react rapidly with \(\text{H}_2\text{O}_2\) (see Chapter 3), it was expected that the \(\text{Mn}^{II}/\text{Fe}^{III}\) form should also react with \(\text{H}_2\text{O}_2\) to form an oxidized Mn/Fe cluster, possibly the \(\text{Mn}^{IV}/\text{Fe}^{III}\) cluster by two-electron oxidation. By contrast, the lack of reactivity toward \(\text{H}_2\text{O}_2\) could be consistent with the \(\{\text{MnFeNO}\}^{10}\) formulation. This complex is not only more oxidized than the \(\text{Mn}^{II}/\text{Fe}^{III}\) complex, but also one of the coordination sites of the Fe\(^{III}\) site is blocked by coordination of the strong NO ligand. Third, reaction of \(\text{Mn}^{III}/\text{Fe}^{III}-\text{R2}\) with a stoichiometric amount of NO results in generation of a small amount (\(\sim 10\%\)) of a quadrupole doublet in the Mössbauer spectra with parameters similar to those observed for the HU-treated \(\text{Mn}^{IV}/\text{Fe}^{III}\) cluster. Further experiments are required to unambiguously unravel the nature of the diamagnetic Fe\(^{III}\)-containing complex. These could include reaction of \(\text{Mn}^{IV}/\text{Fe}^{III}\) with other N-OH-containing molecules, in order to define the functional group required for generation of the diamagnetic Fe\(^{III}\)-containing complex. Commercially available examples include acetylhydroxamic acid and \(N,N'\)-dimethylhydroxylamine. In addition, low-temperature \(\gamma\)-irradiation, also known as cryoreduction, may convert the diamagnetic species to an EPR-active form with half-integer
electron spin ground state. If an EPR-signal is detected, a more detailed characterization by ENDOR/ESEEM methods may provide additional insight into the structure of this form of the cofactor.
Chapter 5
Probing the Effects of Two Strictly Conserved, Class Ic-Specific Residues in *Ct* R2
Nordlund and co-workers were the first to note that two of the amino acid residues that are strictly conserved in class I R2s are changed in Ct R2. The changes are the replacement of the radical-harboring Y (Y122 in Ec R2) with a redox-inactive F (F127 in Ct R2), and the substitution of the carboxylate ligand to metal site 1 (D84 in Ec R2) with E (E89 in Ct R2) (17). These Y→F and D→E substitutions were also noted in the seven other Y•-less R2s available in the sequence databases at that time (17). Moreover, they are also strictly conserved in the ~25 Y•-less R2s that have been identified by BLASTP searches (see Chapter 6). We speculated that they are essential in formation and function of the MnIV/FeIII cofactor To test this hypothesis, the E89D and F127Y variants of Ct R2 have been studied. The results reveal that both changes are crucial to generate and maintain the active MnIV/FeIII cofactor of Ct R2.

**Studies of E89D Ct R2**

Our studies of E89D Ct R2 reveal that the generation of the heterobinuclear Mn/Fe cluster is significantly more disfavored toward the homobinuclear diiron form than in wt Ct R2. Therefore, the reaction of the diiron form is an essential control for the reaction of Mn/Fe form of the E89D variant, and is described first.

The 4.2-K/53-mT Mössbauer spectrum of the product of O2 activation at the FeII/II-Ct R2-E89D cluster yields three quadrupole doublets (Figure 5-1A). The doublet residing in the

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31 The construction of vector encoding Ct R2-E89D variant, the protein overexpression and purification, and activity measurement were all carried out by Monique Maslak Gardner, an undergraduate student working in our lab.

32 The construction of vector encoding Ct R2-F127Y variant, the protein overexpression and purification, the activity measurement were all carried out by Hanne Nørgaard, an exchange Ph. D. student from the Technical University of Denmark (Lyngby, Denmark).
interior of the spectrum has an isomer shift ($\delta$) of 0.50 mm/s and a quadrupole splitting parameter ($\Delta E_Q$) of 0.84 mm/s. It accounts for ~53% of the total intensity (blue line). The parameters of this quadrupole doublet are similar, but not identical, to those of the product of diiron form in $Ct$ R2-wt (Figure 5-1B, hashed lines). The difference most likely arises from the perturbation of site 1 by the modified carboxylate ligand. As for $Ct$ R2 wt, this component is assigned to a $bis$-$\mu$-hydroxo Fe$_{\text{III/III}}$ cluster, as suggested by the crystal structure (17). The remainder of the spectrum can be simulated with two symmetrical quadrupole doublets of equal intensity (Figure 5-1A, red line) with parameters ($\delta_1$ = of 0.54 mm/s, $\Delta E_{Q,1}$ = 1.71 mm/s, $\delta_2$ = of 0.47 mm/s, and $\Delta E_{Q,2}$ = 2.41 mm/s) that are nearly identical to those of the $\mu$-oxo-Fe$_{\text{III/III}}$ cluster of $Ec$ R2 [see Figure 5-1C for comparison] (26, 34). The magnitude of $\Delta E_Q$ of the two components strongly suggests the presence of a $\mu$-oxo-group. Thus, the E89D substitution has a

![Figure 5-1: The 4.2-K Mössbauer spectra collected in external magnetic field of 53 mT oriented parallel to the $\gamma$-beam. (A) The spectrum of the final product of O$_2$ activation at Fe$_{\text{II/II}}$-$Ct$ R2-E89D. The black solid line is the simulation. The blue and red lines on top are the simulation of the sub-spectra assigned to $bis$-$\mu$-hydroxo and $\mu$-oxo Fe$_{\text{II/II}}$ clusters, respectively. (B) The spectrum of the final product of O$_2$ activation at Fe$_{\text{II/II}}$-$Ct$ R2-wt. The blue line is the sub-spectrum assigned to $bis$-$\mu$-hydroxo cluster from (A). (C) The spectrum of the final product of O$_2$ activation at Fe$_{\text{II/II}}$-$Ec$ R2-wt. The red line is the sub-spectrum assigned to $\mu$-oxo cluster from (A). (D) The spectrum of the final product of H$_2$O$_2$ reaction at Fe$_{\text{II/II}}$-$Ct$ R2-E89D. The blue line, which is the sub-spectrum assigned to $bis$-$\mu$-hydroxo cluster from (A), is scaled to have the same area for comparison.](image-url)
profound effect on the outcome of the reaction, yielding \( \sim 50\% \) of the putatively \( \mu\)-oxo-\( \text{Fe}^{III/III} \) cluster. As a control, the spectrum of the final product of the reaction of \( \text{Fe}^{II/II} \) R2-E89D with \( \text{H}_2\text{O}_2 \) is shown in Figure 5-1D. It is essentially composed of an unusually broad quadrupole doublet, with apparent parameters of \( \delta = 0.51 \text{ mm/s} \) and \( \Delta E_Q = 0.86 \text{ mm/s} \). The sub-spectrum assigned to \( \text{bis-\( \mu\)-hydroxo-Fe}^{III/III} \) cluster from O2-activation in E89D is overlaid for comparison (blue line in Figure 5-1D). Their Mössbauer parameters are very similar and suggestive of a \( \text{bis-\( \mu\)-hydroxo-Fe}^{III/III} \) core structure, but the linewidth of the spectrum of the \( \text{H}_2\text{O}_2 \)-treated sample is much greater, which indicates conformational heterogeneity of the cluster. The upper limit for the amount of the \( \mu\)-oxo \( \text{Fe}^{III/III} \) cluster in the product is estimated to be \( \sim 10\% \), which could also be generated from contaminating O2 generated by decomposition of \( \text{H}_2\text{O}_2 \).

The absence of the \( \mu\)-oxo \( \text{Fe}^{III/III} \) cluster in the \( \text{H}_2\text{O}_2 \)-treated sample suggests that the required pathway for the formation of the \( \mu\)-oxo bridged cluster occurs (almost) exclusively during O2 activation, possibly via the \( \text{Fe}^{III/IV} \) intermediate, \( \text{X} \). The direct detection of \( \text{X} \) during O2 activation in E89D is obscured by the unfavorable kinetics, but the observation of its accumulation in reaction of wt, as well as other variants (Y222F, Y338F, W51F) strongly suggests that it is a common intermediate during O2 activation in \( \text{Fe}^{II/II} \) R2. We prefer the model of \( \text{X} \) with \( \mu\)-oxo/\( \mu\)-hydroxo core structure proposed by Solomon group (47), and our DFT calculations on \( \text{Mn}^{IV}/\text{Fe}^{III} \) R2 cluster, the cognate of \( \text{X} \) in redox state, also suggests the same structure (117). The two outcomes of O2 activation may depend on different sites for protonation of \( \text{X} \) (Scheme 5-1). If the oxo-bridge is protonated, the product is a bis-\( \mu\)-hydroxo cluster as labeled with blue color. Conversely, if the protonation occurs at the hydroxo-bridge, the resulting...
μ-oxo/μ-aqua complex may convert to a μ-oxo/terminal water cluster (shown in red), which is the crystallographically characterized form of the Fe$_2^{III/III}$ form of Ec R2 (26). Obviously, the carboxylate ligand plays an essential role to tune the location of protonation in this scheme.

The spectra of the final product of O$_2$ activation at the Mn$_{II/II}$-Ct R2-E89D in zero-field and 53-mT are shown in Figure 5-2A. The sample was prepared in the same fashion as for wt protein (see Appendix B), which typically leads to the formation of ~90% Mn$^IV$/Fe$^{III}$ cluster. However, the main species in the sample of “Mn/Fe” E89D Ct R2 are still Fe$_2^{III/III}$ clusters. Therefore, it is much more difficult for Mn to be incorporated into the E89D protein than in wt, which might imply that site 1, to which E89 serves as a ligand, is the Mn-binding site. The slight broadening of 53-mT spectrum of the final product suggests that there is still a small fraction of Mn$^IV$/Fe$^{III}$ cluster. In order to quantify it, the sub-spectra (from Figure 5-1) of 35% of the μ-oxo (red) and 40% bis-μ-hydroxo Fe$_2^{III/III}$ (blue) clusters have been removed from the zero-field and 53-mT spectra.\textsuperscript{33} The remaining spectra, which turn out to be ~20% in both cases, are considered to be the reference spectra for Mn$^IV$/Fe$^{III}$-Ct R2-E89D cluster (Figure 5-2A and B, \textsuperscript{33} The spectra of a diamagnetic species, e.g., Fe$_2^{III/III}$ cluster, are essentially the same at 0 mT and 53 mT.)
hashed lines). They are quite similar to those of wt, with essentially the same $\delta$ (0.54 mm/s) and a slightly smaller $\Delta E_Q$ (1.11 mm/s). The 53-mT spectrum was simulated with the parameters ($\delta$ and $\Delta E_Q$) from the zero-field spectrum and the remaining parameters from those determined for the Mn$^{IV}$/Fe$^{III}$ of wt Ct R2 (see Chapter 2, green lines in Figure 5-1A). When all components are added up, the resulting spectra match the experimental data reasonably well in both 0-mT and 53-mT (black lines in Figure 5-1A).

The presence of the Mn$^{IV}$/Fe$^{III}$ cluster in Ct R2-E89D is also indicated by EPR spectroscopy. When the final product of the “Mn/Fe” reaction of R2-E89D prepared with $^{56}$Fe is reduced under the turnover condition by dithionite, a well-structured EPR signal similar to the one from

![Figure 5-2](image-url): (A) The 4.2-K Mössbauer spectra of the final product of O$_2$ activation at Mn$^{II}$/Fe$^{II}$-Ct R2-E89D under the indicated external field. The blue and red lines are reference spectrum for 40% bis-μ-hydroxo and 35% μ-oxo Fe$_{2}^{III/II}$ clusters generated from analysis in Figure 5-1. The green lines and black lines are simulations for the Mn$^{IV}$/Fe$^{III}$-Ct R2-E89D cluster and the whole sample, respectively, which are generated as described in the text. (B) The comparison of the 4.2-K Mössbauer reference spectra from Mn$^{II}$/Fe$^{II}$-Ct R2 cluster in E89D (hashed lines) and wt (solid line). The applied external field and its orientation are as indicated for the top and middle spectra while the bottoms are the difference spectra of the two above. (C) X-band EPR spectra arising from the Mn$^{III}$/Fe$^{III}$-Ct R2:R1:CDP:ATP cluster in wt (top, solid line) and E89D (bottom, solid line). The simulations for both are shown in dashed lines. Spectrometer conditions: $\nu$ = 9.45 GHz; $T$ = 14 K; $P$ = 200 mW; modulation frequency, 100 kHz; modulation amplitude, 10 G; time constant, 0.167 s
Mn$^{IV}$/Fe$^{III}$-R2:R1:CDP:ATP in wt Ct R2 develops. A careful comparison (Figure 5-2C) and simulation show that the values of $A_{Mn}$-tensor decreases from (269, 392, 314) MHz in wt to (240, 365, 285) MHz in E89D, which is almost certainly from the perturbation of the ligand switch. The detection of a well-resolved EPR-signal of the Mn$^{III}$/Fe$^{III}$ cluster is interesting, because in wt Ct R2 it is associated with the functional form of the cofactor after PCET has occurred.

The activity measurement of the E89D sample gives a value of 0.02-0.03 s$^{-1}$, remarkably lower than wt (0.3 ± 0.05 s$^{-1}$) under the same conditions, which must be partially attributed to the low yield of heterobinuclear cluster. Since the Mn content probably varies among different preparations, it is difficult to compare the activity of the real active form, the Mn$^{IV}$/Fe$^{III}$-Ct R2 cluster, in wt and E89D. The activity of Ct R2-E89D can be determined, but it requires the quantification of the active Mn$^{IV}$/Fe$^{III}$ form. These studies are ongoing.

**Studies of F127Y Ct R2**

The reaction of a solution of the “Mn$^{II}$/Fe$^{II}$” form of Ct R2-F127Y was studied by SF-absorption spectroscopy first. A transient absorption feature at ~390 nm that may suggest the accumulation of the Mn$^{IV}$/Fe$^{IV}$ intermediate is observed. However, in contrast to the wt enzyme, the decay of A390 is biphasic and more complex (Figure 5-3A). FQ-EPR confirms the transient formation of the Mn$^{IV}$/Fe$^{IV}$ intermediate (Figure 5-3B). In addition to the signature of the Mn$^{IV}$/Fe$^{IV}$ intermediate, there is additional intensity on the fourth line, which is at $g = 2$. This may suggest the presence of an organic radical, and is tentatively attributed to Y127•, or a decay
product thereof. After a reaction time of \( \sim 140 \) s, the intensity of the EPR signals has almost completely decayed, but after even longer reaction times (20 min), a broad and unfeatured signal similar to that of the \( \text{Mn}^{\text{III}}/\text{Fe}^{\text{III}} \)-R2 complex is observed (Figure 5-3C). Mössbauer characterization of the final product (Figure 5-3D, black marks) suggests that the sample contains up to 45% of total Fe in form of the \( \text{Mn}^{\text{III}}/\text{Fe}^{\text{III}} \) cluster. The remaining part is mainly a paramagnetic species reminiscent of high-spin \( \text{Fe}^{\text{III}} \). More careful work is required to fully understand the reaction in F127Y. Nevertheless, the preliminary data suggests the meta-stability of the \( \text{Mn}^{\text{IV}}/\text{Fe}^{\text{III}} \) complex in the variant protein.

This hypothesis is further supported by the activity measurement (Figure 5-4). The content
of Mn$^{IV}$/Fe$^{III}$ complex, the active form identified in wild type Ct RNR required for catalysis, can be directly reflected by the activity. In order to observe the decay of Mn$^{IV}$/Fe$^{III}$ complex during O$_2$ activation in F127Y, an alternative strategy is applied. The apo R2 (200 µM) was preloaded with 1 equiv of Mn$^{II}$ and 1 equiv of Fe$^{II}$ anaerobically and mixed with 5 volume equivalents of air-saturated buffer at ambient temperature to initiate the O$_2$ activation. The aliquot of this common R2 solution was transferred to the assay mixture containing excess R1, DTT, CDP and ATP (same as the standard protocol) at the x th minute and the assay was quenched by HCl at the (x+1) th minute. The mixture was then filtered and analyzed by mass spectrometry. The activity of wt during one hour after O$_2$ activation is consistently ~0.35 s$^{-1}$. The F127Y variant, however, exhibits activity comparable to that of wt Ct R2 within the first minute but the activity decreases continuously, and the enzyme is almost inactive after one hour. Thus, the replacement of Y with the redox-inactive phenylalanine in Mn/Fe RNR is required for efficient catalysis.

**Discussion**

Previous work on the D84E variant of the Ec R2 had shown a marked effect on the kinetics of the O$_2$ reaction and Mössbauer spectrum of its product (137, 138). The results from the E89D
variant of $Ct$ R2 in both $O_2$ reactions at the $Fe_{II/III}$ and $Mn_{II}/Fe_{II}$ clusters further support the important role of this carboxylate ligand. In particular, it is much more difficult for Mn to be incorporated into the E89D protein than in wt, indicating that this ligand switch is a crucial (but probably not the unique) chemical rationale for the enzyme to adopt a Mn/Fe cofactor. Conversely, the F127Y variant can assemble the active $Mn_{IV}/Fe_{III}$ form of the cofactor (although we have not yet obtained direct evidence for this form by Mössbauer spectroscopy) and is initially as active as wt $Ct$ R2. However, the activity decays within $\sim 1$ h completely with formation of the one-electron-reduced $Mn_{III}/Fe_{III}$ form. It is our hypothesis that the $Mn_{IV}/Fe_{III}$ form is not stable and may decay by oxidation of Y127.

These studies are the first step toward one of the long-term goals of this project, the rational reprogramming of a class Ic RNR to conventional class I RNR, and vice versa. This topic will be discussed in the last chapter in more detail.
Chapter 6
Outlook: New Opportunities to Study a Challenging Class of Enzymes
By a combination of activity assay, kinetic analysis, and various spectroscopic methods, it has been demonstrated that Ct RNR employs a high-valent, heterobinuclear Mn$^{IV}$/Fe$^{III}$ cluster for activity, which is the first example for a redox-active Mn/Fe cofactor in biology. During turnover, the Mn$^{IV}$/Fe$^{III}$ cluster is transiently reduced to the Mn$^{III}$/Fe$^{III}$ state to (presumably) generate the C• in the R1 subunit. So the Mn$^{IV}$/Fe$^{III}$ cluster, specifically, the Mn$^{IV}$ site, functionally replaces the Y• of a conventional class I RNR. The Mn$^{IV}$/Fe$^{III}$ cofactor is generated by reaction of O$_2$ with the reduced Mn$^{II}$/Fe$^{II}$ cluster. A Mn$^{IV}$/Fe$^{IV}$ intermediate accumulates and undergoes one-electron reduction of the Fe$^{IV}$ site. The electron to reduce the Fe$^{IV}$ site is shuttled by the near-surface residue, Y222, which has no functional cognate in the best-studied conventional class I RNRs (e.g., from *E.coli* and *Mus musculus*). The Mn$^{IV}$/Fe$^{III}$ cofactor can also be produced from the Mn$^{III}$/Fe$^{III}$ cluster by H$_2$O$_2$, an important physiological reactive oxygen/nitrogen species [RO(N)S$^-$], via the formation of the Mn$^{IV}$/Fe$^{IV}$ intermediate. Alternatively, H$_2$O$_2$ can react with the Mn$^{II}$/Fe$^{II}$ form to generate the active Mn$^{IV}$/Fe$^{III}$ form by two consecutive two-electron oxidation steps to yield Mn$^{III}$/Fe$^{III}$ and Mn$^{IV}$/Fe$^{IV}$ states, respectively, followed by one-electron reduction to Mn$^{IV}$/Fe$^{III}$. These results are summarized in Figure 6-1 (bottom) and compared to the reactions in *Ec* RNR (top).

There are two most obvious differences between the class I and class Ic RNRs. The first concerns the reactivity to H$_2$O$_2$. In *Ec* R2, the met-R2 (Fe$_2$$^{III/III}$-Y form, the cognate of Mn$^{III}$/Fe$^{III}$-Ct R2) can only react with H$_2$O$_2$ very inefficiently, with ~30% yield of Fe$_2$$^{III/III}$-Y• in 1.5 hours (136), which probably rules out the importance of this pathway under physiological conditions. Adventitious reduction of the Y• in the conventional R2s is known to occur (144),

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Figure 6-1: Activation, role in catalysis, and maintenance of the Fe\textsuperscript{III}/Fe\textsuperscript{III}-Y• cofactor of \textit{Ec} R2 (top) and of the Mn\textsuperscript{IV}/Fe\textsuperscript{III} cofactor of \textit{Ct} R2 (bottom). The red boxes indicate EPR-active cofactors. States encircled with a grey dotted line have not been directly detected. Selected EPR and Mössbauer spectra of various states are shown. The C’3-H-cleaving C• radical in the R1 active site was never detected directly, but use of the substrate analogue N\textsubscript{3}-ADP allowed for accumulation of an N-centered radical in the active site. The spectrum of the N-centered observed in \textit{Ec} RNR has been adapted from (9).
and the re-activation of this form requires the complete reduction of Fe_{2}^{III/III} to Fe_{2}^{II/II}, a process termed as “the maintenance pathway”, followed by O_{2} activation. The reduction step is believed to be mediated by YfaE, a [2Fe-2S] ferrodoxin (57). In Ct RNR, H_{2}O_{2} can activate Mn^{III}/Fe^{III} to the active form quickly and quantitatively, which might confer a selective advantage to the pathogens. The study on the effects of other reactive oxygen/nitrogen species, e.g., superoxide, nitric oxide (NO), peroxynitrite, will reveal more about the reactivity of Ct RNR to RONS as part of the host’s immune systems.

Second, the residue of Y222, an additional electron-transfer element during O_{2} activation in Ct R2, is absent in Ec enzyme. Although the mechanism of this process is fairly clear (see Chapter 3), its biological and evolutionary rationale is less apparent. The Y222F variant successfully assembles the Mn^{IV}/Fe^{III} cofactor and is then fully catalytically active. Thus, in vitro and upon a single activation event, Y222 is completely dispensable. The conservation of Y or W at this position among most of the presumptively Mn/Fe-dependent R2s of an electron-transfer-competent residue at this position might reflect a selective advantage conferred by ensuring that the protein is stable to repeated activation events occurring in vivo. Reactivation of the catalytically inactive Mn^{III}/Fe^{III} form as the maintenance pathway in Ct, either by reduction to Mn^{II}/Fe^{II} followed by reaction with O_{2} or by direct reaction with H_{2}O_{2}, would obviate the more costly de novo re-synthesis of R2. The extra ET element may be conserved because it prevents deleterious side reactions that might otherwise lead to progressive inactivation during this redox cycling of the protein in vivo. The conservation could also reflect the existence of a specific accessory protein to deliver the extra electron in vivo, e.g., the cognate
of Ec protein YfaE (51). Y222 in Ct R2 seems ideally positioned to interact with a functionally homologous protein in C. trachomatis. Prospecting for genes that might encode such a factor is in progress.

**Ct RNR: The Best Opportunity to Dissect Proton-Coupled Electron Transfer**

**PCET-gating mechanism(s)**

Nocera, Stubbe, and co-workers proposed a PCET mechanism for Ec RNR involving proton transfers (PTs) from the Fe1-coordinated water to the Y122 phenol oxygen and from the indole N-H of W48 to its hydrogen-bonding partner, D237 (blue arrows in Figure 6-2A) (10). The former PT would increase the effective reduction potential of the Y122• and the latter would diminish the effective potential of a W48 radical. Together, they could permit the migration of the "hole" from its resting position on Y122 onto W48, toward R1, thus the PCET-gate opens. The crucial principle of this mechanism is that the proton coupling is strictly local (i.e., there is no net proton-translocation into the cofactor site), permitting the highly reversible electron transfer. The PTs can be coupled to the conformational

![Figure 6-2: X-ray structures of the Fe_{III/III} forms of E. coli R2 (A) and C. trachomatis R2 (B), which were generated using the coordinates available in pdb files 1MXR and 1SYY, respectively. Proposed proton transfer steps are indicated with blue arrows.](image-url)
change upon the assembly of the holoenzyme.

From our tentative model for the Mn$^{IV}$/Fe$^{III}$ cofactor (see Chapter 2) (117), derivative mechanisms can be proposed for PCET gating in Ct RNR. The structure of Fe$_2^{III/III}$ Ct R2 and the computational model for the Mn$^{IV}$/Fe$^{III}$ cofactor both have solvent ligands to metal site 1, which is essentially at the same site occupied by the Y122 proton donor in the Nocera/Stubbe mechanism (17, 117). The proton donor could thus be conserved in the two systems. Obviously, the acceptor must be different. The model of the Mn$^{IV}$/Fe$^{III}$ suggests that the µ-oxo ligand should be crucial to stabilizing the high-valent Mn$^{IV}$ site. This ligand bridges are highly asymmetric, 1.73 Å from the Mn$^{IV}$ and 1.95 Å from the Fe$^{III}$. Proton donation to the µ-oxo should lengthen the Mn–O bond and destabilize the Mn$^{IV}$ relative to its Mn$^{III}$ PCET product. Thus, conformational change upon the holoenzyme assembly which redirects a proton from the water ligand toward the oxo-bridge could be part of the PCET gate (Figure 6-2B).

Irrespective of the atomic details, Figure 6-2B underscores the unique opportunity to understand the PCET step that the class Ic RNR presents. The Mn$^{III}$/Fe$^{III}$ cluster as the product of PCET is an EPR active species that should be amenable to ENDOR spectroscopy to determine the number and positions of protons. By contrast, the PCET product in the conventional class I system presumably has an ordinary tyrosine residue and an EPR-silent Fe$_2^{III/III}$ cluster. In Chapter 4, we have shown the distinct EPR signals (products) generated by dithionite or HU reduction of the Mn$^{IV}$/Fe$^{III}$ form in free R2 or under turnover conditions. In particular, the substrate binding is the key to generate the species exhibiting the sharp and featured EPR spectrum, although the binding site is believed to be ~35 Å away from the di-metal cluster. This phenomenon almost
certainly reflects structural changes at the cofactor site that are part of the PCET-gate.

The understanding of gating mechanism can also be approached by studies in model chemistry. Different from organic radicals, the redox potential of \( \text{Mn}^{IV} \) in \( \text{Mn}^{IV}/\text{Fe}^{III} \) cluster at (de)protonated state is less well known. To our knowledge, there is no such report and no direct evidence to show that the \( \text{Mn}^{IV} \) site, a moderate oxidant under general considerations, could generate \( \text{C}^\bullet \). Our hypothesis is that the moderate oxidant (\( \text{Mn}^{IV}=\text{O} \)) can be tuned to a strong one (\( \text{Mn}^{IV}\text{-OH} \)) via coupling to PT. In particular, we expect that the \( \text{Fe}^{IV} \) site lacks the tuning ability due to the intrinsic differences of Mn and Fe atoms, thus further ruling out the possibility that \( \text{Fe}^{II/III} \) cluster can initiate the PCET catalytically.

**Pathway Radicals**

The \( \text{Mn}/\text{Fe} \)-dependent RNR should also afford great opportunities to dissect the radical-hopping ET that takes place after the gate opens. For the \( \text{Y}^\bullet \)-dependent RNRs, the direct observation of the pathway radical is obscured by the unfavorable kinetics and the absence of a convenient spectroscopic “handle” (see Chapter 1). Stubbe and co-workers substituted \( \text{Y}^{356} \) in R2, \( \text{Y}^{730} \) and \( \text{Y}^{731} \) in R1 subunit with unnatural amino acids surmount these obstacles, which have diminished radical-reduction potentials and distinct spectroscopic signatures (77-82).

By contrast, in the \( \text{Mn}/\text{Fe} \)-dependent system, the radical propagation step converts the integer-spin \( \text{Mn}^{IV}/\text{Fe}^{III} \) cofactor, an EPR-silent species, to states containing the \( \text{Mn}^{III}/\text{Fe}^{III} \) cluster and a pathway radical, both of which are \( S = 1/2 \) components and exhibit sharp \( g = 2 \) EPR
signatures.\textsuperscript{34} The great sensitivity of EPR and the production of two equivalents of "spin" by each excursion of the hole from the Mn\textsuperscript{IV} site should permit detection of even low levels (< 1%) of an accumulating intermediate state, which in turn could allow important details of the PCET step and gating thereof to be probed by time-dependent EPR and ENDOR methods.

An interesting observation was obtained when the Mn\textsuperscript{II}/Fe\textsuperscript{II} form was exposed to an air-saturated solution containing R1, ATP, and CDP. In addition to the Mn\textsuperscript{IV}/Fe\textsuperscript{IV} intermediate, a new \(g = 2\) signal was detected by EPR spectroscopy (Figure 6-3, top spectrum). The hyperfine structure suggests that it is a \(Y^\bullet\), which might be located on any of the \(Ys\) of the proposed PCET pathway or the novel, branched ET pathway. To further test this hypothesis, each of the proposed residues on two pathways was changed (C672S, Y990F, and Y991F in \(Ct\)-R1 and Y338F, W51F, and Y222F \(Ct\)-R2). In addition to these single variants, the Y222F/Y338F \(Ct\)-R2 double variant was studied (Figure 6-3).\textsuperscript{35} The \(Y^\bullet\) signal detected in Y338F variant is markedly different from others, suggesting a different location of the radical, which can be Y222. When both Y338 and Y222 are replaced, the radical is essentially diminished. The simplest interpretation is that the “hole” generated by Mn\textsuperscript{IV}/Fe\textsuperscript{IV} intermediate during O\textsubscript{2} activation, is propagated along the proposed PCET pathway or the branched pathway. However, the detection of \(Y^\bullet\) in variant W51F does not support this hypothesis. The incorporation of the unnatural amino acids into \(Ct\) RNR will be a powerful tool to pinpoint the location of the radical and map out its hopping

\textsuperscript{34} The dipolar coupling between the two \(S = 1/2\) entities is expected to be small, due to the \(\sim 1/r^3\) distance dependence.

\textsuperscript{35} The EPR spectra have been scaled to the similar intensity for qualitative characterization. The variation of the intensity of the signal can be attributed to the handling by different people, the varying hand-quenching time, etc. Repeat of these experiments under more strict control is required for quantitative comparison.
pathway(s).

**Figure 6-3.** X-band EPR spectra showing the formation of organic radicals presumably generated by Mn<sup>IV</sup>/Fe<sup>IV</sup> intermediate under the turnover conditions. An O<sub>2</sub>-free solution of Mn<sup>II</sup>/Fe<sup>II</sup>-Ct R2 (3 mM R2 monomer, 1.5 equiv Fe<sup>II</sup> and 1.5 equiv Mn<sup>II</sup>) was quickly mixed with 9-volume of air-saturated solution, which gives 900 μM R1, 4mM CDP, 1mM ATP, and 10mM DTT after mixing. The reaction was hand-quenched by cold isopentane at ~15-20s. Wild type R1 and R2 proteins were used in the “wt” sample (top). All the samples containing R1 variants (2nd to 4th) were mixed with R2 wt, and all the samples with R2 variants (bottom four) were mixed with R1 wt. The right panel is the narrow scan of the corresponding sample on the left panel around $g = 2$ position to show the hyperfine structure of the radical. Spectrometer conditions were: $T = 14.0 \pm 0.2K$; $P = 20 \mu W$; $\nu = 9.45$ GHz; modulation frequency: 100 kHz; time constant: 167ms modulation amplitude: 10G (left) or 1G (right); scan times: 1 (left) or 10 (right).

**Rational Reprogramming of Class I and Class Ic RNRs**

One of the intriguing experiments is the rational manipulation of metalloenzyme reactivity, based on the in-depth understanding of the reaction mechanism. Previous work on *Ec* R2 revealed that the reactivity of the Fe<sub>2</sub> cluster can be re-engineered by two amino acid substitutions into a self-hydroxylating monooxygenase (see Chapter 1) (65). The similarity between conventional class I and the novel class Ic RNRs offers a great chance to explore the possibility of rationally redesigning their redox cofactors, i.e., whether a class I RNR can use the
Mn\textsuperscript{IV}/Fe\textsuperscript{III} cofactor or a class Ic RNR can use the Fe\textsuperscript{II}/III-Y• cluster to carry out catalysis, with minimal substitutions of the amino acids.

In addition to the replacement of the radical-harboring Y-residue with F, one obvious difference between the Ec and Ct R2 cofactor sites is one of the ligands to metal site 1, where Ec D84 corresponds to Ct E89 (16, 17). Previous work on the Ec R2-D84E (56, 137, 138) and our recent work on Ct R2-E89D variant (see Chapter 5) have both shown that this seemingly innocuous substitution has a marked effect on the cofactor assembly. A blastp search of the NCBI non-redundant protein sequence database with Ct R2 as the query sequence identifies > 25 additional (hypothetical) R2 proteins with F at the position aligning with the radical Y of the conventional R2 proteins (Figure 6-4). All of these sequences have an E residue aligning with Ec D84/Ct E89 (thus named “E/F” R2s), and, conversely, all sequences retaining the radical Y have D at this position (“D/Y” R2s). This strict one-to-one correlation let us to assume that all the E/F

![Figure 6-4: Results of a BLASTP search using the Ct R2 sequence as query, analyzed with CLUSTALW, and displayed as unrooted dendrogram. Putative class Ic “EF” R2s are shown in red and selected class I “DY” R2s in black. The gray-shaded area represents the group of 10 “DY” R2s and 5 “EF” R2s with great sequence similarity and is expanded for clarity on the right. R2s from species discussed in this chapter are highlighted with bold lines.](image-url)
R2s (if functional) will be found to use Mn/Fe cofactors and the D/Y proteins use conventional Fe$_2$-Y• cofactors.

Although the Ec and Ct proteins are the best-characterized class I (D/Y) and class Ic (E/F) R2s, respectively, they are (presumably) not the best choice for rational reprogramming, because their sequences are only 24% identical and 47% similar. Analysis of the sequences of all E/F R2s that we identified and those of selected D/Y R2s reveals one group of 15 sequences containing 5 E/F and 10 D/Y hypothetical R2s (shaded area in Figure 6-4), in which every pair of E/F and D/Y sequences shares as much as ~70% identity and ~85% similarity [e.g., the D/Y *Methylococcus capsulatus* (Mc) and E/F *Saccharopolyspora erythraea* (Se) proteins]. Preliminary data reveal that Mc R2 yields a Y• after O$_2$ reaction at Fe$_{II/II}$ cluster, and Se R2 transiently generates the Mn$^{IV}$/Fe$^{IV}$ intermediate (Figure 6-5) during O$_2$ reaction at Mn$^{II}$/Fe$^{II}$ cluster. The reverse reactions in each protein do not occur.\textsuperscript{36} These results supports our assumption for cofactor employment based on the primary sequence as indicated above, and imply that there may be relatively few essential structural differences between the two classes. Therefore, the

\textsuperscript{36} These experiments were carried out by Allen J. Easton, a postdoc fellow, and Laura Dassama, a graduate student in our lab.
rational reprogramming may be feasible.

**Hypothesis for Evolution of Class I RNRs**

There is consensus that DNA (thus RNRs) existed before the accumulation of oxygen in the primitive atmosphere by photosynthesis (15, 30). Therefore, the O_2-dependent class I RNRs could not have been the first strategy to evolve. Evolution of the first class I enzyme from a class II or III precursor would have involved recruitment of an extant protein (e.g., a primitive diiron oxygenase/oxidase) to bind to the extant RNR and act as radical-generating cofactor subunit. By obviating the expensive synthesis of AdoCbl (in the case of evolution from class II) or permitting function in the presence of O_2 (for evolution from class III), this development would have provided a profound advantage, driving its evolutionary fixation.

The discovery of the Mn/Fe-dependent R2(s) raises the questions of which O_2-dependent system evolved first and whether they arose sequentially or divergently from a common ancestor. Both systems employ elaborate and sophisticated machinery to generate and stabilize a high-potential hole that can be used for generation of the cysteiny l radical in a conformationally gated fashion, which probably evolved independently with the development of R1 subunit from its ancestor (the class II or class III precursor). The primordial R2 subunit could undergo complete redox cycling of its metal cluster in every turnover in the manner of a modern bacterial multicomponent monooxygenase (54). A meta-stable high-valent intermediate (IV/IV or III/IV) could be generated when the reduced metal cluster of the ancestral R2 reacted with O_2. Transient association with the ancestral R1 could have allowed for irreversible extraction of one electron
by the high-valent intermediate, initiating turnover. Reductive quenching of the C• by a reductant following each turnover would have required that the R2 cofactor cycle through the II/II state and react with O₂ each time. Thus, six bioenergetically valuable reducing equivalents per deoxyribonucleotide [four to reduce O₂ to 2 H₂O and two to reduce the ND(T)P to the dND(T)P] were required. The subsequent evolutionary events could allow the stabilization of the hole, by either on the metal cluster itself or on a side-chain radical, and a gating mechanism thereof. It would have allowed to increase numbers of turnovers per O₂ reaction, pushing the e-/dND(T)P ratio to the optimized value of two that is approached by at least some modern RNRs.

According to this view, the next question is whether the cofactor-cycling, ancestral R2 used an Fe₂ cluster, a Mn/Fe cluster, or some other cofactor. We suggest that it used an Fe₂ cofactor and was a relative of the modern diiron oxygenases, perhaps complete with a specific reductase protein to enable the necessary cofactor cycling (Figure 6-6C). The proteins YfaE (51) and NrdI (145), recently shown to reduce the Fe₂III/III clusters in the "radical maintenance pathways" of the class Ia (Ec) and Ib (Streptococcus pyogenes) R2s, respectively, could be descendants of this specific reductase. An introduction of a Y residue at the appropriate location in the primitive R2 (as well as adjustment of the subunit interface and PCET pathway) would have led to the Fe₂-Y• lineage. Conversely, substitution of the metal ion under the influence(s) of assembly factors, structural adaptations favoring binding of and O₂ reactivity with Mn²⁺, changes in metal bioavailability, etc. would have initiated the Mn/Fe R2 lineage. It is noteworthy that this hypothesis invokes a primordial class I RNR functioning in exactly the manner initially proposed for Ct RNR by Gräslund, Nordlund, McClarty, and co-workers (17, 104, 105).
It is possible that relics of the primordial class I enzyme might still exist in the form of Fe₂-dependent R2s that either lack the Y• or stabilize it only ineffectively. Indeed, the Y• of
*Pseudomonas aeruginosa* (*Pa*) R2 reported by Torrents, et al. is only meta-stable, and the catalytic activity is believed to be independent of its initial Y• content (146), suggesting that its Fe₂ cofactor can readily undergo cycling under turnover conditions. Coincidentally, the *Pa* R2 is one of the D/Y enzymes in the group of 15 as mentioned above (Figure 6-4), and our preliminary results also indicate that the formation of a meta-stable Y• in *Mc* R2. The D/Y R2s in the group might therefore be viewed as a less evolved cousin of class I and class Ic R2s that more effectively stabilize the PCET hole. Whether other Fe₂-dependent R2s might even lack the Y• altogether remains to be seen. Analogously, it is possible that relics of the Mn/Fe lineage, in the form of R2s that can function with either a Fe₂ or a Mn/Fe cofactor, might still exist.
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Appendix A: Materials and Methods

**Materials.** Culture media components (yeast extract and tryptone) were purchased from Marcor Development Corporation (Hackensack, NJ). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Biosynth International (Naperville, IL). Kanamycin, antifoam A emulsion, phenylmethylsulfonyl fluoride (PMSF), tris-(hydroxymethyl)aminomethane (Tris), 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), 3-(N-Morpholino)propanesulfonic acid (MOPS), imidazole, ferrous ammonium sulfate hexahydrate, Dithiothreitol (DTT), methyl viologen (1,1’-dimethyl-4,4’-bipyridinium dichloride), sodium dithionite (sodium hydrosulfite) ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine], hydroxyurea, disodium adenosine-5’-triphosphate (ATP), cytidine 5’-diphosphate sodium salt hydrate (CDP), 4-aminoantipyrine, vanillic acid (4-Hydroxy-3-methoxybenzoic acid), Mahma NONOate [6-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine], were purchased from Sigma (St. Louis, MO). Glycerol, 30% hydrogen peroxide solution, peroxynitrite and sodium chloride were purchased from EMD (Gibbstown, NJ). Magnesium acetate and (ethylenedinitrilo)tetraacetic acid (EDTA) were purchased from J. T. Baker (Phillipsburg, N.J.). Manganous chloride tetrahydrate was purchased from Fisher Scientific (Fair Lawn, NJ). Oligonucleotide primers were purchased from Invitrogen (Frederick, MD) and Integrated DNA Technologies (Coralville, IA). Reagents for the polymerase chain reaction (PCR), restriction enzymes, T4 DNA ligase, and the buffers for these enzymes were purchased from New England Biolabs (Beverly, MA). *Escherichia coli* DH5α and BL21(DE3) cells and pET vectors were purchased from Novagen (Madison, WI). Lyophilized horseradish peroxidase was purchased
from BD Biosciences (San Jose, CA). Peroxynitrite was purchased from Calbiochem (Ann Arbor, Michigan). Quikchange XL site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). Sephadex G-50 fine was purchased from GE Healthcare (Piscataway, NJ). Ni-NTA agarose and QIAquick gel extraction kit were purchased from Qiagen (Valencia, CA). Wizard plus minipreps DNA purification system was purchased from Promega (Madison, WI). \(^{57}\)Fe metal was purchased from Advanced Materials and Technology, Incorporated (New York, NY). Genomic DNA from *Chlamydia trachomatis* serovar *D* was provided by Professor Richard Stephens (University of California, Berkeley). \(N_3\)-ADP (2'-azido-2'-deoxy-adenosine 5'-diphosphate) was provided by Professor JoAnne Stubbe (Massachusetts Institute of Technology).

**Construction of Vectors to Over-express His\(_6\)-affinity-tagged R2 Subunits of C. trachomatis**

**RNR.** Plasmid pET28a-Ct R2, which contains the *Chlamydia trachomatis nrdB* gene inserted into the pET28a expression vector (Novagen), was constructed by the following procedure. The gene was amplified from *C. trachomatis serovar D* genomic DNA by using the polymerase chain reaction (PCR) with primers 1 (5'-TTA ACG GTT CAT ATG CAA GCA GAT ATT TTA GAT GG-3'; *NdeI* restriction site in bold) and 2 (5'-ATG CTC GAG GCT TTC TCC TAT TTT GAT ATC AGG-3'; *XhoI* site in bold). The 1040 base-pair (bp) PCR fragment was purified by gel electrophoresis, extracted from the gel by using the Qiagen (Valencia, CA) QIAquick system as instructed by the manufacturer, restricted with *NdeI* and *XhoI*, gel-purified again, and ligated with pET28a that had been digested with the same enzymes and gel-purified. The sequence of the entire coding region of the vector was verified by ACGT, Inc (Wheeling, IL).
Construction of Vectors to Over-express His₆-affinity-tagged Truncated R1 Subunits of C. trachomatis RNR. Plasmid pET28a-Ct R1 (full-length), which contains the C. trachomatis nrdA gene inserted into the pET28a expression vector, was constructed as follows. The gene was amplified in three fragments by PCR with C. trachomatis serovar D genomic DNA. Fragment 1 of the gene (170 bp) was amplified by using primers 3 (5'-GGC AGC CAT ATG GTC GAT CTA CAA GAA AAG CAA TGC ACA ATT GTT AAG CGC AAT GG-3') and 4 (5'-GGA ACT TTC TAG ATC TTC AGG CAA AGG CAT GTG A-3'). Primer 3 has an NdeI site for ligation with pET28a. Primer 4 removes an endogenous NdeI site by introducing a single, translationally silent T to C substitution (complement of underscored) and contains an XbaI site (in bold) for ligation with fragment 2. Fragment 1 was gel-purified, restricted with NdeI and XbaI, and re-purified. Fragment 2 of the gene (1039 bp) was amplified with primers 5 (5'-TCA CAT GCC TTT GCC TGA AGA TCT AGA AAG TTC C-3') and 6 (5'-GAT TTT GGA TCC CCA TGT AAG AAA ACT GTA GAT CGC GAG-3'). Primer 5 removes the NdeI site (underscored) and introduces the XbaI site (bold) for ligation with fragment 1. Primer 6 removes an internal XhoI site by a translationally silent A to C substitution (complement of underscored) and introduces a BamHI (bold) site for ligation with fragment 3. Fragment 2 was gel-purified, restricted with XbaI and BamHI, and re-purified. Fragment 3 of the gene (2033 bp) was amplified by using primers 7 (5'-CGC GAT CTA CAG TTT TCT TAC ATG GGG ATC CAA AAT CTG-3') and 8 (5'-TTA CTC GAG TAA TTA CTG ACA TGC TTC ACA CCC TTT C-3'). Primer 7 removes the XhoI site (underscored) and contains the BamHI site for ligation with fragment 2. Primer 8 contains an XhoI site for ligation with pET28a. Fragment 3 was gel-purified, restricted with
BamHI and XhoI, re-purified, and ligated in a single step with the digested fragments 1 and 2 and pET28a that had previously been restricted with Ndel and XhoI and gel-purified. The sequence of the entire coding region of the pET28a-Ct R1 (full-length) plasmid in the ligation product was verified.

A His₆-affinity-tagged, Δ(1-248) truncated variant of R1 was used throughout the study of this dissertation because it is more stable and soluble than the full-length R1 (1). The plasmid directing over-expression of the variant R1 was produced by the following procedure. Primers 9 (5'-GGT TCT ACC TAT CAT ATG ACG CAT TCG CAG TTG TTG G-3'; Ndel restriction site in bold) and 10 (5'-GTG GTG GTG GTG CTC GAG TAA TTA CTG ACA TGC TTC AC -3'; XhoI site in bold) were used to amplify a 2436 base pair fragment of the pET28a-Ct R1 (full-length) plasmid encoding residues 249-1047 of R1. The fragment was gel-purified, restricted with Ndel and XhoI, re-purified, and ligated with pET28a that had been digested with the same enzymes and gel-purified. The sequence of the entire coding region of the pET28a-Ct R1-Δ(1-248) plasmid was verified.

Construction of Vectors to Over-express His₆-Affinity Tagged Versions of Ct R2 Variants: Y338F, Y222F, W51F, E89D and F127Y. The Y338F substitution was introduced by PCR by using pET28a-CtR2 as the template and primers 1 (5'-TTA ACG GTT CAT ATG CAA GCA GAT ATT TTA GAT GG-3'; Ndel restriction site in bold) and 11 (5'-GGT GGT G CTC GAG TAA TTA CTG ACA TGC TTC AC -3'; XhoI site shown in bold) to introduce the desired substitution (underlined). The 1086 bp PCR fragment was gel purified, restricted with Ndel and XhoI, re-purified, and ligated with Ndel/XhoI-restricted
pET28a-CtR2. The sequence of the coding region of the vector was verified.

The vectors to direct over-expression of the other four variants were all constructed by using the Quikchange XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) with pET28a-CtR2 as the template. The vector encoding Y222F variant was generated with primers 12 (5'-GGA GAA CAA TAT CAA TTC ATC TTA AGA GAT GAG ACA ATC C-3'; *AflII* site shown in bold) and 13 (5'-GGA TTG TCT CAT CTC TTA AGA TGA ATT GAT ATT GTT CTC CAA TAC C-3'; *AflII* site in bold) encoding the desired substitution (underlined in primer sequences). The vector encoding W51F variant was generated with primers 14 (5'-GGC TGC GCA AAT AAC TTT CCT ACC GAC ATC CCC ATG GGG AAA GAC ATC G-3'; *NcoI* site shown in bold) and 15 (5'-CTT TCC CCA TGG GGA TCT CTG TAG GGA GAA GT TAT TTG CGC AGC C-3'; *NcoI* site shown in bold) introducing the desired substitution (underlined). The vector encoding E89D variant was generated with primers 16 (5'-GGT TTT TTC AGC A CG GCC G AC AGC TTG GTT GGG-3'; *EagI* site shown in bold) and 17 (5'-CCC AAC CAA GCT GT C GG C T GCT GAA AAA ACC TAA ATT C-3'; *EagI* site shown in bold) encoding the desired substitution (underlined in primer sequences). The vector encoding F127Y variant was generated with primers 18 (5’-CAC ACG CAC ACA TAT TTG TAC ATT TGT GAG-3’; *BsrGI* site shown in bold) and 19 (5’-5’-CTC ACA AAT GTA CAA ATA TGT GTG CGT GTG-3’; *BsrGI* site shown in bold) encoding the desired substitution (underlined in primer sequences). The sequences of the coding regions of all vectors were verified by ACGT, Inc. (Wheeling, IL).
Construction of Strains to Over-express His₆-affinity-tagged Ct R1 and R2\textsuperscript{37} in E. coli. Each constructed vector was used to transform BL21(DE3) cells to generate the over-producing strain for the His₆-affinity-tagged protein. The appended affinity tag has a full amino acid sequence of MGS₂H₆S₂GLVPRGSH and is fused to the N-terminal methionine residues of each protein.

Over-expression and Purification of R1 and R2 Proteins. Cultures of the over-producing strains were grown at 37 °C in rich LB broth (containing 35 g/L tryptone, 20 g/L yeast extract, 5 g/L NaCl) supplemented with 0.05 g/L kanamycin to an OD₆₀₀ of 0.7-1.0. They were cooled rapidly to 18 °C by incubation on ice for 15 min, induced by addition of IPTG (50 µM for R1, 200 µM for R2), and grown for 16-18 h at 18 °C. The cells were harvested by centrifugation and the cell pellet was frozen in liquid N₂ and stored at -80 °C. A typical yield was ~ 15 g of wet cell paste per liter of culture.

The frozen cell paste was thawed in 5 mL/g 50 mM Tris-HCl buffer (pH 7.6) containing 10% (v/v) glycerol (buffer A) with 10 mM imidazole and 0.25 mM PMSF. The cells were lysed as previously described (2). The lysate was centrifuged at 10,000g for 20 minutes. The supernatant was stirred gently with Ni-NTA agarose (Qiagen; ~ 1 mL of resin per 10 mL of supernatant). The slurry was then loaded into a column. The column was washed with buffer A containing 20 mM imidazole (four column volumes), followed by buffer A containing 250 mM imidazole (four column volumes) to elute the protein. Fractions containing the desired protein, as identified by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) with coomassie

\textsuperscript{37} The procedures for overexpression, purification and reconstitution for wild-type Ct R2 and its variants are the same unless specified.
staining, were pooled (typically, a total of three column volumes). The pooled fractions were concentrated to approximately ~ 90 mg/mL in an Amicon ultrafiltration cell with a YM30 membrane. The proteins were judged to be ~ 95% pure by SDS-PAGE with coomassie staining.

R1 was dialyzed to equilibrium against 2 L of 100 mM HEPES buffer (pH 7.6), 10% (v/v) glycerol (buffer B) containing 10 mM EDTA (to remove contaminating manganese) followed by two changes of buffer B to remove the EDTA. R2 was subjected to the procedure to remove iron and manganese described in the following paragraph.

Removal of Metal Ions from R2. R2 emerges from this purification procedure with ~ 0.75 equiv (per monomer) of bound Fe (or ~0.3 equiv for variants W51F), as determined by a previously described colorimetric assay based on the ferroin chelator, ferrozine (3). The bound iron was reduced to FeII by a ~ 1 h incubation with sodium dithionite (6 mM) and methyl viologen (20 μM) at room temperature in the absence of O2. The blue color of the reduced form of methyl viologen served as a redox indicator. A ten-fold molar excess (with respect to iron) of ferrozine was added, and the intense purple color of the FeII-(ferrozine)3 complex developed over several seconds to a few minutes. At this point, exclusion of O2 was no longer necessary. The protein was separated from the FeII-(ferrozine)3 complex by chromatography on Sephadex G-50 (GE Healthcare). Fractions containing the R2 protein (as judged by their absorption spectra) were pooled, and the volume of the pool was reduced to give [R2] > 80 mg/mL. This solution could be frozen in liquid N2 and stored at − 80 °C. Recovery of the R2 was typically ~ 70% in the chelation step. After chelation, the iron content was ~ 0.02 equiv. To remove trace manganese, the protein was dialyzed to equilibrium against buffer B containing 10 mM EDTA. It was then
dialyzed to equilibrium against two changes of buffer B to remove the EDTA.

**Determination of Protein Concentrations.** The concentrations of metal-depleted proteins were determined by measuring the absorbance at 280 nm by assuming monomeric molar absorptivities ($\varepsilon_{280}$) of 138,660 M$^{-1}$cm$^{-1}$ for truncated R1, 57,750 M$^{-1}$cm$^{-1}$ for the R2 wt and E89D, 56,470 M$^{-1}$cm$^{-1}$ for the Y222F and Y338F, 52,060 M$^{-1}$cm$^{-1}$ for the W51F, and 60,850 M$^{-1}$cm$^{-1}$ for the F127Y as calculated by the method of Gill and von Hippel (4).

**Preparation of Mn$^{II}$/Fe$^{II}$-Ct R2 Samples.** O$_2$ was removed from protein samples as previously described (5) and all the following procedures were carried out in an MBraun anoxic chamber. Mn$^{II}$ stock solution was prepared by dissolving MnCl$_2$•4H$_2$O into 5mM H$_2$SO$_4$ solution. Fe$^{II}$ stock solution with natural abundance Fe was prepared by dissolving commercial Fe(NH$_4$SO$_4$)$_2$•6H$_2$O into 5mM H$_2$SO$_4$ solution. $^{57}$Fe$^{II}$ enriched (~95%) stock solution was prepared by dissolution of $^{57}$Fe$^0$ in H$_2$SO$_4$, as previously described (6). Two different strategies for metal addiction were applied. For experiments in Chapter 2 before the EXAFS section, two metal stock solutions were pre-mixed in the desired ratio, and the metal mixture was then added to the metal-depleted R2. For all the other experiments, the Mn$^{II}$ was always added before the slow addition of the Fe$^{II}$ (usually over the period of 1-2 min).

**Preparation of Mn$^{IV}$/Fe$^{III}$-Ct R2 Samples.** For experiments in Chapter 2 before the EXAFS section, Mn$^{IV}$/Fe$^{III}$-Ct R2 was prepared by mixing of Mn$^{II}$/Fe$^{II}$-Ct R2 solution with an O$_2$-containing buffer at 5 °C for 30 min. Mössbauer analysis showed that it typically generated a mixture of ~75% Mn$^{IV}$/Fe$^{III}$ cluster and 25% Fe$^{III}$/Fe$^{III}$ cluster in wt reaction. To favor the generation of the heterobinuclear form, an alternative strategy was applied in other experiments.
To an air-saturated solution of 370 μM (monomer concentration) apo R2 at 5 °C were added 1.5 equiv of Mn^{II} and 5 mM sodium ascorbate. Fe^{II} (0.75 equiv per monomer) was added slowly over a period of 20 min. After 1 h at 5 °C, unbound metal was removed by dialysis against 10 mM EDTA in buffer B. The EDTA was then removed from the protein by dialysis against buffer B twice. Removal of > 95% of the free Mn^{II} was verified by EPR spectroscopy. Mössbauer analysis indicated the presence of ~85%-90% of the active Mn^{IV}/Fe^{III} form and ~10% of the inactive Fe^{II/III} form in the product of wt reaction. This ratio can vary a lot in the variant E89D due to its intrinsic properties.

Preparation of Mn^{III}/Fe^{III}-R2. Mn^{IV}/Fe^{III}-R2 was reduced in an MBraun anoxic chamber with 1.2 molar equiv (2.4 electron equiv) of sodium dithionite for 60 min and subsequently used for the kinetic and spectroscopic experiments. The Mn^{III}/Fe^{III}-R2 used for the activity assays was prepared by treatment of the active protein with 7.5 molar equiv of sodium dithionite to completely eliminate activity. EPR spectroscopy showed that > 80% of the protein was converted to the one-electron-reduced Mn^{III}/Fe^{III} state (i.e, that less than 20% was further reduced by the excess dithionite).

Measurement of the Catalytic Activity of Ct R2 with Excess R1. The activity assays were carried out in a total volume of 0.30 mL with 1.0-80 μM R2 monomer (varied to give reliable quantitation throughout the range of observed R2 activities), 10 R1/R2, 2.5-5.0 ATP/R1, 2 mM CDP, 10 mM DTT, 12 mM magnesium acetate, and 1 mM EDTA in 20 mM Na-Hepes buffer, pH 7.6 (buffer C). Reactions were initiated by addition of Mn^{II}/Fe^{II}-Ct R2 or Mn^{IV}/Fe^{III}-Ct R2 (depends on different purposes) into the air-saturated assay solution containing R1, DTT, ATP
and CDP and terminated by addition of HCl to a final concentration of 100 mM. Precipitated protein was removed by filtration through a Microcon YM-3 (Millipore Corporation). A 10 µL aliquot of the filtrate was injected with a mobile phase of 10% acetonitrile/90% water/0.5 mM HCl running at 0.05 mL/min onto a Waters Micromass ZQ 2000 mass spectrometer (Milford, MA) with an electrospray ionization probe operating in the negative ion mode. Spectrometer conditions were: capillary voltage, 4.00 kV; cone voltage, -50 V; extractor voltage, -2 V; RF lens voltage, 0 V; source temperature, 80 °C; desolvation temperature, 450 °C; desolvation gas flow, 150 L/h; cone gas flow, 60 L/h. The ion currents at m/z = 402 and m/z = 386 (M− for CDP and dCDP, respectively) were continuously and simultaneously monitored after injection. Triplicate injections of each reaction sample were performed. The ratio of the heights of the CDP and dCDP peaks to the sum of these peak-heights was multiplied by the initial concentration of CDP (2 mM) to give the concentrations of substrate and product in each reaction sample. Validation of the assay method is provided in Figures B-1, B-2 and B-3.

**Figure B-1:** Validation of the mass-spectrometric method for quantification of CDP (substrate) and dCDP (product) in the activity assays. Samples containing all components of the *C. trachomatis* RNR reaction except the Δ(1-248) R1 (Δi) and CDP were constituted. CDP and dCDP were added at varying mole fractions to a final [CDP + dCDP] of 2 mM. The samples were then analyzed as activity assay samples ("quenched" with acid, filtered, and injected on the mass spectrometer; see Materials and Methods for details) to obtain experimental quantification of CDP and dCDP (y-axis) to compare to the known values (x-axis). The solid line is a "fit" with a slope of 1.0. In a related experiment, samples containing all components but R2 were constituted. To one of
these samples, dCDP was added to 2 mM (equal to the [CDP]), and the sample was subjected to the analysis. A mole fraction dCDP of 0.47 ± 0.04 was obtained in the triplicate measurements (theoretical 0.50). To a second of these samples, R2 was added to initiate turnover, and the reaction was allowed to proceed to completion. After termination with acid, CDP was added to 2 mM (equal to the initial [CDP]), and the sample was subjected to the analysis. A mole fraction dCDP of 0.50 ± 0.03 was obtained in the triplicate measurements (theoretical 0.50).

**Figure B-2:** Verification of the linear dependence of dCDP produced on the reaction time even at high fractional conversions (> 0.6). This particular experiment was performed in 2006, early in our studies of Ct RNR, before its Mn-dependence had been recognized. The R2 was used as-isolated from *E. coli* [i.e., it had not been activated as in our later work by metal chelation followed by reaction with MnII, FeII and O2] at a [R2 monomer] of 20 µM. The solid line is a linear fit, giving v/[R2 monomer] (turnover number) of 0.038 s⁻¹ (37 °C).

**Figure B-3:** Verification of the linear dependence of turnover velocity on [R2]. These experiments were carried out over many months on several different preparations of R2, which had all been activated by the metal-chelation and MnII/FeII/O2 activation treatments. For the assays at 37 °C (circular points, solid fit line), the points shown are for 1 and 20 µM R2 monomer (in addition to 0 µM) and give values of v/[R2] (turnover number) of 0.60 and 0.58 s⁻¹, respectively. For 22 °C (square points, dashed fit line), the points shown are for 4, 20, and 40 µM R2 monomer and give v/[R2] = 0.35, 0.40, and 0.40 s⁻¹, respectively. Time dependence was not explicitly interrogated in these experiments. Rather, a single reaction time appropriate to give 20-60% conversion was selected for each combination of [R2 monomer] and temperature.

**Quantification of Hydrogen Peroxide.** The H₂O₂ concentration was determined by a spectrophotometric assay based on the oxidation of 4-aminoantipyrine catalyzed by horseradish peroxidase (HRP) with H₂O₂ as oxidizing co-substrate (7). The product of this oxidation reacts with vanillic acid and forms a red quinone imine dye, which exhibits a broad absorption band...
centered at 490 nm with molar absorptivity of 6990 M\(^{-1}\) cm\(^{-1}\). The assay mixture was composed of 40 U/ml HRP, 5 mM 4-aminoantipyrine and 10 mM vanillic acid in 0.2 M MOPS buffer (pH 7.5) containing 0.2 M sodium chloride. Addition of a solution for which \([\text{H}_2\text{O}_2]\) was to be determined initiated the reaction. Development of the red color was complete in seconds.

A 30% (v/v, I assume) \(\text{H}_2\text{O}_2\) stock solution (EMD, Gibbstown, NJ) was diluted 1000-fold with the standard R2 protein buffer (100 mM Hepes, 10% glycerol, pH 7.6). To mimic how the \(\text{H}_2\text{O}_2\) solution is treated in stopped-flow and freeze quench experiments, this solution was allowed to stand at room temperature for one hour prior to its assay for \([\text{H}_2\text{O}_2]\). The measured concentration of \(\text{H}_2\text{O}_2\) stock was 8.3 M, similar to the theoretical concentration (8.8 M).

**Preparation and Quantification of Peroxynitrite.** Peroxynitrite (PN) stock was purchased from EMD (Gibbstown, NJ). It is provided in 4.7% sodium hydroxide at a nominal concentration of 160-200 mM. Fresh-made manganese dioxide was used to remove the contaminating \(\text{H}_2\text{O}_2\). The MnO\(_2\) was prepared as previously described. An 8 g portion of potassium permanganate was dissolved in 50 ml water. A 500 ml aliquot of 95% ethanol was added slowly (over ~20 minutes) and the reaction was stirred overnight. The dark brown precipitate was collected the next day by vacuum filtration and washed with 3 L of water. It was then dried under vacuum overnight before use. To decompose contaminating \(\text{H}_2\text{O}_2\) in the PN stock solution, 0.2 g of MnO\(_2\) was incubated with 2 ml of PN stock for 15 min on ice. The MnO\(_2\) was removed by filtration. The concentration of PN was determined by its absorption at 302 nm (molar absorptivity of 1670 M\(^{-1}\)cm\(^{-1}\)). After removal of \(\text{H}_2\text{O}_2\) by the MnO\(_2\) treatment, [PN] was measured to be 195 mM and residual \([\text{H}_2\text{O}_2]\) was determined to be 6.0 mM by the assay described above. Thus, the PN
solution retains a residual 3% H$_2$O$_2$ contamination, relative to PN concentration. This stock solution was divided into aliquots and stored at -80 °C until needed. After thawing, any unused PN stock was discarded (i.e., not used again).

**Stopped-flow Absorption Spectroscopy.** The stopped-flow apparatus has been described (5). SF experiments in this thesis were carried out in an Applied Photophysics (Surrey, U.K.) SX18.MV apparatus housed in the MBraun anoxic chamber and equipped with either photodiode array detector (with the white light source) or photomultiplier detector (with the monochromatic light source). It typically gives the dead time of ~ 1.3 ms. The experiments were carried out either at 5 °C or ambient temperature as indicated in the figure legends. The analysis of data was described in the text.

**Freeze-quench Experiments.** Same as described before (8), the rapid FQ instrument used in this thesis is from Update Instruments (Madison, MI) with a Model 715A computer controller, which typically gives the minimum quenching time of ~ 20 ms.

**EPR Spectroscopy.** EPR spectra were recorded on an ESP300 spectrometer from Bruker (Billerica, MA) equipped with an ER 041 MR Microwave Bridge and a 4102ST X-band Resonator from Bruker as described before (5).

**Simulation of the EPR Spectra of Mn$^{IV}$/Fe$^{IV}$- and Mn$^{III}$/Fe$^{III}$-R2.** The simulations are based on the commonly used spin Hamiltonian formalism (9) and were carried out with respect to the total spin of the electronic ground state, $S_{\text{Total}} = 1/2$. For simulation of the EPR spectra, Equation B-1 was used. The first term is the electron Zeeman effect and the second and third terms are the
hyperfine couplings between the electron spin and the $^{55}$Mn nuclear spin ($I = 5/2$) and between the electron spin and the $^{57}$Fe nuclear spin ($I = 1/2$), respectively.

\[
\hat{H} = \beta \mathbf{S} \cdot \mathbf{g} \cdot \mathbf{B} + \mathbf{S} \cdot \mathbf{A}_{\text{Mn}} \cdot \mathbf{I}_{\text{Mn}} + \mathbf{S} \cdot \mathbf{A}_{\text{Fe}} \cdot \mathbf{I}_{\text{Fe}}
\]  

(B-1)

The program SimFonia (Bruker, Billerica, MA) was used to simulate EPR spectra by the second-order-perturbation method for powder spectra. The full-matrix diagonalization program, SIM, which was written by Høgni Weihe (University of Copenhagen) (10), was used to simulate the spectra by an independent method to verify the parameters obtained with SimFonia.

**Mössbauer Spectroscopy.** As described before (5), Mössbauer spectra were recorded on spectrometers from WEB research (Edina, MN) operating in the constant acceleration mode in a transmission geometry. Spectra were usually recorded with the temperature of the sample maintained at 4.2 K unless specified. For low-field spectra, the sample was kept inside a SVT-400 dewar from Janis (Wilmington, MA). The magnitudes of the static magnetic fields for the low-field Mössbauer spectra were determined using a Digital Tesla meter (model 132D) with a Hall probe LPT 130-20S (Group3 Technologies Inc., Auckland, NZ). For high-field spectra, the sample was kept inside a 12SVT dewar (Janis), which houses a superconducting magnet that allows for application of variable magnetic fields between 0 and 8 T parallel to the $\gamma$-beam. The isomer shifts quoted are relative to the centroid of the spectrum of a metallic foil of $\alpha$-Fe at room temperature. Details of reaction and spectroscopy conditions are provided in the appropriate figure legend or in the figure itself.

**Simulation of the Mössbauer Spectra.** For simulation of the Mössbauer spectra, the program WMOSS (Web Research, Edina, MN) was used. All simulations were carried out with the
assumption that the fluctuation rate of the electron spin is slow compared to the $^{57}$Fe Larmor frequency. The details for analysis were described in the text.

**Derivation of Kinetic Equations.** The reaction of a single irreversible step $A \rightarrow B$ with the first-order rate constant $k_1$ is governed by the first order differential equation:

$$-\frac{d[A]}{dt} = k_1 \cdot [A]$$

which gives:

$$[A]_t = [A]_0 \cdot \exp (-k_1 \cdot t)$$

For a reaction composed of two irreversible steps $A \rightarrow B \rightarrow C$ with the first-order rate constant $k_1$ and $k_2$, respectively:

$$\frac{d[B]}{dt} = k_1 \cdot [A]_t - k_2 \cdot [B]_t = k_1 \cdot [A]_0 \cdot \exp (-k_1 \cdot t) - k_2 \cdot [B]_t$$

which gives:

$$[B]_t = \left[ \frac{k_1}{k_2 - k_1} \right] \cdot [A]_0 \cdot \exp (-k_1 \cdot t) + C \cdot \exp (-k_2 \cdot t)$$

when $t = 0$, $[B]_0 = 0$

$$\Rightarrow C = - \left[ \frac{k_1}{k_2 - k_1} \right] \cdot [A]_0$$

Therefore,

$$[B]_t = \left[ \frac{k_1 \cdot [A]_0}{k_2 - k_1} \right] \cdot \left[ \exp (-k_1 \cdot t) - \exp (-k_2 \cdot t) \right]$$
References


Appendix B: Abbreviations

5′-dA•: 5′-deoxyadenosyl radical
AdoCbl: 5′-deoxyadenosylcobalamin
AdoMet: S-adenosylmethionine
ATP, adenosine-5′-triphosphate
CDP, cytidine-5′-diphosphate
Ct: Chlamydia trachomatis
C•: cysteinyI radical
dCDP, 2′-deoxycytidine-5′-diphosphate
dNDP, 2′-deoxyribonucleoside 5′-diphosphate
DFT: density functional theory
DT: dithionite
DTT: dithiothreitol
Ec: Escherichia coli
EDTA: ethylenediamine-tetraacetate
ENDOR: electron-nuclear double resonance
EPR: electron paramagnetic resonance
ET: electron transfer
EXAFS: extended X-ray absorption fine structure
FQ: freeze-quench
G•: glycyl radical
HU: hydroxyurea
MCD: magnetic circular dichroism
N3-ADP: 2′-azido-2′-deoxyadenosine-5′-diphosphate
NDP: ribonucleoside diphosphate
NHE: normal hydrogen electrode
NTP: ribonucleoside triphosphates NTP
PCET: proton-coupled electron transfer
PELDOR: pulse electron-electron double resonance
PT: proton transfer
RO(N)S: reactive oxygen(nitrogen) species
RNR: ribonucleotide reductase
SF: stopped-flow
sMMOH: soluble methane monooxygenase hydroxylase
wt: wild-type
Y•: tyrosyl radical
ZFS: zero-field splitting
Appendix C: Publications
Paper I
Permissions Letter

DATE: Wednesday, September 24, 2008

TO: Wei Jiang
the Pennsylvania State University
Dept. of Biochem. and Molec. Biol.
University Park, PA 16802
United States

FROM: Elizabeth Sandler, Rights and Permissions

RE: Your request for permission dated 09/23/08 (submission id 37498) regarding Wei Jiang et al., SCIENCE 316:1188-1191 (25 May 2007)

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Science 316, 1188 (2007);
DOI: 10.1126/science.1141179

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A Manganese(IV)/Iron(III) Cofactor in Chlamydia trachomatis Ribonucleotide Reductase

Wei Jiang,† Danny Yun,†,§ Lanah Saleh,†,‡ Eric W. Barr,† Gang Xing,† Lee M. Hoffart,† Monique-Anne Maslak,∥ Carsten Krebs,⊥⊥ J. Martin Bollinger Jr.⊥⊥⊥

In a conventional class I ribonucleotide reductase (RNR), a diiron(III/IV) cofactor in the R2 subunit reacts with oxygen to produce a diiron(III/IV) intermediate, which generates a stable tyrosyl radical (Y*). The Y* reversibly oxidizes a cysteine residue in the R1 subunit to a cysteinyl radical (C•), which abstracts the 3'-hydrogen of the substrate to initiate its reduction. The RNR from Chlamydia trachomatis lacks the Y*, and it had been proposed that the diiron(III/IV) complex in R2 directly generates the C* in R1. By enzyme activity measurements and spectroscopic methods, we show that this RNR actually uses a previously unknown stable manganese(IV)/iron(III) cofactor for radical initiation.

Ribonucleotide reductases (RNRs) provide all organisms with 2' deoxyribonucleotides for DNA synthesis (1, 2). All known RNRs are thought to initiate ribonucleotide reduction by using a cysteine thiol radical to abstract the hydrogen atom from the 3'-carbon (3, 4). Three distinct strategies to generate the initiating cysteinyl radical (C*) have been described and are, in part, the basis for division of the RNRs into three classes. Class II and III RNRs use strategies involving the 5'-deoxyadenosyl radical, generated either by homolysis of the Co-C bond of 5'-deoxyadenosylcobalamin (class II) or by reductive cleavage of the 5'-C=S-bond of S-adenosyl-L-methionine by a separate acti vating protein (class III), as the ultimate oxidant for cysteine activation. The 5'-deoxyadenosyl radical either generates the C* directly (class II) or generates a stable glycol radical (G*) that reversibly oxidizes the cysteine (class III) (3, 5, 6). In a conventional class I RNR (e.g., from Homo sapiens, Saccharomyces cerevisiae, or aerobic Escherichia coli), a binuclear iron center in its cofactor subunit, R2, reacts with oxygen to oxidize a tyrosine residue by one electron to a stable tyrosyl radical (Y*). The Y* in R2 generates the C* in the catalytic subunit, R1, where nucleotide reduction occurs (7).

An unexpected adaptation of the class I functional architecture was revealed by the recent characterization of the RNR from the bacterium Chlamydia trachomatis (8), an obligate intracellular parasite and important human pathogen. The presence of a phenylalanine in place of the tyrosine residue in R2 that normally harbors the essential initiating Y* was revealed first by sequence comparisons (8) and subsequently by x-ray crystallography (9). Consistent with these findings, no evidence for a Y* was found in biochemical studies (9–11). Sequences of R2 genes from other organisms revealed that the absence of the Y* is not specific to the chlamydial RNRs (9). Notably, the presence of genes encoding such R2 proteins in the genomes of other pathogens (e.g., Mycobacterium tuberculosis) suggested that the novel RNRs might have arisen as an adaptation to the host's immune response (9) and might present specific targets for design of new antibacterial drugs.

To explain how the C. trachomatis RNR can function without the essential Y*, Nordlund, Gräslund, and co-workers suggested that an Fe2Ill-R2 complex was sufficient (11). Although the reported induction and stabilization of the Fe2Ill-R2 complex were consistent with the earlier Nordlund/Gräslund hypothesis, the fact that this form was never enriched to greater than ~30% of the total protein (10) left open the possibility that a different form was responsible for the modest activity observed. After preparing His6-affinity-tagged forms of C. trachomatis R2 and the N-terminally truncated At1 to 248-R2 (12) reported by McClarty and co-workers to be more stable than full-length R1 (8), we noted a distinct lack of correlation between the catalytic activities and iron contents of different preparations of R2 (13).

By reductive chelation of iron from the purified protein and subsequent dialysis against ethylenedinitritotetraacetate (EDTA) (12), R2 was isolated with less than 0.02 equivalents (equiv) iron (I4) and very low catalytic activity [velocity (v)/[R2] ≤ 0.01 s⁻¹] (15). The metal-depleted R2 so obtained was not detectably activated by addition of 2 equiv of Fe5. By contrast, addition of both Fe5 and MnIII was found to activate the metal-depleted R2 by a factor of more than 50 (Fig. 1). A MnIII:Fe5 ratio of unity gave maximal activation (Fig. 1A), and a total of two divalent metal ions per monomer was sufficient for ~85% of maximal activation (Fig. 1B).

The results in Fig. 1, in particular the 1:1 Mn:Fe ratio giving maximal activity, suggest the use of a MnFe cofactor rather than a Fe2 cofactor by C. trachomatis RNR. Because the raison d'être of the R2 subunit and its metallocofactor is to transiently oxidize the cysteine residue in R1 by one electron (7), the fully reduced complex formed upon addition of the divalent metal ions to the protein (MnIII:Fe5-R2) cannot be active but, by analogy with other class I RNRs, might react with O2 to produce an oxidized state that functions in catalysis. Indeed, we observed no turnover after addition of an O2-free solution containing R2, MnII, and Fe5 (0.75 equiv
each metal) to an O₂-free RNR reaction solution, but we did observe activity when the assay solution to which the MnIV/FeIII-R2 was added contained O₂ and when the MnIV/FeII-R2 solution was first exposed to O₂ before it was added to the assay solution. In the latter case, activity did not require O₂ in the assay solution, indicating that previous exposure of the MnIV/FeII-R2 to O₂ activates the subunit.

The active form exhibits no obvious electron paramagnetic resonance (EPR) signal (in X-band, perpendicular mode). A sample prepared with 0.5 equiv Fe and 1.0 equiv Mn (to disfavor formation of Fe₃/III/III product) exhibits a Mössbauer quadrupole doublet at 4.2 K and zero field (Fig. 2A). The parameters [isomer shift (δ) = 0.52 mm s⁻¹; quadrupole splitting parameter (ΔE₂) = 1.32 mm s⁻¹] establish that the iron site is converted to FeII by the reaction with O₂. The sharp [line width (σ) = 0.30 mm s⁻¹] doublet in zero magnetic field and marked broadening in a weak (53 mT) field (Fig. 2B) reveal that this complex has a paramagnetic ground state with an integer value of the total electron-spin quantum number, Stotal. This characteristic distinguishes the C. trachomatis MnFeII/II-R2 product from the previously characterized product Fe₂III/III clusters in other R2 proteins, which are diamagnetic (S_total = 0) as a result of antiferromagnetic coupling between the two S = 5/2 Fe₃III ions. The presence of a Mn ion coupled to the FeIII site is demonstrated by the EPR spectra of the one-electron reduced form of the complex produced by a brief (~2 min at 22°C) treatment with 20 mM dithionite. These spectra show hyperfine coupling to both an I (nuclear spin quantum number) = 5/2 ⁵²Mn nucleus (Fig. 3A) and an I = 1/2 ⁵⁷Fe nucleus (compare Fig. 3, A and B). The EPR spectra establish that the reduced form has a coupled Mn/Fe cluster with S_total = 1/2. The Mössbauer spectrum of the reduced complex acquired at zero field and 190 K (Fig. 2C) is a broad (Γ ~ 0.5 mm s⁻¹) quadrupole doublet (16) with parameters (δ = 0.43 mm s⁻¹, ΔE₂ = 0.81 mm s⁻¹) that indicate that the Fe site remains in the +III oxidation state. Thus, the dithionite treatment reduces the Mn site but not the FeIII site of the active state. The reduced Mn site must have an even number of valence electrons for coupling with the odd-electron FeIII site to give S_total = 1/2. MnIII (S = 2) is the only chemically reasonably possible possibility, establishing that the Mn in the active state is +IV. Most likely, antiferromagnetic coupling between the MnIV (S Mn = 3/2) and high-spin FeIII (S Fe = 5/2) ions gives S_total = 1, consistent with the observed Mössbauer characteristics (broadening in a 53-mT magnetic field) of the active form.

The EPR spectrum at 4 K of the MnIV/FeIII-R2 is perturbed in the presence of R1, cytidine 5'-diphosphate (CDP), and adenosine 5'-triphosphate (ATP) (compare Fig. 3, A and C). This observation indicates that binding of R1 to MnIV/FeIII-R2 (and possibly binding of the nucleotides to the R1-R2 complex) affects the structure of the buried cofactor, a phenomenon not previously observed in a class I RNR. The spectra at 14 K (Fig. 3, D to G, solid curves) are particularly sharp and featured, and show hyperfine coupling to ⁵⁷Fe (compare Fig. 3, D and E) as well as ⁵²Mn. By simulation of the spectrum of the ⁵⁷Fe-containing sample (Fig. 3D, dashed curve), the g-tensor of the S = 1/2 ground state (2.030, 2.020, 2.015) and the ⁵²Mn hyperfine coupling tensor, A_Mn [269, 392, 314] MHz, were determined. The marked anisotropy of A_Mn is consistent with the assigned +III oxidation state (17, 18). Additional evidence for the +III iron valence is provided by the ⁵⁷Fe hyperfine coupling (Fig. 3E, solid curve), which can be reproduced (Fig. 3E, dashed curve) with an isotropic A_Fe [−64.5, −64.5, −64.5] MHz typical of high-spin FeIII (19).

The substrate analog, 2'-azido 2'-deoxyadenosine 5'-diphosphate (N₃-ADP), was used to confirm the conclusion that the MnIV/FeIII cluster is the functional cofactor. It has been shown that treatment of a class I or class III RNR with a 2'-azido–substituted nucleotide results in irreversible loss of the C• generator (20, 21) as a result of aberrant reactions, beginning with loss of the azido moiety (either as N₃₋ or N₃) from the initial 3'-centered radical (22–24). Thus, the Y• or G• is irreversibly reduced (20, 21) instead of being regenerated, as it is at the end of a normal turnover. We predicted from these precedents that treatment of C. trachomatis RNR should lead to irreversible conversion of the EPR-silent MnIV/FeIII cluster to the EPR-active MnIII/FeIII state. Indeed, treatment with N₃-ADP generates the same EPR signal seen upon dithionite reduction of MnIII/FeIII-R2 in the presence of R1, CDP, and ATP (Fig. 3, F and G). This signal does not develop when the inactivator is replaced by the natural substrate, CDP. The additional features in the 3330 to 3420 G region (marked by arrows; see also fig. S1) that are not part of the spectrum of MnIII/FeIII-R2-R1 (dashed simulations) are attributable to the nitrogen-centered radical previously shown to accumulate during N₃-NDP-mediated inactivation of conventional class I RNRs (25–26). The formation of the MnIV/FeIII cluster and free radical upon reaction with N₃-ADP confirms the activity of the MnIV/FeIII cluster.

Scheme 1 summarizes our working hypothesis for how the C. trachomatis RNR functions without a Y• initiator. By using manganese in place of one of the ions of the conventional R2 metal center, C. trachomatis R2 is able to generate an oxidized cluster that possesses both kinetic stability and sufficient oxidative potency to generate the C• in R1 when triggered to do so by the protein(s). The marked perturbation to the EPR signal of the MnIV/FeIII cluster caused by binding of R1 (and perhaps nucleotides) provides a tool not present in conventional class I RNR systems for investigating the triggering process.

We attempted to reconcile our conclusion that MnIV/FeIII-R2 is the active form with previous observations suggesting that Fe₃III/IV-R2 is active (9–11). We were unable to reproduce the reported induction of the Fe₃III/IV complex from Fe₂III/III R2 under turnover conditions (11), even though we did verify that turnover was occurring (at 4 to 5% of the rate of the Mn/Fe-activated R2). Addition of a solution containing R2 and 1.5 equiv FeIII to a solution containing O₂, R1 (2 equiv relative to R2), CDP, and ATP at 22°C did, as previously reported by the Gräslund group (10), result in generation and stabilization
of the Fe$_2^{III/IV}$ cluster (0.15 equiv that decayed by less than 10% over 10 min) (see fig. S2). However, quantification of the 2'-deoxy-CDP (dCDP) product from these samples showed that turnover was occurring at the same rate in the samples that contained the Fe$_2^{III/IV}$ complex as in the identical samples (prepared with Fe$_2^{III/III}$-R2) that lacked detectable levels of this complex. In other words, we find that the activity does not correlate with the Fe$_2^{III/IV}$ content of the R2. Moreover, replacement of CDP with N3-ADP in the experiment giving the stabilized Fe$_2^{II}$ active MnIV/FeIII-R2 (cluster by dithionite reduction of MnIII/FeIII-R2 might result from a Mn contaminant present in the stabilization buffer). This complex confirms the incompatibility of the Fe$_2^{III/IV}$ cluster for radical initiation.

Suspecting that the modest activity that we and others (8–11) observe without adding MnII to R2 might result from a Mn “contaminant” (in R2 or another assay component), we tested for development of the very distinctive EPR signal of the MnIII/FeIII-R2•R1 complex after treatment of our putatively iron-only protein with either dithionite or N3-ADP in the presence of R1 and ATP. Indeed, in both cases, the spectrum was readily detected (fig. S3A). Its intensity relative to that exhibited by the purposefully generated MnIII/FeIII-R2 sample (~4 to 5%) (compare to fig. S3B) correlated with the ratio of enzymatic activity of the two R2 forms.

We suggest that the activity previously attributed to the Fe$_2^{III/IV}$-R2 complex (9–11) resulted from a MnIII/FeIII-R2 contaminant that escaped detection. First, Fig. 1A shows that only ~0.2 equiv contaminating Mn would have been required to give the maximum R2 activity previously reported (230 nmol min$^{-1}$ mg$^{-1}$, $v_2/R2$ = 0.155 s$^{-1}$) (10). Whereas this quantity is five times the ~0.04 equiv detected in our preparations, the different methods of subunit preparation, and perhaps different enzyme concentrations employed in the activity determinations [not reported in (10) but expected to have been lower than in our assays because of their use of the more sensitive radiometric assay], could have resulted in a greater Mn-R2 equivalency in the previous study. Second, much lower activities of <75 nmol min$^{-1}$ mg$^{-1}$, $v_2/R2$ < 0.05 s$^{-1}$, similar to the activities of some of our preparations of R2 before activation with MnIII and FeIII, were reported in the subsequent study by the same group (11). This large variation in specific activity seems inconsistent with the authors’ belief that addition of only FeII should have fully activated R2 but could be explained by a variable Mn contaminant in their preparations. If MnII had been present, R2 would [as reported in (10)] have been activated by addition of only FeII in the presence of O$_2$, but the extent of activation would have depended on the quantity of the contaminating Mn. Third, by contrast to all other (conventional) class I RNRs, the active R2 state has no obvious EPR signal. Thus, if MnII had been present, addition of FeII in the presence of O$_2$ would have trapped the Mn in an EPR-silient state, allowing it to escape detection by the reported EPR experiments (10, 11). We suggest that these considerations can reconcile all previous observations with our proposal that MnIII/FeIII-R2 is the sole active form.

The demonstrated in vitro activity of the MnIII/FeIII form of C. trachomatis R2 (produced in E. coli) strongly suggests that this previously unknown cofactor functions in vivo in the pathogen’s RNR. It is possible that other metals might substitute for Mn (or Fe) in vivo, but the subtlety of the cofactor’s function (gated electron transfer by a stable high-valent complex) makes this possibility remote. The results raise the questions of why and in what other species this distinctive radical-initiation strategy evolved. Given that the RNR subunits from C. trachomatis and other chlamydiae are more similar in sequence to their counterparts from mammalian species such as H. sapiens and Mus musculus than to the subunits from other bacterial RNRs (with the exception of those from the opportunistic human pathogen, Pseudomonas aeruginosa) (8), it seems likely that the parasitic bacteria co-opted their host’s RNR genes and then evolved them for selective advantage. Without knowledge of the manganese requirement, Nordlund, Gräslund, and co-workers suggested that this selective advantage might have been increased resistance to oxidants (e.g., superoxide and nitric oxide) produced as part of the host’s immune response (9). This hypothesis, plausible in view of previous evidence that the Y’s of conventional class I RNRs are targets for these chemical defenses (27–29), seems even more attractive in light of our results: The demonstrated substitution of one iron ion by manganese is strikingly reminiscent of the induction of the MnIII/FeIII-R2•R1 silent state, allowing it to escape detection by the reported EPR experiments (10, 11). We suggest that these considerations can reconcile all previous observations with our proposal that MnIII/FeIII-R2•R1 is the sole active form.

**Fig. 3.** X-band EPR spectra illustrating formation of the MnIII/FeIII cluster by dithionite reduction of active MnIV/FeIII-R2 (A to E) or by treatment of the holoenzyme (MnIV/FeIII-R2•R1•ATP) with N3-ADP (F and G). The dithionite reduction was carried out either in the absence [(A) and (B)] or in the presence [(C) to (E)] of R1, CDP, and ATP. For (A), (C), (D), and (F), the R2 samples were prepared with natural-abundance iron (91.7% $^{55}$Fe with $I = 0$); for (B), (E), and (G), $^{57}$Fe-enriched iron (95%, $I = 1/2$) was used. All spectra had a microwave frequency of 9.45 GHz and a modulation frequency of 100 kHz. Other spectrometer conditions were [(A) to (C)] temperature, 4 K (nominal temperature indicated on Oxford cryostat); microwave power, 20 mW; modulation amplitude, 10 G; scan time, 167 s; time constant, 167 ms; resolution of field axis, 1024 points; scans per spectrum, 1; [(D) to (G)] temperature, 14.0 $\pm$ 0.2 K; microwave power, 0.20 mW; modulation amplitude, 4 G; scan time, 334 s; time constant, 167 ms; resolution of field axis, 2048 points; scans per spectrum, 10. The sharp signal at 3380 G is from the spectrometer cryostat and is prominent in (A) to (C) because of the low temperature and high power used for these spectra. In (D) to (G), a minor contribution from free MnII has been removed by subtraction of the spectra of control samples either not treated with dithionite [(D) and (E)] or treated with CDP instead of N3-ADP [(F) and (G)]. The dashed, lighter-colored traces in (D) to (G) are simulations of the spectra of MnIII/FeIII-R2•R1 (12) with the g and A tensors as in the text.

**Scheme 1**

**ACTIVATION**

![Scheme 1](image)

**CATALYSIS**

![Scheme 1](image)
by *E. coli* in response to oxidative stress of a Mn-dependent paralog to the constitutively expressed, Fe-dependent superoxide dismutase (30). Examination of the reactivity of the distinctive Mn/Fe cofactor toward these oxidants may thus provide a biochemical rationale for its evolution.

References and Notes

12. Materials and methods are available as supporting material on Science Online.
13. For example, preparations from *E. coli* cultures grown in rich medium had the same iron content as preparations from iron-supplemented minimal medium (~0.75 equiv; all metal equilibrations and activities are on a per monomer basis) but ~10 times the activity (velocity *v*/[R2] = 0.035 ± 0.01 s⁻¹, compared with 0.003 ± 0.001 s⁻¹). Conversely, R2 from rich medium to which the cell-permissive Fe⁷ chelator, 1,10-phenanthroline, was added immediately before induction of overexpression emerged with much less iron (<0.05 equiv) but ~70% of the activity (*v*/[R2] = 0.025 ± 0.01 s⁻¹) of the R2 from equivalent cultures lacking the chelator.
14. All metal equilibrations and activities for the homodimeric R2 protein are reported on a per monomer basis.
15. To reduce the residual R2 activity to this low level, it was necessary also to dialyze the R1 used in the activity assay against EDTA.
16. The S = 1/2 complex exhibits a magnetically split Mössbauer spectrum at low temperature, but the use of this higher temperature makes electronic relaxation fast compared with the ⁵⁷Fe nuclear precession frequency and collapses the spectrum into a quadrupole doublet.
31. This work was supported by grants from NIH (GM55365 to J.M.B.), the Arnold and Mabel Beckman Foundation (Young Investigator Award to C.K.), and the Camille and Henry Dreyfus Foundation (Teacher Scholar Award to C.K.). The authors thank R. Stevens (University of California, Berkeley) for generously providing *Chlamydia trachomatis* serovar D genomic DNA and J. Stubble (Massachusetts Institute of Technology) for the kind gift of *N₂*-ADP.

Supporting Online Material

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Figs. S1 to S3

References

12 February 2007; accepted 19 April 2007
10.1126/science.1141179

Probing Transcription Factor Dynamics at the Single-Molecule Level in a Living Cell

Johan Elf,¹* Gene-Wei Li,²* X. Sunney Xie³†

Transcription factors regulate gene expression through their binding to DNA. In a living *Escherichia coli* cell, we directly observed specific binding of a lac repressor, labeled with a fluorescent protein, to a chromosomal lac operator. Using single-molecule detection techniques, we measured the kinetics of binding and dissociation of the repressor in response to metabolic signals. Furthermore, we characterized the nonspecific binding to DNA, one-dimensional (1D) diffusion along a short DNA segment, and 3D translocation among segments through cytoplasm at the single-molecule level. In searching for the operator, a lac repressor spends ~90% of time nonspecifically bound to and diffusing along DNA with a residence time of <5 milliseconds. The methods and findings can be generalized to other nucleic acid binding proteins.

In all kingdoms of life, transcription factors (TFs) regulate gene expression by site-specific binding to chromosomal DNA, preventing or promoting the transcription by RNA polymerase. The lac operon of *Escherichia coli*, a model system for understanding TF-mediated transcriptional control (1), has been the subject of extensive biochemical (2–4), structural (5), and theoretical (6, 7) studies since the seminal work by Jacob and Monod (8). However, the in vivo kinetics of the lac repressor, and all other TFs, has only been studied indirectly by monitoring the regulated gene products. Traditionally, this was done on a population of cells (9), in which unsynchronized gene activity among cells masks the underlying dynamics. Recent experiments on single cells allow investigation of stochastic gene expression (10–15). However, direct observation of TF-mediated gene regulation (16) remains difficult, because it often involves only a few copies of TFs and their chromosomal binding sites. Here we report on a kinetics study of how fast a lac repressor binds its chromosomal operator and dissociates in response to a metabolic signal in a living *E. coli* cell.

Single-molecule detection also makes it possible to investigate how a TF molecule searches for specific binding sites on DNA, a central question in molecular biology. Target location by TFs (and most nucleic acid binding proteins) is believed to be achieved by facilitated diffusion, in which a TF searches for specific binding sites through a combination of one-dimensional (1D) diffusion along a short DNA segment and 3D translocation among DNA segments through cytoplasm (17). However, real-time observation in living cells has not been available because of technical difficulties. Here we report on such an investigation, providing quantitative information of the search process.

The lac repressor (LacI) is a dimer of dimers. Under repressed conditions one dimer binds the major lac operator, O1, and the other dimer binds one of the weaker auxiliary operators, O2 or O3 (18) (Fig. 1A). LacI binding to O1 prevents RNA polymerase from transcribing the lac operon (lacZYA). Upon binding of allolactose, an intermediate metabolite in the lactose pathway, or a nondegradable analog, such as IPTG (isopropyl β-D-thiogalactopyranoside), the repressor’s affinity for the operator is substantially reduced to a level comparable to that of nonspecific DNA interaction (19).

To image the lac repressor, we expressed it from the native chromosomal lacI locus as a C-terminal fusion with the rapidly maturing (~7 min) yellow fluorescent protein (YFP) Venus (A206K) (15, 20) (Fig. 1A). The short maturation time prevents the lac operator sites from being occupied by immature fusion proteins. The C-terminal fusion avoids interference with the N-
The Active Form of *Chlamydia trachomatis* Ribonucleotide Reductase R2 Protein Contains a Heteronuclear Mn(IV)/Fe(III) Cluster with S = 1 Ground State

Wei Jiang, J. Martin Bollinger, Jr.,* and Carsten Krebs*

Department of Biochemistry and Molecular Biology and Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

Received April 11, 2007; E-mail: ckrebs@psu.edu

The reduction of nucleotides to 2′-deoxynucleotides by ribonucleotide reductases (RNRs) begins with the abstraction of the 3′-hydrogen atom of the substrate by a transient cysteine thyl radical (C•). Class I, II, and III RNRs differ in subunit composition and the nature of the cofactor used to generate the 3′-H-abstraction C•. A conventional class I RNR uses a stable tyrosyl radical (Y•), which is introduced into the enzyme’s homodimeric subunit (also denoted β2) by reaction of O2 with an adjacent carboxylate-bridged non-heme diiron cluster, to generate the C• in its R1 subunit (also denoted α2) via long-range (≈35 Å) proton-coupled electron transfer (PCET). The identification in *Chlamydia trachomatis* (Ct) and other species of pathogenic bacteria of RNRs having the class I subunit architecture but the Y•-harboring tyrosine replaced by phenylalanine raised the question of how such an RNR might function without the initiating Y•. We recently showed that Ct RNR uses a stable MnIV/FeIII cofactor, generated by reaction of the MnIV/FeII−R2 complex with O2 for radical initiation (Scheme 1). Although heteronuclear MnIV/Fe complexes of various oxidation states, including one example of an inorganic MnIV/FeIII complex, have been reported, Ct RNR is, to our knowledge, the first case in which an enzyme characterizes this novel cofactor by Mössbauer spectroscopy, showing that it has a triplet (Sground state = 1) ground state resulting from antiferromagnetic coupling of its MnIV (Spn = 3/2) and high-spin FeIII (SFe = 5/2) constituents and providing parameters to calibrate calculation of its geometric and electronic structure.

**Scheme 1.** Radical-Generating Cofactor of Ct RNR (left) and Conventional Class I RNR (right)

![Scheme 1](image)

The 4.2 K/zero-field Mössbauer spectrum of a sample prepared by reaction of a solution of R2, MnIII, and FeIII with O2 (Figure 1, hashed marks) comprises two quadrupole doublets with similar isomer shifts (δ) but different quadrupole splittings (ΔEQ). The minor doublet (25% of the total intensity) matches the spectrum of the FeII35 Å) proton-coupled electron transfer (PCET). The other parameters in the simulations are the asymmetry parameter, η, the axial and rhombic zero-field splitting (ZFS) parameters of the Sground state = 1 ground state, D(S=1) and (E/D)(S=1), respectively, and the hyperfine tensor for the FeIII ion, (A(FeβββFe)).

The fact that the overall splitting of the spectrum is larger at 4 T than at 8 T reveals that the electronic Zeeman term dominates the ZFS interaction at these magnetic field strengths. As a consequence, the spin expectation value of the ground state (S) and the internal magnetic field [Bint = \((S)(A(FeβββFe))\)] approach their limiting values (corresponding to (S) = 1) at these applied fields, and the hyperfine tensor can be determined accurately from the spectra. In a spin-coupled cluster, the A-vector for the iron ion with respect to the Sground state = 1 ground state (AFe) is given by eq 1, in which cFe and aFe represent the vector coupling coefficient and the intrinsic hyperfine tensor, respectively.

\[
A_{Fe} = c_{Fe} a_{Fe}
\]  

(1)

For high-spin FeIII sites, (A(FeβββFe)) is dominated by the Fermi contact term and exhibits nearly isotropic values of ~21 T. For the Sground state = 1 state, cFe = +7/4. Thus, (A(FeβββFe)) is expected to be nearly isotropic with values of ~37 T. Indeed, analysis of the spectra yielded a nearly isotropic hyperfine tensor, (A(FeβββFe)) = (~40.2, -38.9, -38.0) T, similar to the expected value.

With the AFe determined from the 4 and 8 T spectra, the ZFS parameters were then determined accurately from the spectra with lesser applied magnetic fields, under which conditions the ZFS is significant compared to the electronic Zeeman term. Simulation of the spectra allowed for determination of the magnetic field dependence of (S), which determines the magnitude of the internal magnetic field and the resultant splitting in each spectrum. From the field-dependent spectra, D(S=1) = −1.9 cm−1 and (E/D)(S=1) = 0.33 were found. D(S=1) is related to the intrinsic D values of the
Mn IV and Fe III ions via eq 2, with the assumption that the total spin states are well separated in energy (i.e., \( J \gg D \)).

\[
D_{\text{S=1}} = 14/S D_{\text{Fe}} + 3/10 D_{\text{Mn}}
\]

(2)

Figure 2. The 4.2 K Mössbauer spectra of Mn IV/Fe III–R2 derived by component analysis of the experimental spectra. The solid lines are simulations according to the spin Hamiltonian given in the Supporting Information and the following parameters: \( S_{\text{Fe}} = 1 \), \( D_{\text{Fe}} = -1.9 \) cm\(^{-1} \), \( E(D)_{\text{Fe}} = 0.33 \), \( \delta = 0.52 \) mm/s, \( \Delta E_{Q} = 1.32 \) mm/s, \( \eta = -2.6 \). \( \Delta (\text{Fe})/\Delta (\text{Fe}) = (-40.2, -38.9, -38.0) \) T.

Mn IV and Fe III ions via eq 2, with the assumption that the total spin states are well separated in energy (i.e., \( J \gg D \)).

\[
D_{\text{S=1}} = 14/S D_{\text{Fe}} + 3/10 D_{\text{Mn}}
\]

(2)

The Mn IV and Fe III ions could conceivably couple ferromagnetically to yield an \( S_{\text{Total}} = 4 \) ground state. However, the inherent anisotropy of \( \langle S \rangle \) of the \( S_{\text{Total}} = 4 \) system precludes simulation of all the experimental spectra with a single set of spin-Hamiltonian parameters. Specifically, comparison of the 4 and 8 T spectra establishes that \( \langle S \rangle \) along each of the three molecular axes nearly reaches saturation in an applied field of \( 
\sim 4 \) T. \( D_{\text{S=4}} \) would therefore have to be small (\( \sim 0.5 \) cm\(^{-1} \), see Figure S2). Conversely, with \( D_{\text{S=2}} \) of this small magnitude, \( \langle S \rangle \) (for \( D_{\text{S=4}} < 0 \)) saturates at \( \sim 30 \) mT (Figure S2), resulting in much greater magnetic hyperfine splitting in the weak-field spectra than is observed (Figure S3). Thus, ferromagnetic coupling to yield \( S_{\text{Total}} = 4 \) can be ruled out.

The demonstration by Mössbauer spectroscopy that the Mn IV/Fe III cluster in the active form of Cr R2 has an \( S_{\text{Total}} = 1 \) ground state arising from antiferromagnetic coupling between the \( S_{\text{Mn}} = 3/2 \) and \( S_{\text{Fe}} = 5/2 \) ions represents an important first step toward defining its electronic structure. Moreover, parameters obtained in the analysis can now be used to calibrate density functional theory calculations to extract deeper insight.20,21 This insight will permit an important unresolved aspect of class I RNR function—how electron transfer to the oxidized cofactor in Cr R2 is “ gated” so as to occur only in the active holoenzyme complex—to be addressed. Direct interrogation of the radical initiation step in conventional class I RNRs has proven to be extremely challenging.2 The R2 product of radical transfer does not accumulate during turnover because of unfavorable kinetics.2,7 Accumulation of the reduced cofactor can be promoted by use of radical-trapping substrate analogues or R1 variants,23–26 but extracting atomic-level insight into the changes to the cofactor upon reduction is not straightforward. The reduced Y lacks a useful spectroscopic signature. The Fe III/III cluster is either unchanged by the radical-transfer step or, at most, may transfer a proton to the Y from a coordinated water ligand, a change not obviously demonstrable by the spectroscopic methods to which the EPR-silent cluster is amenable. The use of the novel cofactor by Cr RNR affords a unique opportunity to dissect the initiation step. The metal cluster undergoes reduction from Mn IV/Fe III to Mn III/Fe III, which is, by contrast to the reduced cofactor of a conventional class I RNR, EPR active.11 Moreover, although the reduced form might, as in E. coli RNR, not accumulate during turnover, it can be prepared in stable form and its structure is affected by binding of R1 and nucleotides, as demonstrated by marked changes to its EPR spectrum.11 The structural changes caused by formation of the complex are almost certainly elements of the gating mechanism. By defining what they are and extending the description of the geometric and electronic structure of the Mn IV/Fe III cofactor initiated by this work, insight into the mechanisms of radical transfer and conformational gating thereof may be obtained.

Acknowledgment. This work was supported by the National Institutes of Health (GM-55365 to J.M.B.), the Beckman Foundation (Young Investigator Award to C.K.), and the Dreyfus Foundation (Teacher Scholar Award to C.K.).

Supporting Information Available: Rationale for method of sample preparation, component analysis to resolve the spectra of Mn IV/Fe III–R2 shown in Figure 2, analysis of these spectra for the hypothetical case of an \( S_{\text{Total}} = 4 \) ground state, selected plots of the spin expectation values, and the spin Hamiltonian used for analysis of the Mössbauer spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Abbreviations used: Ct. Chlamydia trachomatis; PCET, proton-coupled electron transfer; RNR, ribonucleotide reductase; ZFS, zero field splitting.


(16) An anisorec solution of R2 (3.0 (mm), MnII (3.0 mm), and FeIII (1.5 mm) in 100 mm HEPES (pH = 7.6) containing 10% (v/v) glycerol was mixed at 5 °C with an equal volume of an O2-saturated solution of the same buffer, and the reaction was allowed to proceed for 10 min before the sample was frozen. The control sample was prepared identically, except the MnII was omitted and the concentration of FeIII was doubled in the protein reaction solution. See the Supporting Information for a discussion of the logic underlying the method of sample preparation.


(19) High-spin FeIII and MnIV have well-isolated, nondegenerate orbital ground states and typically exhibit small intrinsic D values.


(22) Ge, J.; Yu, G.; Aitor, M. A.; Stubbe, J. Biochemistry 2003, 42, 10071–10083.


JA072528A

J. AM. CHEM. SOC. • VOL. 129, NO. 24, 2007 7505
A Manganese(IV)/Iron(IV) Intermediate in Assembly of the Manganese(IV)/Iron(III) Cofactor of Chlamydia trachomatis Ribonucleotide Reductase†

Wei Jiang, ‡ Lee M. Hoffart, ‡ Carsten Krebs,* ‡ J. Martin Bollinger, Jr. * ‡ §

Department of Biochemistry and Molecular Biology and Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

Received May 11, 2007; Revised Manuscript Received June 12, 2007

ABSTRACT: We recently showed that the class Ic ribonucleotide reductase from the human pathogen Chlamydia trachomatis uses a MnIV/FeIII cofactor to generate protein and substrate radicals in its catalytic mechanism [Jiang, W., Yun, D., Saleh, L., Barr, E. W., Xing, G., Hoffart, L. M., Maslak, M.-A., Krebs, C., and Bollinger, J. M., Jr. (2007) *Science* *316*, 1188–1191]. Here, we have dissected the mechanism of formation of this novel heterobinuclear redox cofactor from the MnIV/FeIII cluster and O2. An intermediate with a g = 2 EPR signal that shows hyperfine coupling to both 55Mn and 57Fe accumulates almost quantitatively in a second-order reaction between O2 and the reduced R2 complex. The otherwise slow decay of the intermediate to the active MnIV/FeIII–R2 complex is accelerated by the presence of the one-electron reductant, ascorbate, implying that the intermediate is more oxidized than MnIV/FeIII. Mössbauer spectra show that the intermediate contains a high-spin FeIV center. Its chemical and spectroscopic properties establish that the intermediate is a MnIV/FeIV–R2 complex with an S = 1/2 electronic ground state arising from antiferromagnetic coupling between the MnIV (∆Smn = 3/2) and high-spin FeIV (∆SFe = 2) sites.

A conventional class I ribonucleotide reductase (RNR), such as the RNR from *Escherichia coli* or *Homo sapiens*, activates O2 at a carboxylate-bridged FeII cluster in its R2 subunit to oxidize a nearby tyrosine (Y) residue to a stable tyrosyl radical (Y•) (1). The Y• in R2 oxidizes a cysteine residue in the R1 subunit by a long-distance (~35 Å), intersubunit, proton-coupled electron transfer (PCET), generating a transient cysteine thyl radical (C•) (2, 3). The C• in R1 initiates reduction of the ribonucleoside diphosphate (NDP) substrate by abstracting the hydrogen atom from C3 (4, 5). After reduction of the substrate 3′ radical to the 2′-deoxy (product) 3′ radical by two additional cysteine residues in R1 (which become oxidized to a disulfide), the hydrogen originally abstracted from C3 is returned to this position, regenerating the C• and yielding the 2′-deoxyribonucleoside diphosphate (dNDP) product. The C• then reoxidizes the Y• in R2 back to the stable Y•.

When McClarty and co-workers identified the genes encoding the class I RNR subunits from several species of Chlamydiae, they noted that the R2 proteins had phenylalanine (F) residues at the position aligning with the Y•-harboring tyrosine residues of the other R2s (6). They found that the Chlamydia trachomatis (Cr) RNR was, nevertheless, catalytically active. Subsequent biochemical and structural characterization of the Cr R2 confirmed the absence of a Y• and location of F at the site normally harboring the Y• (7). Analysis of genome sequences suggested that Y•-less R2 subunits might also be present in other bacteria (including the important human pathogen, *Mycobacterium tuberculosis*) and archaea, and a new subclassification of these RNRs as class Ic was proposed. It was further suggested that a FeIV,III cluster, formed by reaction of O2 with the FeII form of R2 and similar to the intermediate, cluster X, which had previously been shown to generate the Y•s in the R2 proteins from *E. coli* and *Mus musculus* (8, 9), directly generates the C• in these class Ic RNRs. Two subsequent studies on Cr RNR provided support for this hypothesis by showing that the oxidized diiron cluster could be stabilized for several minutes (10) and even induced to accumulate from FeIII,III–R2 (11) in a complete RNR reaction solution (containing R1, R2, CDP, ATP, Mg2+, and DTT). However, the relatively meager R2 activity (e.g., <3% that of *E. coli* R2) and marked variation thereof (230 units/mg in ref 11 but only <75 units/mg in ref 11) reported by these authors suggested that another, minor form of the protein, present in varying amounts in different preparations, might be responsible for the observed activity. Indeed, we recently demon-
strated that a MnIV/FeIII cluster can be assembled in Ct R2 and that this heterodinuclear form exhibits much greater activity than had been observed in the previous studies (12). Importantly, the reduction of the cofactor to the MnII/FeIII oxidation state and formation of the well-characterized, nitrogen-centered free radical upon incubation of the enzyme with the radical-trapping mechanism-based inactivator, 2′-azido-2′-deoxyadenosine 5′-diphosphate, established that the MnIV/FeIII cluster is the radical-initiating cofactor of Ct RNR. Additional experiments showed that the active cofactor forms by reaction of the reduced (MnIV/FeIII) cluster with O2, but the activation process was not studied in detail (12). In this work, we have examined the mechanism of activation of Ct R2 by stopped-flow absorption and freeze–quench EPR and Mössbauer spectroscopies. The results show that the activation mechanism entails (1) the rapid formation of a MnIV/FeIV–R2 intermediate in a bimolecular reaction between the reduced complex and O2 and (2) the slow decay of the intermediate by reduction of the iron site to FeIII. Kinetic characteristics of the decay step suggest that it may be mediated by the protein, perhaps by the same residues required for the intersubunit radical transfer that initiates turnover (2).

**MATERIALS AND METHODS**

**Expression and Purification of Ct R2.** The protein used throughout this study has an additional 20 amino acids (MGS,H5S,GFPGS) appended to the N-terminal methionine residue of Ct R2. The appendage contains a His6 element to permit purification of the protein by metal ion affinity chromatography. Preparation of the plasmid that directs overexpression of this protein in E. coli, growth of the overexpression strain, and purification of the metal-depleted form of the R2 protein have been described (12).

**Preparation of the MnII/FeII–R2 Complex.** In an MBraun anoxic chamber, MnII and FeII (natural abundance FeII, which contains 91.8% 56FeII and is hereafter referred to as 56FeII, or ~95% enriched 57FeII, which is hereafter referred to as 57FeII) were added to the metal-depleted R2 to form the O2-reactive MnIV/FeIII–R2 complex. It was determined in the course of this study that R2 containing only MnII does not react with O2 (not shown), whereas previous studies had established that the FeII–R2 complex reacts rapidly (7, 10). The reaction of FeII–R2 results in development and decay of the absorption spectrum and sharp, isotropic, g = 2.00 EPR signal of the FeII–R2 cluster (7, 10). To minimize this undesired reaction, stopped-flow absorption and freeze–quench EPR experiments employed a 3-fold excess of Mn over Fe. MnII (1.5 equiv relative to the R2 monomer) was added to the metal-depleted R2 protein first, and the solution was incubated at ambient temperature (22 °C) to allow for binding. FeII (0.5 equiv) was then added slowly. This solution was loaded into the stopped-flow or freeze–quench apparatus. For preparation of the freeze–quenched sample for Mössbauer characterization of the MnIV/FeIV–R2 intermediate, a 2-fold excess of MnII over FeII was employed. The divalent metal ions (1.0 equiv of MnII, 0.5 equiv of 57FeII) were preincubated before being added to the metal-depleted R2. The MnIV/FeII–R2 solution was then loaded into the freeze–quench syringe.

**Stopped-Flow Absorption and Freeze–Quench EPR and Mössbauer Experiments.** The stopped-flow and freeze–quench apparatus and procedures and the EPR and Mössbauer spectrometers have been described (13). The magnitudes of the static magnetic fields for the low-field Mössbauer spectra were determined using a Digital Tesla meter (model 132D) with a Hall probe LPT 130-20S (Group3 Technologies Inc., Auckland, New Zealand). Details of reaction and spectroscopy conditions are provided in the appropriate figure legend or in the figure itself.

**Simulation of the EPR and Mössbauer Spectra of MnIV/FeIV–R2.** The simulations are based on the commonly used spin Hamiltonian formalism (14) and were carried out with respect to the total spin of the electronic ground state, Stotal = 1/2. For simulation of the EPR spectra, eq 1 was used.

\[
\vec{H} = \beta S \cdot \vec{g} \cdot \vec{B} + S \cdot A_{\text{Mn}} I_{\text{Mn}} + S \cdot A_{\text{Fe}} I_{\text{Fe}}
\]  

(1)

The first term is the electron Zeeman effect, and the second and third terms are the hyperfine couplings between the electron spin and the 59Mn nuclear spin (I = 1/2) and between the electron spin and the 55Fe nuclear spin (I = 1/2), respectively. The program SimFonia (Bruker, Billerica, MA) was used to simulate EPR spectra by the second-order perturbation method for powder spectra. The full-matrix diagonalization program, SIM, which was written by Høgni Weihe (University of Copenhagen) (15), was used to simulate the spectra by an independent method to verify the parameters obtained with SimFonia. For simulation of the Mössbauer spectra, the program WMOSS (Web Research, Edina, MN) was used. All simulations were carried out with the assumption that the fluctuation rate of the electron spin is slow compared to the 57Fe Larmor frequency. The first two terms of eq 1 were solved first with the parameters obtained from analysis of the EPR spectra. It was necessary to consider AFe explicitly, because it affects the splitting in the weak-field (B < ~150 mT) spectra. Coupling between the electron spin of the ground state, S = 1/2, and the 55Mn nuclear spin, I = 1/2, leads to two states with spins F = 2 and F = 3 (with F = S + I). For B > 150 mT, the electron Zeeman effect dominates the S·AFe·I term, the states are “pure” and characterized by the Ms values, and the state expectation values are at their maxima, |S⟩ = 0. With weaker applied fields, the electron Zeeman interaction and AFe are comparable, leading to mixing of the states. From the solution of the first two terms in eq 1, the spin expectation value, |S⟩, was calculated. Equation 2, in which all symbols have their usual meaning (14), was then used to compute the Mössbauer spectrum. All tensors were assumed to be collinear.

\[
\langle S \rangle \cdot A_{\text{Fe}} I_{\text{Fe}} = g_{\text{Fe}} B \cdot I_{\text{Fe}}
\]  

(2)

**RESULTS**

**Freeze–Quench EPR Evidence for Accumulation of an Oxidized MnFe Intermediate.** The EPR spectrum of the O2-reactive MnIV/FeII–R2 complex, resolved as the difference of the spectra taken before and after exposure of the reactant solution to O2 (Figure S2), exhibits a broad resonance centered at g ~ 2 that shows hyperfine coupling characteristic of an I = 1/2 59Mn nucleus. Optimal detection of this signal requires relatively low temperature and high power (e.g., 4.2
K and 20 mW, as in Figure S2). Spectra of samples freeze–
quenched during the reaction of this complex with O2
were recorded under less stringent conditions (14 K and 20 µW)
to eliminate the contribution from the reactant complex. By
subtracting the spectrum of the reactant solution under these
conditions from the spectra of the freeze-quenched samples,
the contribution of free Mn^{II} (resulting from the use of excess
Mn^{II} in preparation of the reactant complex) was also
removed. The time-dependent spectra (Figure 1A) illustrate
that an intermediate with a sharp EPR signal develops
rapidly upon reaction with O2 and then decays slowly. The
spectrum of this intermediate has six lines separated by ~80
G that reflect hyperfine coupling to a single 55Mn nucleus. When
the intermediate is formed from the Mn^{III}/Fe^{III}→R2
reactant containing 57Fe, the sextet signal also shows hyper-
fine coupling to this 1/2 nucleus (compare the first and
third spectra in Figure 1B). Simulation of these spectra
(Figure 1B, second and fourth spectra) together with the
Mössbauer spectra to extract electronic structural parameters
(including the 55Mn and 57Fe hyperfine coupling tensors, A_{55Mn}
and A_{57Fe}) is presented below.

**Figure 1:** X-band EPR spectra at 14.0 (±0.2) K of the Mn^{IV}/Fe^{IV}
intermediate in activation of Ct R2. (A) Spectra of samples freeze–
quenched at various reaction times (indicated on the figure) after
mixing at 5°C of an O2-free solution of Mn^{II}/Fe^{III}→R2 (0.40 mM
R2 monomer, 0.5 equiv of Fe, 1.5 equiv of Mn) with an equal
volume of O2-saturated buffer. The spectrum of the recovered
product (completion) sample has been scaled to account for the
fact that it was manually frozen rather than being freeze–quenched
(×0.6, the packing factor typical of freeze–quenched samples). In
addition, the appropriately scaled spectrum of the reactant sample
(×0.5 because it was not diluted and ×0.6 because it was manually
frozen) was subtracted from the experimental spectrum of each
sample to generate the spectra shown. (B) Spectra of samples pre-
pared by manual mixing of an O2-free solution of Mn^{II}/Fe^{III}→R2
(3.0 mM R2 monomer, 0.75 equiv of each metal ion) at ambient
temperature (22 ± 2°C) with 9 equiv of O2-saturated buffer and freezing after 20 ± 2 s. The first and third traces are the
experimental spectra of the samples prepared with 56Fe and
57Fe, respectively. Spectrometer conditions: microwave frequency,
9.45 GHz; microwave power, 20 µW; modulation frequency, 100
kHz; modulation amplitude, 10 G; scan time, 167 s; time constant,
167 ms. The second and fourth traces are simulations of the
experimental spectra generated as described in Materials and
Methods with the g, A_{55Mn}, and A_{57Fe} tensors given in Table 1.

**Figure 2:** Kinetics of the activation of Ct R2 by stopped-flow
absorption spectroscopy. (A) Spectra acquired at the indicated
reaction times after mixing at 5°C of an O2-free solution of
Mn^{II}/Fe^{III}→R2 (0.40 mM R2 monomer, 0.5 equiv of Fe, 1.5 equiv
of Mn) with an equal volume of O2-saturated buffer. (B) Dependence
of the kinetics of the reaction on [O2]. An equivalent
Mn^{II}/Fe^{III}→R2 solution was mixed with 100% (black), 50% (red),
or 25% (blue) O2-saturated buffer. The black circles are the EPR
signal intensities from the experiment of Figure 1A (which had
identical reaction conditions) scaled for direct comparison to the
absorbance changes. The inset shows the apparent first-order rate
constant for the formation phase of the reaction (obtained by fitting
a double-exponential equation to the data) versus [O2], which gives
a second-order rate-constant (slope) of 13 (±3) mM⁻¹ s⁻¹. (C) Dependence of the kinetics on the concentration of ascorbate. The
otherwise equivalent Mn^{II}/Fe^{III}→R2 reactant solution, which
contained ascorbate at a concentration sufficient to give the indicated
[ascorbate] after mixing, was mixed with 100% O2-saturated buffer.
The inset shows the apparent first-order rate constant for the decay
phase versus [ascorbate], which gives a limiting reduction rate
constant (asymptote of hyperbolic fit) of 0.7 (±0.1) s⁻¹.

**Kinetics of the Reaction by Stopped-Flow Absorption Mea-
surements.** Accumulation of the Mn/Fe intermediate complex
is also apparent in the time-dependent absorption spectra
from the reaction (Figure 2A). An intense feature at ~390
nm develops rapidly (black, red, and blue traces) and then
decays slowly, leaving the spectrum of the Mn^{IV}/Fe^{III}→R2
product (green) (16). An overlay of the scaled intensities of
the EPR spectra from the experiment of Figure 1A (Figure
2B, filled circles) with the absorbance-versus-time trace from
the stopped-flow experiment with the same reaction condi-
tions (Figure 2B, black open diamonds) illustrates that the g
~ 2 EPR signal and 390 nm absorption feature arise from
the same intermediate. The kinetics of the intermediate were
declared, and the effects of variation of [O2] and inclusion of
a reductant (ascorbate) were interrogated by additional
stopped-flow experiments. Formation of the intermediate is
kinetically first order in [O2] (Figure 2B). The replots of
the apparent first-order rate constant, obtained by fitting the
Similarly, the Mn IV/Fe III cofactor of active
been shown that ascorbate can donate this electron (17)
the magnetic field). The other five packets either are somewhat
units more oxidized than the Mn II/Fe II
acceleration of its decay to the Mn IV/Fe III state by a reductant
mediate in a concentration-dependent fashion without af-

Table 1: Spin Hamiltonian Parameters of the Mn IV/Fe IV—R2
Intermediate

<table>
<thead>
<tr>
<th>parameter</th>
<th>Fe IV site</th>
<th>Mn IV site</th>
</tr>
</thead>
<tbody>
<tr>
<td>g (MHz)</td>
<td>2.017, 2.030, 2.027</td>
<td></td>
</tr>
<tr>
<td>δ (mm/s)</td>
<td>−55.9, −59.3, −40.5v</td>
<td>247, 216, 243</td>
</tr>
<tr>
<td>ΔE0 (mm/s)</td>
<td>0.17 (6)</td>
<td>−0.75</td>
</tr>
<tr>
<td>η</td>
<td>−10</td>
<td></td>
</tr>
</tbody>
</table>

* Sign determined by Mössbauer spectroscopy.

It is generally considered appropriate to invoke the pseudo-first-
other approximation implicit in this fitting analysis only when one
reactant is in at least 10–20-fold excess over the other. In these
experiments, O2 is in excess over the theoretical concentration of the
reactive Mn IV/Fe III—R2 complex by a minimum of 2.3-fold and a
maximum of 9.0-fold. However, within this range, the apparent first-
order rate constant still behaves as a nearly linear function of the
concentration of the excess reactant (see Figure S3), and the error
introduced into the second-order rate constant by the approximation is
small (~10%) in comparison with other sources (e.g., ~25% in the
values of |O2|).

equation for two parallel first-order reactions to the data,2 versus [O2] gives a second-order rate constant (slope) of 13
(±3) mM−1 s−1 (Figure 2B, inset).

Formation of the Y• and Fe II/III cluster of a conventional
determination of the
Mn for the Mn IV site of Mn II/IV

characterization of the Intermediate by EPR and Möss-
bauer Spectroscopy. The electronic structure of the inter-
mediate was probed further by EPR and Mössbauer spec-

specifically, analysis of the spectrum of the 0.09 s sample shows that the dominant features in the latter spectrum develop with the same kinetics
for the g ~ 2 EPR signal and 390 nm absorption feature
and are therefore associated with the same intermediate.
Specifically, analysis of the spectrum of the 0.09 s sample
suggests that ~40% of the intensity of the spectrum is
attributable to the intermediate.

The 4.2 K/53 mT Mössbauer spectra (Figure S4) of the
Mn IV/Fe IV—R2 complex (top spectrum) and samples prepared
by reacting this complex at 5 °C with O2 for 0.090 s (second
spectrum from top), 2.0 s (near the time of maximal
accumulation of the intermediate; third spectrum from top),
or 10 min (completion; bottom two spectra) before freezing
illustrate the accumulation of the intermediate to a high level
and its subsequent decay to the previously characterized
Mn IV/Fe III—R2 product (16). Importantly, comparison of the
spectra of the 0.09 and 2 s samples shows that the dominant features in the latter spectrum develop with the same kinetics
as for the g ~ 2 EPR signal and 390 nm absorption feature
and are therefore associated with the same intermediate.
Specifically, analysis of the spectrum of the 0.09 s sample
suggests that ~40% of the intensity of the spectrum is
attributable to the intermediate.

The 4.2 K/variable-field Mössbauer spectra of the 2 s
sample are dominated (~70% of the absorption area) by
features of the intermediate (Figure 3). Ideally, the spectral contributions of the minor species would be removed by subtraction of appropriate reference spectra in order to
resolve the spectrum of the intermediate for detailed simul-
analysis. However, in this case, even though the
accumulation of the intermediate compares favorably with the best cases that we have encountered in previous studies,
the multiplicity and unknown identities of the minor species
make removal of their contributions impossible. The major
“contaminant” is a high-spin Fe II species of unknown
identity.4 Its presence is most clearly revealed in the weak-
field (B < 53 mT) spectra by peaks at ~0.2 and +2.8 mm/s
(~17% intensity; Figure 3, middle spectrum, blue line). The
magnetic field dependence of the Fe II-associated spectral
component is unknown, precluding its removal. Fortunately,
it is clear that, as expected for high-spin (S = 2) Fe II, the
Mössbauer features become broader and contribute little to
the overall line shape of the experimental spectrum of the 2
s sample with increasing field strengths. The remaining contaminants are predictable from the previous characteriza-
tion of the product of the reaction (16), which showed that
it contains ~80% Mn IV/Fe III—R2 and ~20% of the homodi-
nuclear Fe II/III product. Thus, the iron in the 2 s sample not
associated with the Mn IV/Fe IV intermediate and Fe II con-

3 The Mn IV sites of the Mn IV/Fe IV cluster of catalase and the Mn IV/Fe IV intermediate in Cr R2 have the same spin projection factors, and therefore the magnitudes of the A Mn tensors with respect to the total spin of the S = 1/2 ground states can be directly compared.

4 Candidates for the Fe II complexes are aqueous Fe II, complexes in which the divalent metal is bound to R2 either in mononuclear fashion or in a homodinuclear or heterodinuclear cluster, or some combination of these. The Fe II-associated features remaining in the spectrum of the 2 s sample are different from the spectrum of the reactant complex and thus cannot simply be removed by subtraction of this spectrum.

1 The Mn IV sites of the Mn IV/Fe IV cluster of catalase and the Mn IV/Fe IV intermediate in Cr R2 have the same spin projection factors, and therefore the magnitudes of the A Mn tensors with respect to the total spin of the S = 1/2 ground states can be directly compared.
the experimental spectra can accommodate a small contribution from the product species (≤9% total), their contribution could be much less or even negligible. The spectra do reveal the presence of a small quantity of the Fe$_{IV}$Fe$_{IV}$ complex. In particular, two features of X are nearly fully resolved in the 13 mT spectrum and can be used to estimate the contribution from the complex (~11%; Figure 3, bottom spectrum, red line). In addition, the highest energy lines of the subspectra of the Fe$_{IV}$ and Fe$_{III}$ sites are coincident at 3.7 mm/s in the 8 T spectrum, resulting in a more intense line that reveals the presence of the complex (Figure 3, top spectrum, red line).

Despite this heterogeneity, the predominance of the Mn$_{IV}$/Fe$_{IV}$ intermediate makes the positions and shapes of its features sufficiently clear for simulations to be used to extract spectroscopic parameters (Table 1 and Figure 3, solid black lines). In particular, the contributions of species with integer-electron-spin ground states (Fe$_{II}$ species and the Mn$_{IV}$/Fe$_{III}$ and Fe$_{IV}$/Fe$_{III}$ products) are canceled in field orientation dependent spectra (53 mT // 53 mT ⊥), and the contributions of the species with half-integer spin (the Mn$_{IV}$/Fe$_{IV}$ and Fe$_{IV}$/Fe$_{III}$ intermediates) are resolved (14). The contribution from the Fe$_{IV}$/Fe$_{IV}$ intermediate (red line) is small compared to that of the Mn$_{IV}$/Fe$_{IV}$ intermediate (black line). This difference spectrum provides constraints on the parameters of the Mn$_{IV}$/Fe$_{IV}$ intermediate, in particular the isomer shift (δ). Because δ must be determined from magnetically split spectra, the uncertainty in this crucial parameter is fairly large (0.06 mm/s). Nevertheless, even with the large uncertainty, the value of δ (0.17 ± 0.06 mm/s) indicates that the intermediate has an Fe$_{IV}$ site. Indeed, the center of the range is essentially identical with δ of the Fe$_{IV}$/Fe$_{IV}$ complex, Q, in the reaction of soluble methane monoxygenase (22, 23).

The hyperfine tensor for the Fe$_{IV}$ site with respect to the total spin of the ground state (S = $^1/2$), $A_{Fe}$, determines the splitting in the spectra and is given by the product of the intrinsic hyperfine tensor, $a_{Fe}$, and the spin projection factor, $c_{Fe}$, (eq 3) (24).

$$A_{Fe} = c_{Fe} a_{Fe}$$ (3)

The components of $A_{Fe}$ are negative, as revealed by the decrease of the overall splitting with increasing applied magnetic field (e.g., compare the 53 mT, 4 T, and 8 T spectra in Figure 3) (25). The components of the intrinsic hyperfine tensor for iron, $a_{Fe}$, are negative. Thus, $c_{Fe}$ must be positive. A positive value of $c_{Fe}$ requires that $S_{Fe} > S_{Mn}$ ($^1/2$). The Fe$_{IV}$ site must therefore be in the high-spin configuration ($S_{Fe} = 2$). For this spin system, $c_{Fe} = 2$ for the $S = 1/2$ ground

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3 The 4.2 K/53 mT Mössbauer features of the Fe$_{IV}$/Fe$_{IV}$ cluster in C.t R2, which accumulates to a high level in the reaction of the Fe$_{III}$/O$_2$ protein with O$_2$, are almost identical with those of X in E. coli R2 (unpublished results). Therefore, we used the published parameters of E. coli X (26) to simulate the spectrum of the cognate complex in C.t R2.

4 The standard tactic of raising the temperature to make the electronic fluctuation rapid with respect to the nuclear precession frequency and thereby collapse the magnetic spectrum into a quadrupole doublet for more accurate determination of δ and ∆E$_Q$ failed. The 120 K/zero-field spectrum is very broad and featureless, implying that the fluctuation rate of the electronic states is comparable to the Fe Larmor frequency (the intermediate relaxation regime) at this temperature.
state, giving $a_{Fe} = -28.0, -29.7, -20.3$ MHz. These values are almost identical to those of the high-spin Fe$^{IV}$ site of cluster $X$ in $E. coli$ R2 ($a_{Fe} = -27.6, -27.6, -20.6$ MHz) (26), consistent with the assignment of the Fe site of the $Ct$ R2 intermediate as high-spin Fe$^{IV}$.

The $g$ tensor of the $S = \frac{1}{2}$ ground state, given by eq 4 (24), is nearly isotropic as a result of relatively small anisotropy of $g_S$ and $g_{Mn}$, as was observed before for Mn$^{IV}$ and high-spin Fe$^{IV}$ species (20, 26).

$$g_{S=1/2} = 2g_{Fe} - g_{Mn} \quad (4)$$

Comparison of the low-field spectra clearly illustrates the perturbation associated with the hyperfine coupling to the $I = \frac{1}{2}$ $^{55}$Mn nucleus, which is comparable in magnitude to the electron Zeeman term in weak fields. For example, at a field of 13 mT (Figure 3, bottom spectrum), the absolute magnitude of the internal magnetic field [given by $-(S)(A_{Mn}g_S\beta )_{Fe}$] is smaller for some of the states as a consequence of subsaturating values of $S$ (see Figure S1 for plots of the field dependence of the spin expectation values), resulting in reduced splitting and greater intensity in the center of the spectrum. As the field is increased in the 0–150 mT regime, $S$ and the magnetic splitting increase (compare to the 53 mT || spectrum; Figure 3, middle). With much greater fields ($B > 150$ mT), splitting decreases again (compare the 53 mT || spectrum to the 8 T spectrum) because for the ground state the applied field opposes the already saturated internal field from the electron spin.

**DISCUSSION**

The stopped-flow absorption and freeze–quench EPR and Mössbauer data thus establish that the active Mn$^{IV}$/Fe$^{III}$ cofactor of $Ct$ RNR forms via a Mn$^{IV}$/Fe$^{IV}$ intermediate that decays by reduction of the Fe$^{IV}$ site (Scheme 1, top). The Mn$^{IV}$ ($S_{Mn} = \frac{1}{2}$) and high-spin Fe$^{IV}$ ($S_{Fe} = 2$) sites of the intermediate couple antiferromagnetically to yield an $S = \frac{1}{2}$ ground state. Whereas a Mn$^{IV}$/Fe$^{III}$ complex has been reported (27), the $Ct$ R2 intermediate is, to our knowledge, the first example of a Mn$^{IV}$/Fe$^{IV}$ complex. In view of the X-ray crystal structure of the (presumptively) Fe$^{III}$ form of the $Ct$ R2 protein by Högobom et al. (7), which suggested a bis($\mu$-hydroxo)-dimetal core, and previous studies suggesting formation of a ($\mu$-O$_2$)-Fe$^{IV/IV}$ complex, $Q$, in the catalytic cycle of soluble methane monoxygenase (28), we consider it very likely that the Mn$^{IV}$/Fe$^{IV}$ intermediate also has this [M$_2$O$_2$(H)$_8$]$_{4+}$ “diamond core” structure. Its half-integer ($S = \frac{1}{2}$) electron-spin ground state, which contrasts with the $S = 0$ ground state of $Q$, and heterodinuclear rather than homodinuclear nature should afford unique opportunities to test this hypothesis and probe details of the core structure by electron–nuclear double resonance (ENDOR) and X-ray absorption experiments.

$Q$ and the $Y$-generating Fe$_{2}^{III}$ intermediate, $X$, form from the corresponding $\mu$-peroxo-Fe$_{2}^{III}$ intermediates in methane monoxygenase (23) and conventional RNR–R2 proteins (29, 30), respectively. However, no Fe$_{2}^{IV}$ complex has ever been detected in an R2 protein, either because O–O cleavage occurs reductively or because the Fe$_{2}^{IV}$ complex is reduced too rapidly to accumulate. In the best studied R2 reaction, in $E. coli$ R2, tryptophan (W) 48 near the protein’s surface is the proximal electron source for this step, and the resultant W48 cation radical is readily reduced by a variety of compounds (Fe$^{III}$, ascorbate, thiols) (Scheme 1, bottom) (31). A radical of the corresponding W residue in $Ct$ R2 (W. Jiang, L. Saleh, and J. M. Bollinger, Jr., unpublished observations), proving that this residue can function equivalently in the class Ic R2 and could, in principle, rapidly reduce the Mn$^{IV}$/Fe$^{IV}$ cluster to limit its accumulation. Apparently, changes accompanying replacement of one Fe by Mn (e.g., of the mechanistic pathway or reduction potentials of constituent complexes), structural adjustments to the cluster site (e.g., the presence of E89 in $Ct$ R2 in place of the D84 found in $E. coli$ R2), or both allow the IV/IV state to build up uniquely in the $Ct$ R2 protein. Nevertheless, the “saturation” of the observed rate constant in Figure 2C suggests that reduction of the Mn$^{IV}$/Fe$^{IV}$ complex by ascorbate might also take place by a two-step mechanism, with the first step being the oxidation of W51 (or perhaps another residue). A rate constant of 0.7 ± 0.1 s$^{-1}$, the asymptotic value of $k_{obs}$ for decay of the intermediate, for the first step in this hypothetical electron-shuttling mechanism would rationalize the saturation of the decay rate constant at this value. This speculation should be...
testable by use of alternative reductants and variant R2 proteins.

It remains to be seen whether the Mn$^\text{IV}/\text{Fe}^\text{IV}$ intermediate, like Q and X, forms from a (μ-peroxo)-Mn$_2^\text{III}$ intermediate. The stopped-flow and freeze–quench EPR data provide no evidence for the accumulation of such a complex. Thus, as in E. coli R2, it might prove necessary to perturb the reaction kinetics [e.g., by replacement of a ligand, as in the D84E substitution in E. coli R2 that was shown to stabilize the peroxy intermediate (32)] to permit accumulation of a peroxide precursor to the Mn$^\text{IV}/\text{Fe}^\text{IV}$ intermediate.

**SUPPORTING INFORMATION AVAILABLE**

Calculated energies and spin expectation values of the $^{55}$Mn hyperfine interaction in low magnetic fields, EPR spectrum of the O$_2$-reactive Mn$^{\text{IV}}$/Fe$^{\text{IV}}$–R2 complex, analysis proving the applicability of the pseudo-first-order approximation in analysis of the [O$_2$]-dependent stopped-flow data, and a comparison of the 4.2 K/53 mT Mössbauer spectra of the Mn$^{\text{IV}}$/Fe$^{\text{IV}}$–R2 reactant complex and samples frozen at various times after reacting this complex with O$_2$. This material is available free of charge via the Internet at http://pubs.acs.org.

**REFERENCES**


BI700906G
Rapid and Quantitative Activation of *Chlamydia trachomatis* Ribonucleotide Reductase by Hydrogen Peroxide†

Wei Jiang,‡ Jiajia Xie,§ Hanne Nørgaard,∥ J. Martin Bollinger, Jr.,*‡§ and Carsten Krebs*‡§

**Department of Biochemistry and Molecular Biology and Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802**

Received October 16, 2007; Revised Manuscript Received January 24, 2008

**ABSTRACT:** We recently showed that the class Ic ribonucleotide reductase (RNR) from the human pathogen *Chlamydia trachomatis* (*Ct*) uses a MnIV/FeIII cofactor in its R2 subunit to initiate catalysis [Jiang, W., Yun, D., Saleh, L., Barr, E. W., Xing, G., Hoffart, L. M., Maslak, M.-A., Krebs, C., and Bollinger, J. M., Jr. (2007) *Science* 316, 1188–1191]. The MnIV site of the novel cofactor functionally replaces the tyrosyl radical used by conventional class I RNRs to initiate substrate radical production. As a first step in evaluating the hypothesis that the use of the alternative cofactor could make the RNR more robust to reactive oxygen and nitrogen species [RO(N)S] produced by the host’s immune system [Högborn, M., Stemmark, P., Voevodskaya, N., McClarty, G., Gräslund, A., and Nordlund, P. (2004) *Science* 305, 245–248], we have examined the reactivities of three stable redox states of the Mn/Fe cluster (MnII/FeII, MnIII/FeIII, and MnIV/FeIII) toward hydrogen peroxide. Not only is the activity of the MnIV/FeIII→R2 intermediate stable to prolonged (>1 h) incubations with as much as 5 mM H2O2, but both the fully reduced (MnII/FeII) and one-electron-reduced (MnIII/FeIII) forms of the protein are also efficiently activated by H2O2. The MnIII/FeIII→R2 species reacts with a second-order rate constant of 8 ± 1 M−1 s−1 to yield the MnIV/FeIV→R2 intermediate previously observed in the reaction of MnII/FeII→R2 with O2 [Jiang, W., Hoffart, L. M., Krebs, C., and Bollinger, J. M., Jr. (2007) *Biochemistry* 46, 8709–8716]. As previously observed, the intermediate decays by reduction of the Fe site to the active MnIV/FeIII→R2 complex. The reaction of the MnIV/FeII→R2 species with H2O2 proceeds in three resolved steps: sequential oxidation to MnIII/FeIII→R2 (k = 1.7 ± 0.3 mM−1 s−1) and MnIV/FeIV→R2, followed by decay of the intermediate to the active MnIV/FeIII→R2 product. The efficient reaction of both reduced forms with H2O2 contrasts with previous observations on the conventional class I RNR from *Escherichia coli*, which is efficiently converted from the fully reduced (FeII/II) to the “met” (FeIII/III) form [Gerez, C., and Fontecave, M. (1992) *Biochemistry* 31, 780–786] but is then only very inefficiently converted from the met to the active (FeIV/IV–Y′) form [Sahlin, M., Sjöberg, B.-M., Backes, G., Loehr, T., and Sanders-Loehr, J. (1990) *Biochem. Biophys. Res. Commun.* 167, 813–818].

Ribonucleotide reductases (RNRs) catalyze the reduction of ribonucleotides to 2'-deoxyribonucleotides to provide precursors for DNA synthesis and repair. A conventional class I RNR, such as that from *Escherichia coli* (*Ec*) or *Homo sapiens*, harbors in its R2 subunit a cofactor comprising a tyrosyl radical (Y′) and an adjacent carboxylate-bridged Fe2III cluster (I). The cofactor components form together by reaction of O2 with the fully reduced (FeII/II) form of the cluster (I). A FeII/IV intermediate, X, oxidizes the tyrosine residue to the stable Y′ as it is reduced to the product FeIV/III cluster (2–4). In the catalytic cycle, the Y′ in R2 oxidizes a cysteine residue in the R1 subunit by a long-distance (~35 Å), intersubunit, proton-coupled electron transfer (PCET), generating a transient cysteine thiol radical (5, 6). The cysteine radical in R1 initiates reduction of the ribonucleoside diphosphate (NDP) substrate by abstracting the hydrogen atom from C3′ (7, 8). After reduction of the substrate 3′ radical to the product 3′ radical by two additional cysteine residues in R1 (which become oxidized to a disulfide), the hydrogen originally abstracted from C3′ is returned to this position, regenerating the cysteine radical and yielding the dNDP product. The cysteine radical then reoxidizes the Y in R2 to the stable Y′.

Inhibition of RNRs is a proven strategy for combating cancer and some viruses (9–11). Reduction of the catalytically essential Y′ in R2 is part of the mechanisms of action of several RNR-targeting drugs (12–14). For example, the anticancer drug hydroxyurea reduces the Y′ to tyrosine (15, 16),
yielding a state known as met-R2. Adventitious reduction of the potently oxidizing Y to give met-R2 may also occur in the reducing environment of the cell. An enzymatic reactivating system capable of slowly (over many minutes) regenerating the Y by mediating reduction of the FeIII/III cluster in Ec met-R2 to FeIV/III (which then reacts with O2) was reported and studied by Reichard, Fontecave, and co-workers (17, 18), but its physiological relevance has not been established. More recently, Stubbe and co-workers identified a [2Fe-2S] cluster-containing ferredoxin, YfaE, that can reduce Ec met-R2 rapidly (in seconds) to the FeIII/III form, leading to rapid formation of Y upon exposure to O2 (19). This reaction is likely to be relevant to control of the Y content of R2 in vivo. In vitro reactivation of met-R2 by H2O2 has also been reported, but this reaction is very inefficient (regeneration of 30% of the Y in 1.5 h) (20).

When McClarty and co-workers identified the genes encoding the class I RNR subunits from several species of chlamydiae, they noted that the R2 proteins have phenylalanine (F) residues aligning with the radical-harboring tyrosines of the other R2 proteins (21). They found that the R2 from Cr is, nevertheless, catalytically active (21). Subsequent biochemical and structural characterization of Cr R2 by Nordlund, Gräslund, and co-workers confirmed the absence of the Y and the location of F at the corresponding site, and a new subclass (Ic) was established to comprise the Cr enzyme and the hypothetical Y-less RNRs encoded within the genomes of several other bacteria and archaea (22). In that same study and in two subsequent reports from the Gräslund group, the hypothesis was advanced that reaction of O2 with the FeIII/III cluster generates a high-valent Fe2III/IV cofactor (similar to the Y-generating cluster X in the conventional R2 proteins) that functionally replaces the Y as the radical initiator in the class Ic RNRs (22–24). It was further speculated in the case of the Cr RNR that the use of the novel cofactor might render the RNR and bacterium (an obligate intracellular parasite) more robust to reactive oxygen and nitrogen species [RO(N)S] produced in the host’s innate immune response (22). Indeed, several earlier studies had shown that the Y in the conventional class I system can be targeted by RO(N)S (25–30).

We recently verified an essential aspect of the Nordlund–Gräslund hypothesis, the functional replacement of the Y with a high-valent metal cofactor, but showed that the Ct R2 actually uses a heterobinuclear MnIV/FeII cofactor (31, 32), rather than the homobinuclear Fe2III/IV cofactor proposed by these authors, as the radical initiator. The functional cofactor forms via a MnIV/FeIV intermediate in the reaction of the MnIV/FeII form of the protein with O2 (33). The MnIV ion replaces the Y of the conventional class I RNRs as the radical initiator (31). This “metal makeover” (34) does not rule out the second aspect of the Nordlund–Gräslund hypothesis, that the alternative radical initiation system renders the enzyme more resistant to host-generated RO(N)S. In fact, in light of the several precedents for the involvement of Mn in bacterial oxidative stress responses (35–37), its presence in the Ct RNR cofactor would seem to make this hypothesis even more attractive. In this work, we have begun to test the second aspect of the Nordlund–Gräslund hypothesis by examining the reactivity of the Mn/Fe cofactor toward H2O2, a biologically important RO(N)S. Among the biologically important RO(N)S, hydrogen peroxide is the most stable and its chemistry the most simple. Moreover, its production is known to be an important component of innate immunity to bacteria (38). The complete stability of the active MnIV/FeIII−R2 species and rapid, quantitative conversion of both fully reduced (MnIV/FeII) and one-electron-reduced (MnIII/FeII) forms of the protein to the active state upon their treatment with H2O2, when contrasted with previous observations on the reactions of fully reduced (39, 40) and met forms (20) of Ec R2 with H2O2, are consistent with the hypothesis that the novel radical initiation system could be an adaptation to RO(N)S produced by the host.

MATERIALS AND METHODS

Expression and Purification of Ct R2. Ct R2 with an N-terminal 22-residue extension containing a His6 affinity tag was expressed in E. coli, purified by chromatography on Ni-NTA agarose, and converted to the metal-free (apo) form as previously described (31).

Preparation of the MnIV/FeIII−R2 Species. To an air-saturated solution of 370 µM (monomer concentration) apo R2 at 5 °C were added 1.5 equiv of MnII and 5 mM sodium ascorbate. FeII (0.75 equiv per monomer of either natural abundance or ~95% 57Fe-enriched FeII) was added slowly over a period of 20 min. After 1 h at 5 °C, unbound metal was removed by dialysis against 10 mM EDTA [in 100 mM Na-HEPES (pH 7.6) and 10% glycerol]. The EDTA was removed from the protein by dialysis against buffer [100 mM Na-HEPES (pH 7.6) and 10% glycerol]. Removal of >95% of the free MnII was verified by EPR spectroscopy. Mössbauer analysis of 57Fe-labeled samples indicated the presence of 85–90% of the active MnIV/FeIII form and 10–15% of the inactive Fe2III/IV form.

Preparation of the MnIII/FeII−R2 Species. The MnIV/FeII−R2 form was reduced in an MBAuron anoxic chamber with 1.2 molar equiv (2.4 electron equiv) of sodium dithionite for 60 min and subsequently used for the kinetic and spectroscopic experiments. The MnIII/FeII−R2 species used for the activity assays was prepared by treatment of the active protein with 7.5 molar equiv of sodium dithionite to completely eliminate activity. EPR spectroscopy showed that >80% of the protein was converted to the one-electron-reduced MnII/FeIII state (i.e., that <20% was further reduced by the excess dithionite).

Activity Assays. The enzymatic activity supported by samples of Ct R2 in the presence of excess R1, CDP, ATP, and DTT was determined as previously described (31).

Kinetic and Spectroscopic Experiments. The stopped-flow apparatus, the EPR and Mössbauer spectrometers, and the freeze-quench apparatus and procedures have been described previously (41). Absorbance-versus-time traces from the reactions of MnIII/FeII−R2 and MnII/FeII−R2 species with H2O2 were analyzed by nonlinear regression according to eqs 1 and 2, respectively, in which k1−k3 are apparent first-order rate constants, ∆A1−∆A2 are amplitudes for the exponential phases, and A0 is the absorbance at time zero. The assumption of a pseudo-first-order excess of H2O2 inherent in these equations is met by the experimental conditions. The assumption of irreversibility is reasonable because cleavage of H2O2 to water is highly exergonic.

\[
A(t) = A_0 + \Delta A_1 [1 - \exp(-k_1 t)] + \Delta A_2 [1 - \exp(-k_2 t)]
\]  

(1)
RESULTS

\( \Delta(t) = A_0 + A_1[1 - \exp(-k_1t)] + A_2[1 - \exp(-k_2t)] + A_3[1 - \exp(-k_3t)] \) (2)

Colorimetric Assay for H\(_2\)O\(_2\) Concentration. All H\(_2\)O\(_2\) concentrations quoted were calculated by assuming the concentration listed on the commercially obtained stock solution (EMD, Gibbstown, NJ). This concentration was verified by using a previously described colorimetric assay (42). The experimentally determined H\(_2\)O\(_2\) concentration (8.3 mM) agreed with the value quoted by the manufacturer (8.8 mM). The same assay was also used to monitor decomposition of H\(_2\)O\(_2\) in the presence of various forms of Ct R2.

**Characterization of H\(_2\)O\(_2\)-Mediated Reactivation by Mössbauer Spectroscopy.** The activity assays demonstrate rapid and quantitative reactivation of dithionite-reduced (Mn\(^{IV}\)/Fe\(^{III}\)) Ct R2 by H\(_2\)O\(_2\). Mössbauer spectroscopy was used to verify that this reactivation reflects conversion of the inactive Mn\(^{III}\)/Fe\(^{III}\) R2 complex to the active Mn\(^{IV}\)/Fe\(^{III}\) form. First, the Mn\(^{IV}\)/Fe\(^{III}\) R2 complex was converted by dithionite reduction to the Mn\(^{IV}\)/Fe\(^{III}\) R2 complex. The Mössbauer spectra of this sample after its subsequent treatment with 7.5 molar equiv of dithionite revealed a sharp quadrupole doublet at 4.2 K and zero field (Figure 1A). The Mn\(^{IV}\)/Fe\(^{III}\) R2 species exhibits a sharp quadrupole doublet at 4.2 K and zero field. Figure 1A shows that, in this particular sample of the Mn\(^{IV}\)/Fe\(^{III}\) R2 complex, approximately 90% of the iron is in the form of the Mn\(^{IV}\)/Fe\(^{III}\) cluster. Its quadrupole doublet has an isomer shift (\(\delta\)) of 0.52 mm/s and a quadrupole splitting parameter (\(\Delta E_Q\)) of 1.38 mm/s (solid line in Figure 1A). \(\Delta E_Q\) is slightly larger than the value reported previously [\(\Delta E_Q = 1.32 \text{ mm/s (32)}\)]. We attribute this to the presence of a greater concentration of glycerol in this sample (45%, v/v) than in the previous sample (10%, v/v). The sample also contains a minor (~10%) contaminant of the homobinuclear Fe\(^{III}\) complex (red line).

At 190 K and zero field (Figure 1B), the isomer shift of the Fe\(^{III}\) site is slightly diminished (\(\delta = 0.47 \text{ mm/s}\)) by the second-order Doppler effect (43). The quadrupole splitting is temperature-independent (\(\Delta E_Q = 1.37 \text{ mm/s}\)) and identical within the experimental uncertainty (0.03 mm/s) to the value at 4.2 K.

Treatment of this sample with 1.2 equiv of dithionite alters the 190 K/zero field spectrum (Figure 1C) to a much broader quadrupole doublet with parameters (\(\delta = 0.45 \text{ mm/s}, \Delta E_Q\)) 0.47 mm/s, \(\Delta E_Q\)) still characteristic of high-spin Fe\(^{III}\) (43), implying (as previously noted (31)) that the Fe site of the cofactor is not reduced. The 4.2 K/53 mT spectrum of the reduced sample is broad and magnetically split (Figure S1). These observations are consistent with reduction of the Mn\(^{IV}\) site to yield a Mn\(^{III}/Fe^{III}\) cluster with an S = 1/2 ground state, as previously reported (31).

The Mössbauer spectra of this sample after its subsequent treatment with excess H\(_2\)O\(_2\) (300 mM or 100 equiv for 15 min) confirm the essentially quantitative conversion of the dithionite-inactivated (Mn\(^{III}/Fe^{III}\)) R2 back to the active Mn\(^{IV}/Fe^{III}\) R2 complex by H\(_2\)O\(_2\) (data not shown). The Mössbauer spectra of this sample after its subsequent treatment with 1.2 equiv of H\(_2\)O\(_2\) activity (%)

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<td>Mn(^{IV}/Fe^{III})</td>
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\(^*\) The activity of the Mn\(^{IV}/Fe^{III}\) R2 complex is \(\sim 0.4 \text{ s}^{-1}\) or 600 units/mg under the assay conditions that were used (22 ± 2 °C, 10 equiv of R1).

![Figure 1: Mössbauer spectra showing the reduction of the Mn\(^{IV}/Fe^{III}\) R2 complex to the Mn\(^{III}/Fe^{III}\) R2 complex by dithionite and reoxidation to the Mn\(^{IV}/Fe^{III}\) R2 complex by H\(_2\)O\(_2\). The temperature and magnetic field are indicated on the spectra. (A and B) A sample of the Mn\(^{IV}/Fe^{III}\) R2 complex prepared as described in Materials and Methods. (C) A sample after treatment of the Mn\(^{IV}/Fe^{III}\) R2 complex with 1.2 equiv of dithionite for 1 h. (D and E) Dithionite-reduced sample after subsequent treatment with 300 mM H\(_2\)O\(_2\) for 15 min. (F) Samples prepared by exposure of the Mn\(^{IV}/Fe^{III}\) R2 complex (1.5 mM R2 monomer, 1 equiv of Mn\(^{IV}\), and 0.5 equiv of Fe\(^{III}\)) to 300 mM H\(_2\)O\(_2\) (hash marks) or 1 mM O\(_2\) (solid line). The solid black lines plotted over spectra A–E are simulations with parameters quoted in the text. The red line is the experimental spectrum of the Fe\(^{III}\)-mediated R2 form, scaled to 10% of the total intensity of spectrum A.](image)
can again be attributed to the quadrupole doublet of the MnIV/FeIV complex (blue circles and red squares, respectively) vs H2O2 concentration, volume of an H2O2 solution in the same buffer. The green circles to eq 1 reveals that the formation phase has the expected ° (which gives a second-order rate constant (slope) of 8 (blue circles). As the solid lines in D and E indicate, ∼90% of the intensity can again be attributed to the quadrupole doublet of the MnIV/FeIV complex [δ = 0.47 mm/s and ΔE₀ = 1.34 mm/s at 190 K (D), and δ = 0.52 mm/s and ΔE₀ = 1.35 mm/s at 4.2 K (E)].

**Kinetics and Mechanisms of the Reaction of the MnIII/FeIII Complex with H2O2 by Stopped-Flow Absorption and Freeze-Quench EPR Spectroscopies.** Stopped-flow absorption and freeze-quench EPR experiments were used to define the kinetics and mechanism of H2O2-mediated reactivation of the dithionite-generated MnIII/FeIII complex. An intense ∼390 nm absorption band, similar to the feature R2 intermediate in the reaction of the MnII/FeII complex with H2O2. The kinetics of the decay phase (limiting kobs) versus H2O2 concentration [inset of Figure 2A (circles)] gives a second-order rate constant of 8 ± 1 M⁻¹ s⁻¹ for the reaction of the MnIII/FeIII complex with H2O2. The kinetics of the decay phase (limiting kobs = 0.06 ± 0.01 s⁻¹) are consistent with those previously observed for decay of the MnIV/FeIV complex to MnII/FeII complex (33). Thus, both the character of the spectral changes and the kinetics suggest the accumulation of the MnIV/FeIV intermediate.

The accumulation of this complex was directly demonstrated by freeze-quench EPR experiments (Figure 3). The dithionite-reduced R2 reactant exhibits a broad, poorly defined g ~ 2 EPR spectrum (top spectrum) from the antiferromagnetically coupled (S = 1/2) MnIII/FeIII cluster. When this reactant is mixed with H2O2, the well-defined, sharp, six-line signal characteristic of the MnII/FeII complex with H2O2 and then decays slowly (Figure S2). Analysis of A390-versus-time traces for the reaction (Figure 2A) according to eq 1 reveals that the formation phase has the expected first-order dependence on H2O2 concentration, whereas the decay phase is relatively insensitive to H2O2 concentration (inset of Figure 2A). The slope of the plot of the apparent first-order rate constant for the formation phase (kobs) versus H2O2 concentration [inset of Figure 2A (circles)] gives a second-order rate constant of 8 ± 1 M⁻¹ s⁻¹ for the reaction of the MnIV/FeIV intermediate with H2O2. The kinetics of the decay phase (limiting kobs = 0.06 ± 0.01 s⁻¹) are consistent with those previously observed for decay of the MnIV/FeIV complex to MnII/FeII complex (33). Thus, both the character of the spectral changes and the kinetics suggest the accumulation of the MnIV/FeIV intermediate.

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Activation of the Fully Reduced MnIII/FeII–R2 Complex with H2O2. Previous studies have shown that the fully reduced (Fe2II/III) form of Ec R2 reacts with H2O2 to produce met-R2 (39, 40). The cognate reaction in Ct R2 would convert the MnIII/FeII complex to the MnIII/FeIII–R2 complex, which should, as demonstrated above, then react with a second equivalent of H2O2 to yield the active state. Indeed, treatment of the MnIII/FeII–R2 complex with excess H2O2 results in an activity equivalent to that produced by treatment with O2. In addition, the 4.2 K/zero field Mössbauer spectra (Figure 1F) of the products of the H2O2 (hash marks) and O2 (solid line) reactions are nearly identical.3 In both cases, application of the 53 mT magnetic field elicits the same diagnostic broadening of the quadrupole doublet (data not shown), confirming the formation of the MnIV/FeIII–R2 complex as the ultimate product also in the reaction of the fully reduced protein with H2O2.

Kinetics and Mechanism of the Reaction of the MnIII/FeII–R2 Complex with H2O2. The expectation that this reaction proceeds by two sequential reactions with H2O2 via MnIII/FeII–R2 and MnIV/FeIV–R2 intermediates was confirmed by stopped-flow absorption (spectra of selected reaction times are shown in Figure S3) and freeze-quench EPR experiments. A190-versus-time traces from the reaction exhibit two resolved development phases followed by a slower decay phase (Figure 2B). Both development phases exhibit an approximately first-order dependence on H2O2 concentration (inset of Figure 2B). The plot of kobs for the slower of the two phases versus H2O2 concentration (blue circles) gives a second-order rate constant (8 ± 1 M−1 s−1) for combination with H2O2 that is indistinguishable from the value determined for the reaction of the MnIII/FeIII–R2 complex with H2O2. Values of kobs for the decay phase are also indistinguishable from those for the decay phase in the reaction of the MnIII/FeIII–R2 complex at equivalent concentrations of H2O2 (not shown). Thus, the slower development phase and the decay phase reflect conversion of the MnIII/FeII–R2 complex to the MnIV/FeIV–R2 complex (development) and then to the MnIV/FeIII–R2 complex (the decay). The more rapid development phase corresponds to conversion of the MnIII/FeII–R2 complex to the MnIII/FeIII–R2 complex by the first reaction with H2O2. The second-order rate constant for this step, 1.7 ± 0.3 M−1 s−1 [from the inset of Figure 2B (gray diamonds)], is ~200 times that for the second H2O2-mediated cluster oxidation and similar to the rate constant for conversion of fully reduced (Fe2II/III) Ec R2 to met-R2 by H2O2 [6 ± 1 M−1 s−1 (W. Jiang, C. Krebs, and J. M. Bollinger, Jr., unpublished data)].

EPR spectra of samples freeze-quenched during the reaction provide additional evidence for sequential accumulation of MnIII/FeIII–R2 and MnIV/FeIV–R2 intermediates (Figure 4). The spectral features of the first intermediate are broad3 and thus neither as intense nor as easily quantifiable as the features of the MnIV/FeIV–R2 intermediate. Nevertheless, these features can readily be discerned (arrows in Figure 4) at shorter reaction times (e.g., 0.090 and 1.5 s) in the regions outside the sharp six-line spectrum of the MnIV/FeIV–R2 complex and can be seen to decay at longer times (13 s). The much sharper and more well-defined features of

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3 The spectrum is published again here for comparison to the spectra of the R2 sample converted to the MnIV/FeIII state by H2O2.
Scheme 1: Interconversions of Oxidation States of the Mn/Fe Cluster in C. trachomatis R2

**DISCUSSION**

Scheme 1 summarizes the demonstrated redox interconversions of the Ct R2 Mn/Fe cofactor. In considering the potential relevance of the conversions mediated by H₂O₂ to the evolution and function of the novel cofactor, it is important to note that H₂O₂ is but one of several ROS generated by the host’s innate immune response. Others, including nitric oxide (NO), superoxide, and peroxynitrite, are in some respects more reactive and seemingly more likely to target a bacterium’s RNR. Indeed, NO (25, 26, 28, 29), superoxide (27), and peroxynitrite (30) have been reported to target the Y’s of conventional class I RNRs, but we are not aware of any report that H₂O₂ does so. Nevertheless, a plausible mechanism by which H₂O₂ might inhibit a conventional class I RNR can be formulated from published data. As previously noted, it is known that regeneration of the $Y^*$ by in situ reduction of the Fe₃⁺/II cluster to Fe₃⁺/II and reaction of O₂ with the fully reduced cluster occurs in E. coli (18, 19) and probably also in other organisms. The presence of H₂O₂ would lead to a partition of the Fe₃⁺/II form between conversion to active R2 (by O₂) and conversion to met-R2 (by H₂O₂). For the case of the Ec protein, the rate constant for reaction of the fully reduced protein with O₂ is only ~50 times greater than the rate constant for its reaction with H₂O₂. Thus, H₂O₂ in the millimolar concentration range should be capable of competing effectively with ambient O₂ in aerobically growing cells for reaction with the Fe₃⁺/II—R2 complex. The resulting conversion to met-R2 would delay reactivation by requiring (at least) another round of cluster reduction. Reaction of the met form with H₂O₂ to regenerate the active state directly, without the need for cluster reduction, is so inefficient in vitro (20) that it is not expected to be important in vivo. By contrast, trapping of the Mn/II/Fe₃⁺/II form of Ct R2 by H₂O₂ gives a form, Mn/II/Fe₃⁺/II—R2, that reacts efficiently with H₂O₂ to generate the active form. Thus, if inhibition of the ancestral form of the class Ic RNR (which might have been a Fe₃⁺/III-containing enzyme) by H₂O₂ was an important defense mechanism for the ancestral host, then the advent of the Mn/Fe cofactor could have conferred a selective advantage to the mutant bacterium.

The structural changes to R2 (and perhaps also to R1) that might have been needed for this evolutionary “metal makeover” (34) remain to be established and may shed more light on the evolution of the cofactor.

**SUPPORTING INFORMATION AVAILABLE**

A 4.2 K/53 mT Mössbauer spectrum of a sample prepared by treating the Mn/III/Fe³⁺—R2 complex with 1.2 equiv of dithionite, selected time-dependent UV—visible absorption spectra from the reactions of the Mn/III/Fe³⁺—R2 and Mn/III/Fe³⁺—R2 species with H₂O₂, absorbance-versus-time traces from the reaction of the Fe₃⁺/III—R2 complex with H₂O₂, and kinetics of the decomposition of H₂O₂ by various forms of R2. This material is available free of charge via the Internet at http://pubs.acs.org.

**REFERENCES**

Activation of *C. trachomatis* Ribonucleotide Reductase by H$_2$O$_2$

Biochemistry, Vol. 47, No. 15, 2008 4483


Branched Activation- and Catalysis-Specific Pathways for Electron Relay to the Manganese/Iron Cofactor in Ribonucleotide Reductase from *Chlamydia trachomatis*

Wei Jiang,‡ Lana Saleh,‡,§ Eric W. Barr,‡ Jiajia Xie,‡ Monique Maslak Gardner,‡ Carsten Krebs,*,‡,⊥ and J. Martin Bollinger, Jr.*,‡,⊥

Department of Biochemistry and Molecular Biology and Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

Received May 12, 2008; Revised Manuscript Received June 27, 2008

ABSTRACT: A conventional class I (subclass a or b) ribonucleotide reductase (RNR) employs a tyrosyl radical (Y*) in its R2 subunit for reversible generation of a 3'-hydrogen-abstracting cysteine radical in its R1 subunit by proton-coupled electron transfer (PCET) through a network of aromatic amino acids spanning the two subunits. The class Ic RNR from the human pathogen *Chlamydia trachomatis* (Ct) uses a MnIV/FeIII cofactor (specifically, the MnIV ion) in place of the Y* for radical initiation. Ct R2 is activated when its MnIV/FeIII form reacts with O2 to generate a MnIV/FeIV intermediate, which decays by reduction of the FeIV site to the active MnIV/FeIII state. Here we show that the reduction step in this sequence is mediated by residue Y222. Substitution of Y222 with F retards the intrinsic decay of the MnIV/FeIV intermediate by ~10-fold and diminishes the ability of ascorbate to accelerate the decay by ~65-fold but has no detectable effect on the catalytic activity of the MnIV/FeIII→R2 product. By contrast, substitution of Y338, the cognate of the subunit interfacial R2 residue in the R1 ↔ R2 PCET pathway of the conventional class I RNRs [Y356 in *Escherichia coli* (Ec) R2], has almost no effect on decay of the MnIV/FeIII intermediate but abolishes catalytic activity. Substitution of W51, the E. coli cognate of the cofactor-proximal R1 ↔ R2 PCET pathway residue in the conventional class I RNRs (W48 in Ec R2), both retards reduction of the MnIV/FeIV intermediate and abolishes catalytic activity. These observations imply that Ct R2 has evolved branched pathways for electron relay to the cofactor during activation and catalysis. Other R2s predicted also to employ the Mn/Fe cofactor have Y or W (also competent for electron relay) aligning with Y222 of Ct R2. By contrast, many R2s known or expected to use the conventional Y* -based system have redox-inactive L or F residues at this position. Thus, the presence of branched activation- and catalysis-specific electron relay pathways may be functionally important uniquely in the Mn/Fe-dependent class Ic R2s.
oxidation of Y122 by one electron to the radical, and transfer of an “extra” electron (20–22). During activation of Ec R2, the extra electron is transferred to a very reactive adduct (23). Thus, Ec uses a rapid redox equilibrium with exogenous reductants (e.g., FeII or ascorbate) (23). Intermediate X oxidizes the nearby tyrosine residue (Y122) in the final and slowest step of the reaction, yielding the active FeIII/III/Y- form of the protein (19, 20). When the activation reaction is carried out in the presence of > 10 mM Mg2+, W48’+ engages in a rapid redox equilibrium with Y356 (32), the next R2 residue in the proposed R1 → R2 PCET pathway (4, 13, 33). The reversible formation of the Y356’ radical initiates an efficient pathway for decay of W48’+ even in the absence of a facile one-electron reductant (32). Thus, Ec R2 utilizes the same two-residue pathway for electron relay to its cofactor during both activation and catalysis.

We recently showed that the class Ic RNR from Chlamydia trachomatis (Ct) employs a high-valent, heterobinuclear MnIV/FeIII cofactor in place of the FeII/III/Y cofactor of the conventional class I system (34, 35). Use of a mechanism-based inactivator (2′-deoxy-2′-azidoadenosine-5′-diphosphate) provided evidence that the MnIV/FeIII cofactor undergoes reduction to MnIII/FeIII during catalysis (34). Results obtained upon treatment of the enzyme with the well-known class I RNR inhibitor, hydroxurea, provided additional support for this hypothesis (36). Presumably, reduction of the MnIV ion of the cofactor to MnIII generates the C in the Ct R1 subunit via the intersubunit PCET pathway, of which all residues are conserved (Figure 1A, red dotted lines) (37). In analogy to the conventional (FeII/III/Y) R2 proteins, activation of Ct R2 entails reaction of its fully reduced (MnII/FeII) cluster with O2 (34). A MnIV/FeIV intermediate accumulates almost stoichiometrically and then decays by slow
decay step is analogous to the relay of the extra electron to the diiron site by W48 during activation of Ec R2 (23). However, no evidence of accumulation of W+ from the corresponding Ct R2 residue, W51, was obtained (38).

In this study, we showed that Y222, a surface residue aligning with the redox-inactive L233 of Ec R2, cooperates with W51 to relay the extra electron to the MnIV/FeIV intermediate during activation of Ct R2 (Figure 1A, green dotted lines). The relevance of Y222 and the importance of Y338 (the cognate of Ec Y356) for catalytic activity imply that, unlike the Ec protein, Ct R2 uses distinct electron-relay pathways for activation and catalysis (Figure 1A, red dotted lines). The strict conservation of an ET-competent residue (Y or W) at the position corresponding to Y222 in other presumptively MnFe-dependent R2 proteins suggests that the novel, activation-specific, electron relay element is functionally important in the class Ic RNRs.

MATERIALS AND METHODS

Construction of Vectors for Overexpressing Histidine-Tagged Versions of Ct R2 Variants Y222F, Y338F, and W51F. Construction of the plasmid vector, pET28a-CtR2-wt, which directs overexpression of the N-terminally Histagged wild-type (wt) Ct R2 protein, was described previously (34). The vector for directing overexpression of the Y222F variant was constructed by using the Quikchange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), with pET28a-CtR2-wt as the template and primers 1 (5′-GGA CAA TAT CAA TTC AT-3′) and 2 (5′-GGA TCT CTG TAG GGA GAA-3′) showing the positions of residues in the aforementioned electron relay pathways.

FIGURE 1: Branched electron relay pathways in Ct RNR. (A) The catalysis-specific intersubunit pathway between the MnFe cluster in R2 and the conserved cysteine residue in R1, C672, is indicated by the red dotted lines. The conserved residues proposed to participate in electron relay are indicated. The activation-specific pathway is indicated by green dotted lines. This figure was adapted from the docking model for Ec RNR (4, 5). (B) Schematic based on the X-ray crystal structure of Ct R2 (42) showing the positions of residues in the aforementioned electron relay pathways.

2 Presumptive MnFe-dependent R2 proteins were identified by pBLAST queries of the NCBI nr protein database with the sequence of Ct R2. As previously reported (42), R2s with F and E aligning with F127 and E89 of Ct R2 (which are Y122 and D84, respectively, in Ec R2) are assigned to class Ic and are expected to employ MnIV/FeIII cofactors. Among the ~30 presumptive MnFe-dependent class Ic R2s identified by this search, all possess Y or W at the position aligning with Y222 of Ct R2.
Overexpression and Purification of Ct R2 Proteins. The variant Ct R2 proteins were prepared as previously described for the wt protein (34). His-tagged proteins were overexpressed in *E. coli* BL21(DE3) (Novagen, Madison, WI), purified by metal ion affinity chromatography, and depleted of metal ions by the previously described reductive chelation and EDTA dialysis steps (34). The concentrations of apoproteins were determined by absorbance at 280 nm with monomeric molar absorptivities (57750 M⁻¹ cm⁻¹ for the wt, 56470 M⁻¹ cm⁻¹ for Y222F and Y338F, and 52060 M⁻¹ cm⁻¹ for W51F) calculated by the method of Gill and von Hippel (39).

Moessbauer-Spectroscopic Characterization of the Mn₄⁴⁰Fe₃⁹ Complexes of the Ct R2 Proteins. The Mn₄⁴⁰Fe₃⁹ products of the O₂ reactions of wt, Y222F, Y338F, and W51F Ct R2 proteins were prepared with ~95% ⁵⁷Fe-enriched Fe₃⁹ as previously described (40). The Moessbauer spectrometer has been described previously (41).

Stopped-Flow Absorption and Freeze-Quench EPR Kinetics Experiments. The stopped-flow and freeze-quench apparatus and procedures and the EPR spectrometer have been described previously (41). Kinetic traces from the reactions of the Mn₄⁴⁰Fe₃⁹–R₂ complexes with O₂ were analyzed by nonlinear regression according to eq 1, which gives absorbance as a function of time (A₁) for a system of two parallel, irreversible, first-order reactions in terms of rate constants for the two steps (k₁ and k₂), their associated amplitudes (ΔA₁, and ΔA₂), and the absorbance at time zero (A₀).

\[ A₁ = A₀ + ΔA₁[1 - \exp(-k₁t)] + ΔA₂[1 - \exp(-k₂t)] \]  (1)

Although the Ct R2 reaction comprises two sequential irreversible steps, formation of the intermediate is so much faster than its decay (k₁ > 300k₂) that the equation describing the sequential case simplifies to that describing the parallel case. (The approximation of the second-order formation step as a pseudo-first-order step is acceptable with the excess of O₂ employed.) The kinetics of the Mn₄⁴⁰Fe₃⁹–R₂ intermediate predicted by the fit rate constants were calculated according to eq 2, to which eq 1 simplifies in considering concentration rather than absorbance (again, appropriate only for the case of k₁ >> k₂).

\[ [\text{Mn}^{IV}/\text{Fe}^{IV} - \text{R}_2] = [\text{Mn}^{III}/\text{Fe}^{III} - \text{R}_2]_0 \exp(-k₂t) - \exp(-k₁t) \]  (2)

Determination of Catalytic Activities of the Ct R2 Proteins. The same Mn₄⁴⁰Fe₃⁹–R₂ samples characterized by Moessbauer spectroscopy were used to determine the catalytic activities of the wt and variant proteins. Activity was quantified by the previously described mass spectrometric assay (34). In assays of the active wt and Y222F R₂ proteins, 1 μM R₂ monomer was used. To improve the detection limit in assays of the inactive Y338F and W51F variant proteins, 40 μM R₂ monomer was used. Reactions were initiated by addition of R₂. They contained in a final volume of 200 μL a 10-fold excess of ΔA₁(1–248) Ct R₁ (34, 37), 2 mM CDP, 0.5 mM ATP, and 10 mM DTT in 20 mM Na-Hepes buffer (pH 7.6). They were allowed to proceed at 37 °C for 30 min. They were terminated by addition of HCl to a final concentration of 100 mM. Precipitated protein was removed by filtration through a Microcon YM-3 device (Millipore Corp.). A 10 μL aliquot of the filtrate was injected with a mobile phase of 10% acetonitrile, 90% water, and 0.5 mM HCl running at 0.05 mL/min onto a Waters (Milford, MA) Micromass ZQ 2000 mass spectrometer with an electrospray ionization probe operating in the negative ion mode. Spectrometer conditions were as follows: capillary voltage, 4.00 kV; cone voltage, −50 V; extractor voltage, −2 V; RF lens voltage, 0 V; source temperature, 80 °C; desolvation temperature, 450 °C; desolvation gas flow rate, 150 L/h; cone gas flow rate, 60 L/h. The ion currents at m/z 402 and 386 (M⁻ for CDP and dCDP, respectively) were continuously and simultaneously monitored after injection. Triplicate injections of each reaction sample were performed. The ratio of the heights of the CDP and dCDP peaks to the sum of these peak heights was multiplied by the initial concentration of CDP (2 mM) to give the concentrations of substrate and product in each reaction sample. Validation of the assay method is provided in the Supporting Information.

RESULTS

Discovery of Electron Relay by Y222 during O₂ Activation by the Fe₄³⁹ Form of Ct R2. Prior to our discovery that the active form of Ct R2 contains a heterobinuclear Mn₄⁴⁰Fe₃⁹ cofactor that forms by reaction of the Mn₄⁴⁰Fe₃⁹ cluster with O₂ (34), our initial experiments on the catalytically inactive homobinuclear (Fe₂)₃⁹ form of the protein revealed the participation of Y222 in electron relay to the cofactor during O₂ activation. Reaction of the Fe₂₃⁹–R₂ protein (1.5 equiv of Fe₃⁹ per monomer) at 5 °C with O₂ results in rapid development of the sharp ~410 nm absorption signature of a tyrosyl radical (Figure 2A, marked by the arrow). This peak decays within 50 s to yield the featureless spectrum of the Fe₂₃⁹ product (blue spectrum). During activation of *Ec* R₂, a transient W48 cation radical (W48⁺) accumulates under these conditions (23, 24), and the presence of a high concentration (>10 mM) of Mg²⁺ engages a rapid redox equilibrium between W48 and the next residue in the PCET pathway, Y336, resulting in co-accumulation of Y336⁺ and W48⁺ (32). To test whether the transient Y⁺ detected in the Ct R2 reaction resides on Y338, the cognate of W48 in *Ec* R₂, we noted that Y222 is close to W51 (see Figure 1 (41)) rather than the single µ-oxo of the *Ec* cluster (12).

Determinance of Catalytic Activities of the Ct R2 Proteins. The Mn₄⁴⁰Fe₃⁹–R₂ samples characterized by Moessbauer spectroscopy were used to determine the catalytic activities of the wt and variant proteins. Activity was quantified by the previously described mass spectrometric assay (34). In assays of the active wt and Y222F R₂ proteins, 1 μM R₂ monomer was used. To improve the detection limit in assays of the inactive Y338F and W51F variant proteins, 40 μM R₂ monomer was used. Reactions were initiated by addition of R₂. They contained in a final volume of 200 μL a 10-fold excess of ΔA₁(1–248) Ct R₁ (34, 37), 2 mM CDP, 0.5 mM ATP, and 10 mM DTT in 20 mM Na-Hepes buffer (pH 7.6). They were allowed to proceed at 37 °C for 30 min. They were terminated by addition of HCl to a final concentration of 100 mM. Precipitated protein was removed by filtration through a Microcon YM-3 device (Millipore Corp.). A 10 μL aliquot of the filtrate was injected with a mobile phase of 10% acetonitrile, 90% water, and 0.5 mM HCl running at 0.05 mL/min onto a Waters (Milford, MA) Micromass ZQ 2000 mass spectrometer with an electrospray ionization probe operating in the negative ion mode. Spectrometer conditions were as follows: capillary voltage, 4.00 kV; cone voltage, −50 V; extractor voltage, −2 V; RF lens voltage, 0 V; source temperature, 80 °C; desolvation temperature, 450 °C; desolvation gas flow rate, 150 L/h; cone gas flow rate, 60 L/h. The ion currents at m/z 402 and 386 (M⁻ for CDP and dCDP, respectively) were continuously and simultaneously monitored after injection. Triplicate injections of each reaction sample were performed. The ratio of the heights of the CDP and dCDP peaks to the sum of these peak heights was multiplied by the initial concentration of CDP (2 mM) to give the concentrations of substrate and product in each reaction sample. Validation of the assay method is provided in the Supporting Information.
reminiscent of the signature of W48$^{+}$ in the Ec R2 reaction (23, 24). These observations suggest that Y222 is the site of the transient Y$^{+}$ in the reaction of the wild-type Ct R2 protein and that its substitution with F causes accumulation of W$^{+}$, presumably residing on W51. The latter assignment is supported by experiments with the W51F variant (Figure 2D). Reaction of the Fe$^{3+}$/I$^{3+}$ complex of W51F Ct R2 with O$_{2}$ does not result in accumulation of the transient absorption band at ~550 nm. Rather, yet another transient absorption feature, a broad intense band at ~700 nm (arrow) that our previous work on D84E variants of Ec R2 implies can be attributed to a $\mu$-(1,2-peroxo)–Fe$^{3+}$/III complex (43–45), is observed.$^{4}$ Thus, an oxidized diiron intermediate accumulates in place of Y222$^{+}$ (or W51$^{+}$) in the W51F variant. The simplest interpretation of these results is that W51 and Y222 cooperate to relay an electron to the diiron cluster during O$_{2}$ activation, with Y222$^{+}$ being the more stable of the pathway radicals.

**Evidence for Electron Relay by Y222 Also during O$_{2}$ Activation by the Mn$^{II}$/Fe$^{II}$ Form of Ct R2.** To test whether the novel electron relay element, Y222, functions during formation of the active Mn$^{IV}$/Fe$^{IV}$ form of Ct R2, the kinetics of the O$_{2}$ reactions of the aforementioned variant proteins (W51F, Y222F, and Y338F) in their Mn$^{II}$/Fe$^{II}$ forms were compared to those of the wt protein (Figure 3A).$^{5}$ The substitutions have either no effect (Y222F and Y338F) or a minor effect (W51F) on the development of the 390 nm absorption band at the indicated reaction time after mixing of an O$_{2}$-free solution of 0.40 mM R2 (0.80 mM R2 monomer) and 0.60 mM Fe$^{II}$ with an equal volume of O$_{2}$-saturated buffer. For each spectrum that is shown, the 1.3 ms spectrum has been subtracted from the experimental spectrum at the indicated reaction time to illustrate the changes with time.

$^{4}$ The Mössbauer spectra of freeze-quenched samples exhibit the signature of the peroxide complex (43, 46) and confirm this assignment. These results will be presented elsewhere.
absorption of the MnIV/FeIV intermediate, and the Y338F substitution has no effect on the decay of this feature. By contrast, both the W51F and the Y222F substitutions retard decay of this feature by ∼10-fold (Table 1). Freeze-quench EPR experiments on the reactions of the wt, W51F, and Y222F were conducted to confirm that the absorbance-versus-time traces accurately reflect the kinetics of the intermediate (Figure 3B). The kinetics (black squares, blue circles, and green triangles) extracted from the intensities of the sharp six-line EPR feature of the MnIV/FeIV complex (Supporting Information, Figure S2) agree well with traces calculated by using the rate constants extracted from the stopped-flow data (solid black and blue lines), confirming that both the W51F and Y222F mutations stabilize the intermediate by retarding its reduction.

It was previously shown that ascorbate can accelerate the reduction of the MnIV/FeIV intermediate in the reaction of wt Cr R2 (38). Additional stopped-flow absorption experiments were conducted to test whether reduction by ascorbate is mediated by Y222 (Figure 3C). As previously reported, decay of the intermediate is accelerated with a hyperbolic dependence on ascorbate concentration in the reaction of the wt protein (black squares and fit line). The Y338F substitution has no significant effect on this behavior (red diamonds and fit line), consistent with its failure to retard the intrinsic contrast, both the W51F and the Y222F substitutions retard the Y222F variant (Table 1). The results imply that Y222 mediates reduction of the intermediate both in the absence and in the presence of ascorbate.

Verification by Mössbauer Spectroscopy of Formation of the MnIV/FeII Product in the Reactions of the Variant R2s. To verify that, despite the altered kinetics, decay of the MnIV/FeIV intermediate in the W51F and Y222F variants still yields the MnIV/FeII product previously described for the wt protein, Mössbauer spectra of the products were recorded (Figure 4). The MnIV/FeII cofactor has a triplet ($S_{\text{total}} = 1$) ground state resulting from antiferromagnetic coupling of its MnIV ($S_{\text{Mn}} = \frac{3}{2}$) and high-spin FeII ($S_{\text{Fe}} = \frac{5}{2}$) ions. It gives rise to a sharp quadrupole doublet at 4.2 K in zero field and a diagnostic broadening (due to the hyperfine interaction between the $S = 1$ electron spin ground state and the $^{57}$Fe nuclear spin) in the presence of weak external magnetic fields (35). Products of the Y222F, Y338F, and W51F reactions have spectra essentially identical to those of the wt protein in both 0 and 53 mT field, implying that all reactions form the MnIV/FeII cofactor.

Catalytic Activities of wt and Variant Ct R2s Implying Branched Activation- and Catalysis-Specific Electron Relay Pathways. The capacities of the wt and variant R2 products characterized by Mössbauer spectroscopy to support RNR activity in the presence of Ct R1 were quantified to examine the roles of the three aromatic residues in catalysis. The wt and Y222F Ct R2 samples supported indistinguishable activities of 0.60 ± 0.06 and 0.58 ± 0.02 s⁻¹ (mean ± standard deviation of four trials), respectively, implying that Y222 has no important role in the intersubunit PCET step that initiates turnover. By contrast, the activities of the Y338F and W51F variants were less than the detection limit of the assay (0.001 s⁻¹; Table 1), suggesting that Y338 and W51, like their cognates in the mouse and Ec R2 proteins (13, 14, 33), are both essential for the intersubunit PCET step. Thus, Ct R2 uses branched electron relay pathways for activation (W51→Y222) and catalysis (W51→Y338), of which the former is absent in Ec R2.

### DISCUSSION

Previous studies on the activation of the conventional Fe2III/III/Y-dependent Ec and Mus musculus (mouse) R2 proteins have extensively documented the requirement for, and mechanism of, the transfer of the extra electron that balances the four-electron reduction of O2 with the oxidation of two FeII ions and the tyrosine residue by one electron each (19–23, 32, 48). The same requirement applies to activation of the Mn/Fe-dependent Ct R2 (34), but comparison of published data for this reaction (38) with those for the conventional R2 proteins suggests that kinetic details of the steps are quite different. It has been shown that diiron–O2 complexes that are more oxidized than the product (Fe2II/III) complex by two electrons (i.e., peroxo–Fe2II/III or Fe2IV/IV complexes) are fleeting during activation of the conventional R2 proteins (23, 49), at least in part because transfer of the extra electron from the cofactor-proximal PCET tryptophan residue (W48 in Ec R2) is so rapid (>400 s⁻¹ at 5 °C in the Ec R2 reaction) (23). Further evidence suggests that this electron relay step occurs concomitantly (perhaps even concerted) with cleavage of the O–O bond of a (putatively) peroxo–Fe2II/III complex (50). The coupling of the oxidation of the tryptophan to O–O bond cleavage should make this
step thermodynamically favorable, resulting in the observed accumulation of the amino acid radical (W^•) (23, 24). By contrast, previous work on activation of Ct R2 revealed stoichiometric accumulation of the Mn^{IV}/Fe^{IV} complex, in which the O–O bond of O_2 has presumably already been cleaved (without net reduction), and no evidence for the accumulation of an amino acid radical during the subsequent, very slow reduction of the Mn^{IV}/Fe^{IV} complex to the stable, catalytically active Mn^{IV}/Fe^{III} form (38). In this case, the failure of a state containing the Mn^{IV}/Fe^{III} cofactor and an amino acid radical [the cognate of the X–W^{48^-}• “diradical” state in Ec R2 (23)] to accumulate could be explained by the lack of coupling of amino acid oxidation to O–O cleavage and the relatively modest reduction potential of the Mn^{IV}/Fe^{IV} intermediate (Scheme 1, bottom). It appears that substitution of the metal ion is more important than active site tuning in causing these differences, given that addition of O_2 to the Fe^{III} cluster in Ct R2 does result (as in the Ec reaction) in the rapid accumulation of a state containing the Fe^{II} complex, X [shown previously (42, 51–53)], and an amino acid radical (Figure 2A,B and Scheme 1, top). The difference in the location of the transient radical in the diiron reactions (Y222 in Ct R2 vs W48 in Ec R2) can be explained by the lower reduction potential of (neutral) Y^• compared to W^{48^-} (5). The phenolic hydroxyl of Y222 projects outward from the surface of Ct R2 into solution (Figure 1B) and should readily lose its proton to solvent or buffer upon phenol oxidation, localizing the “hole” at this site in preference to the more solvent-protected W51, which hydrogen bonds via its indole NH group to Asp226 (42) and may lose its proton less readily upon oxidation to the cation radical. From a chemical perspective, these considerations can explain the importance of the additional electron relay element in the Mn/Fe-dependent R2: the more favorable oxidation of Y222 (by PCET) may be required for efficient electron relay to the relatively stable Mn^{IV}/Fe^{IV} complex.

The biological and evolutionary rationale for the additional electron relay element is less apparent. The Y222F variant successfully assembles the Mn^{IV}/Fe^{III} cofactor and is then fully catalytically active. Thus, in vitro and upon a single activation event, Y222 is completely dispensable. The conservation among all presumptively Mn/Fe-dependent R2s of an electron relay-competent residue at this position might reflect a selective advantage conferred by ensuring that the protein is stable to repeated activation events occurring in vivo. Adventitious reduction of Y^• in the conventional R2s is known to occur (54), and the corresponding reduction of the Mn^{IV} site in the active form of the class Ic proteins would produce the inactive Mn^{III}/Fe^{III} form. Reactivation of this form, either by reduction to Mn^{III}/Fe^{II} followed by reaction with O_2 or by direct reaction with H_2O_2 (40), would obviate the more costly de novo resynthesis of R2. The extra relay element may be conserved because it prevents deleterious side reactions that might otherwise lead to progressive inactivation during this redox cycling of the protein in vivo. The conservation of Y or W at this position could also reflect the existence of a specific accessory protein for delivering electrons in vivo. Recent studies on the Ec protein YfaE have suggested that it is just such a specific accessory factor, serving either to reduce the Fe^{III} cluster in the inactive “met” (Y^•-reduced) form of the protein to Fe^{II} for subsequent reactivation by O_2, to deliver the extra electron during the activation reaction, or both (55). Y222 in Ct R2 seems ideally positioned to interact with a functionally homologous protein in C. trachomatis (Figure 1). Prospecting for genes that might encode such a factor is in progress.

SUPPORTING INFORMATION AVAILABLE

Comparison of the kinetics of the O_2 reaction of Mn^{II}/Fe^{II} Y222F Ct R2 obtained with the white light source and photodiode array detector to those obtained with the monochromatic light source and photomultiplier detector; time-dependent EPR spectra from the reactions of the wt, Y222F, and W51F R2 proteins; Mössbauer spectra of the final products of the O_2 reactions of the wt, Y222F, Y338F, and W51F proteins; Mössbauer spectra of the final products of the O_2 reactions of the wt, Y222F, Y338F, and W51F proteins recorded over a wide range of Doppler velocities; and figures validating the mass spectrometric activity assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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VITA

**Education**

1999 – 2003       B.Sc.   Nanjing University, CHINA

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