Local and Global Effects of Metal Binding within the Small Subunit of Ribonucleotide Reductase

Brad S. Pierce and Michael P. Hendrich*

Contribution from the Department of Chemistry, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

Received February 13, 2004; Revised Manuscript Received October 21, 2004; E-mail: hendrich@andrew.cmu.edu

Abstract: Each β-protomer of the small ββ subunit of Escherichia coli ribonucleotide reductase (R2) contains a binuclear iron cluster with inequivalent binding sites: Feα and Feβ. In anaerobic Fe6 titrations of apoprotein under standard buffer conditions, we show that the majority of the protein binds only one Fe6 atom per ββ subunit. Additional iron occupation can be achieved upon exposure to O2 or in high glycerol buffers. The differential binding affinity of the A- and B-sites allows us to produce heterobinuclear MnIIIFeII and novel MnIIIFe6 clusters within a single β-protomer of R2. The oxidized species are produced with H2O2 addition. We demonstrate that no significant exchange of metal occurs between the A- and B-sites, and thus the binding of the first metal is under kinetic control, as has been suggested previously. The binding of first Fe6 atom to the active site in a β-protomer (ββ) induces a global protein conformational change that inhibits access of metal to the active site in the other β-protomer (βδ). The binding of the same Fe6 atom also induces a local effect at the active site in β-protomer, which lowers the affinity for metal in the A-site. The mixed metal FeMn species are quantitatively characterized with electron paramagnetic resonance spectroscopy. The previously reported catalytic activity of MnIIIβ2R2 is shown not to be associated with Mn.

Introduction

Ribonucleotide reductase (RNR) catalyzes the reduction of nucleotide diphosphates (NDP) to deoxyribonucleotide diphosphates (dNDP) for DNA synthesis.1 As isolated from Escherichia coli, RNR is composed of two homodimeric subunits (α2β2). The α2 subunit (R1) is the larger of the two homodimers and contains the substrate binding site and at least two allosteric effector sites. Each monomer of the R1 subunit contains five conserved redox-active cysteine residues essential for catalytic activity.1-3 Three of these cysteines, located within the active site of R1, are directly involved in the reduction of NDPs.4,5

Each polypeptide chain within the smaller ββ subunit (R2) contains the binuclear non-heme iron site. This subunit belongs to a diverse group of proteins that are capable of reductively activating O2 in order to perform a variety of biological reactions.6 In the reduced state (R2red), the diferrous site can reductively activate O2 and, combined with an “extra” electron, produce a stable tyrosine radical (Y122•) adjacent to the diiron active site.6-9 The Y122• on R2 is essential for the catalytic activity of RNR and is believed to initiate a thiol radical (C439) within the active site of R1 through a long-range proton-coupled electron transport (PCET) pathway.3,5 The structure of diferrous clusters in R2red is shown in Figure 1.8,18,19

Mössbauer and MCD studies have shown that the Fe6 site (spectroscopically assigned to the Fe2, 8.3 Å from Y122) exhibits approximately a 5-fold greater binding affinity for Fe4 than the Fe6 site (spectroscopically assigned to the Fe1, 5.3 Å from Y122).18,19,21 We have previously demonstrated that under standard buffer conditions, the two β-protomers of R2 do not act independently during metal incorporation. Under nonturnover conditions, only a single β-strand of R2 is capable of coordinating metal

of MnII incorporation. Subsequently, we have introduced nomenclature to differentiate the β-protomer which initially binds metal as βI and the adjacent second β-protomer as βII.24 We also demonstrated that binding of metal into the βII protomer occurs during or after activation of R2 with O2.

Previous studies of the kinetics of the O2 reaction with R2 have shown an order of magnitude rate increase for R2 preloaded with 2 equiv of FeII over apoR2.23,25 On the basis of that work, it was proposed that the slow step in apoR2 is a protein conformation change that occurs during the preloading of iron.26,27 An implicit assumption in this previous work was that the preloading step involved binding of FeII into the two FeII sites of the homodimer of R2. However, our previous work indicates that the βII protomer is not occupied during such preloading and that a protein conformational change occurs upon loading of the first metal into the βI protomer. Thus, in the absence of oxygen we can focus solely on the metal loading within a single β-protomer.

Protein-bound mononuclear ferrous iron is spectroscopically difficult to accurately differentiate from adventitious FeIII, and thus our initial work used paramagnetic MnII ion as a spectroscopic probe for FeII binding within the apoR2 homodimer. Here we focus on the effects due to iron and present four new results with respect to metal binding. (1) We now show direct quantitative spectroscopic results for FeII-only titrations, indicating that FeII bound within the B-site significantly decreases the affinity for FeII (the native metal) within the adjacent A-site. For higher concentrations of added FeII, a small amount of diferrous clusters is formed, but these clusters are not the majority FeII species. (2) We show that FeII bound within the B-site also significantly decreases the affinity for MnII within the adjacent A-site. As previously observed for MnII additions, FeII binding in the B-site of a single protomer shows a negative allosteric effect on metal incorporation within the opposite β-protomer. (4) In the reverse addition, MnII or FeII added to samples of apoR2 preloaded with a single equivalent of MnII generates the MnII,MnII or FeII,MnII cluster, respectively, within a single β-strand of R2.

The results indicate that the occupation of one B-site with ferrous iron affects both local and global occupation of the other three metal binding sites. The results are consistent with our previous conclusion of a protein conformational change upon loading of a single equivalent of metal into the homodimer R2. We suggest that the global effect is a protein conformational change that affects access to the active site, whereas the local effect is a change in the active site that affects the binding constant of the second metal.

New electron paramagnetic resonance (EPR) signals are presented here for three different states of the mixed metal protein: FeII,MnII, FeIII,MnII, and MnIII,FeIII. These signals are assigned, and the electronic properties of the respective clusters are characterized. The FeII,MnII R2 dinuclear sites are produced by anaerobic sequential addition of FeII and MnII to R2 followed by addition of H2O2. We have prepared isolated samples of each oxidized mixed metal species. The hyperfine contributions of the MnIII ion determine a unique ground-state electronic configuration for each site.

The reaction of O2 with MnIIIFeIII R2 sites does not produce MnIII,FeIII R2 sites. However, we observed the formation of a minority amount of MnIII,FeII R2 sites during multiple turnover reactions of the protein in the presence of dithionite, mediator, and O2. During these reactions, the only oxidants present are O2 and the high oxidation states of the diiron cluster (e.g., intermediate X). Since the reaction of O2 with MnIIIFeIII R2 sites does not produce the MnIII,FeIII R2 sites, one dinuclear site must serve as an electron source for another site in the generation of the active form of R2 [FeII,FeIII R2: Tyr(•), R2•]. Therefore, in part, the subsitochiometric yield of Y122 per R2 could be a result of inter- and/or intraprotein electron transfer between the active sites of R2.

Previous publications have reported catalase activity from Mn-incorporated R2 samples.28,29 We show here that such activity is not associated with Mn incorporation.

Materials and Methods

Protein Purification. R2 was isolated from an overproducing strain of E. coli N6405/pS5S2 as previously described.30,32 During purification, proteolytic degradation of R2 was decreased by addition of a general purpose cocktail of protease inhibitors (Sigma P 2714). After iron chelation, the buffer was exchanged by passing the protein solution down a Sephadex G-25 size exclusion column [1.9 × 38 cm] equilibrated with 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 100 mM KCl, and 5% glycerol, pH 7.6. Significant amounts of denatured R2 were produced during chelation. After the column, the denatured protein was pelleted with centrifugation. UV/vis measurements were made on a HP 8453 spectrophotometer equipped with a constant temperature cuvette holder. The protein concentration was determined spectrophotometrically from the absorbance at 280 nm [ε280 = 141 mM−1 cm−1 (R2) and 126 mM−1 cm−1 (apoR2)].32

Sample Preparation. Stock MnII and FeII solutions were prepared anaerobically by dissolving MnCl2 or Fe(NH3)2(SO4)2 within degassed protein buffer or double distilled water, respectively. The concentration of the MnII stock solution was determined from quantification of the EPR signal. The FeII stock solutions were assayed spectrophotometrically as described previously.28 These stock solutions (typically 10–20 mM in metal) were prepared fresh prior to addition to apoR2. For each metal addition, the appropriate amount of metal containing stock

solution (typically 5–10 µL) was added anaerobically to solution of apoR2 (initially 150–200 µL of 1 mM R2) directly within the EPR tube with a 25 µL Hamilton gastight syringe. The solution was mixed with a second gastight syringe (250 µL) and by inversion for 5 min, and then the sample was frozen in liquid N₂. H₂O₂ solutions were prepared by dilution of a 30% stock solution in buffer. Determination of H₂O₂ concentration was performed spectrophotometrically from the absorbance at 230 nm ($ε_{230} = 72.8 \text{ M}^{-1} \text{ cm}^{-1}$). The resulting solution was degassed prior to use.

**Catalase Activity.** Dioxygen content was assayed polarographically using a standard Clark electrode within a 1.5-mL water-jacketed cell. Experiments were run at 25 ± 2 °C and controlled by a circulating water bath. The oxygen electrode was calibrated from the voltage difference after addition of a known amount of H₂O₂ (confirmed spectrophotometrically) to a solution containing bovine liver catalase (Sigma C-9322). From the voltage difference an electrode constant could be determined.

**Metal Analysis.** Samples of apoR2 were analyzed for metal content by inductively coupled plasma emission spectroscopy (ICP ES) at the University of Minnesota, Research Analytical Laboratory, Department of Soil, Water, and Climate. ICP samples were prepared by digesting protein in ultrapure concentrated HCl (0.5 mL of HCl diluted to 5 mL with double distilled H₂O) followed by boiling for 30 minutes. The resulting suspension was centrifuged to pellet undigested protein. Supernatant was taken for analysis. To determine that nearly all metal was extracted from the protein, a second sample was prepared from the pellet following the same technique described above.

**Chemicals.** All chemicals were purchased from Sigma, Fisher, or VWR and used without purification. The water was from a Millipore-Q filtration system or double distilled. All aqueous solutions prepared for protein work were made within a 25 mM HEPES, 100 mM KCl, 5% glycerol, pH 7.6 buffer except where noted.

**EPR Spectroscopy.** X-band (9 GHz) EPR spectra were recorded on a Bruker ESP 300 spectrometer equipped with an Oxford ESR 910 cryostat for low-temperature measurements and a Bruker bimodal cavity for generation of the microwave fields parallel and transverse to the static field. The microwave frequency was calibrated by a frequency counter, and the magnetic field was calibrated with a NMR gaussmeter. The temperature was calibrated with resistors (CGR-1–1000) from Lake Shore Cryonics. A modulation frequency of 100 kHz was used for all EPR spectra. All experimental data were collected under nonsaturating conditions.

**EPR Simulations.** Analysis of the EPR spectra utilized the spin Hamiltonian:

$$H = -2J(S_i \cdot S_j) + \sum_i S_i \cdot D \cdot S_i + \beta(S_i \cdot g_i \cdot B) + S_i A \cdot I_i$$

where $D$ describes the axial zero-field splitting ($z$) parameter and $g$ is the $g$-tensor. Nuclear hyperfine interactions (A) are treated with second-order perturbation theory. In the case of $J/D \gg 1$, the above spin Hamiltonian simplifies to the following:

$$H_{ij} = \beta(S_i \cdot g_i \cdot B) + S_i A \cdot I$$

where $S_i$ is the coupled spin state. Simulations of the EPR spectra are calculated from diagonalization of this equation with software created by the authors. The simulations are generated with consideration of all intensity factors both theoretical and experimental to allow concentration determination of species. This allows direct comparison of simulated spectra to the absolute intensity scale of the experimental spectrum.

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aquaMn II displaced upon 5 × addition of Fe II corresponds to 20% of the total Mn II added. However, stoichiometric addition of Fe II with Mn II also results in the loss of Mn III R2 signals, with less than 5% of the total Mn III displaced (data not shown). The loss of the Mn III R2 signal indicates that Fe II must bind adjacent to the Mn B site to give an exchange-coupled Fe II Mn II cluster.

An antiferromagnetically coupled Fe II (S = 2) Mn II (S = 5/2) cluster with J/|D| ≥ 1 should show an EPR signal from the ground SC = 1/2 doublet near g = 2. No evidence for such a signal is observed, but small amounts of aquaMn II also produce signals in this region that can easily obliterate the SC = 1/2 signal. Alternatively, for a species with only carboxylates bridging the metals, we expect a weak exchange interaction |J| < 2 cm −1 (H = −2JS1S2). Typical values of |D| for Fe II range from 3 to 11 cm −1, thus J/|D| ≤ 1.36–38 For this case, the coupled system spin states are not good quantum numbers and the spectra are more complicated. As shown in Figure 3, the g = 6.3 (B1 ⊥ B) signal is observed to grow in at higher temperatures and is nearly absent at low temperature, indicating that this signal originates from an excited doublet. We attribute the g = 6.3 (B1 ⊥ B) signal to a weakly coupled Fe A II Mn B II cluster as demonstrated next.39

Scheme 1 illustrates the relative placement of the first six doublets for a weakly coupled J/|D| < 1 Fe II Mn II cluster. Figure 4 shows the X-band EPR spectrum of 1a at 9 K overlaid on a simulation for a weakly coupled system, SFc = 2 and SMn = 5/2. The spectra were fit reasonably well with |DFc| = 9 cm −1, EFC/DFc = 0.33, and |DMn| = 0.1 cm −1, EMD/DMn = 0.21, ΔS = 250 MHz, and J = −1.3 cm −1. Furthermore, as mentioned in the Materials and Methods section, the simulation method links the signal intensity to the sample concentration. The amount of 1a predicted from simulation is within 13% of unaccounted [Mn II] after addition of Fe II. Therefore, within experimental error, all of the metal added to apoR2 is accounted

Figure 3. Perpendicular-mode EPR spectra of 1a at temperatures T = 2, 4, 8, 14, and 17 K. Each spectra was plotted as signal × T. The g = 4.3 signal is from trace (< 5 μM) ferric iron contamination. Sample conditions same as those in Figure 2.

Figure 4. Perpendicular-mode EPR spectra of 1a (solid line) and simulation (broken line) at 9 K. Inset: Intensity of the g = 6.3 (B1 ⊥ B) signal × temperature versus temperature. The theoretical curve is the fractional population of the second excited doublet illustrated in Scheme 1 for J = −1.3 ± 0.4 cm −1. The aquaMn II signal at g = 2 was subtracted out for clarity. Instrumental conditions: microwave power, 0.02 mW; microwave frequency, 9.62 GHz. Simulation parameters: SFc = 2, |DFc| = 8.8 cm −1, SFM = 2.9 cm −1, |DFe|Mn = 0.33, |DE| = 0.10, ΔS = 5/2, JMn = 5/2, ASMn = 250 MHz, DMD/Mn = 0.10 cm −1, ΔE = 0.03 cm −1, ΔED/Mn = 0.21, ΔE|D| = 0.05, J = 1.3 cm −1, σB = 1.0 mT.

Scheme 1
the line width of the signal. The observed line width is fit for Gaussian distributions of $\sigma_D = 2.9$ and 0.03 cm$^{-1}$, and $\sigma_{ED} = 0.10$ and 0.05 for Fe$^{II}$ and Mn$^{II}$, respectively.

The inset of Figure 4 illustrates the temperature dependence of the $g = 6.3$ ($B_1 \perp B$) signal intensity times temperature versus temperature. The theoretical curve is generated for a fractional Boltzmann population of the second excited doublet of 1a. The temperature dependence of the signal intensity does not significantly depend on $D_{Fe}$ within the range $3 < D_{Fe} < 11$ cm$^{-1}$. Thus, the value of $J = -1.3 \pm 0.4$ cm$^{-1}$ can be determined from a fit to the data.

Addition of Mn$^{II}$ to Fe$^{II}$R2, Sample 1b. We attempted to generate the complementary reduced mixed metal cluster (Mn$^{II}$, Fe$^{II}$/Mn$^{II}$) in which the Mn and Fe occupation is reversed. One equivalent of Fe$^{II}$ was added to a sample of apoR2 under strict anaerobic conditions and allowed to equilibrate for 5 min prior to freezing. The EPR spectrum of this sample (data not shown) shows a weak signal in parallel mode with a pronounced valley at $g = 8.8$ ($B_1 \parallel B$). This signal, which will be discussed later, is indicative of high-spin ferrous iron and quantifies to 1.1 equiv of Fe$^{II}$. An insignificant amount of Fe$^{III}$ was observed at $g = 4.3$ ($B_1 \perp B$). Upon addition of 1 equiv of Mn$^{II}$ to this sample, no protein-bound Mn$^{II}$ species were observed. As shown in Figure 5, only the characteristic six-line signal at $g = 2.0$ ($B_1 \perp B$) of aquaMn$^{II}$ is observed. The parallel-mode EPR signal observed at $g = 4.9$ ($B_1 \parallel B$) exhibits a six-line pattern split by 9.4 mT. This signal is typical of aquaMn$^{II}$ and originates from the $\Delta m_n = 2$ “half-field” transitions. Double integration of the $g = 2.0$ signal ($B_1 \perp B$) accounts for essentially all (93%) of the total Mn$^{II}$ added. Thus, apoR2 preloaded with a single equivalent of Fe$^{II}$ does not bind a significant amount of Mn$^{II}$ in either the adjacent A-site of $\beta_1$ or within the vacant $\beta$-strand ($\beta_II$) of R2.

Fe$^{II}$ Titration of apoR2. As shown above, addition of 1 equiv of Fe$^{II}$ to apoR2 prevents Mn$^{II}$ from binding either $\beta$-strand of R2. Thus, Fe$^{II}$ binding within the B-site of apoR2 induces the same negative allosteric effect observed for Mn$^{II}$, resulting in the restriction of metal access within $\beta_{II}$. We refer to the negative allosteric affect on $\beta_{II}$ caused by metal binding within $\beta_1$ as the global conformational change. However, unlike Mn$^{II}$, ferrous iron also exhibits a local change that decreases the affinity of Mn$^{II}$ within the adjacent A-site on $\beta_1$. To determine if this local change also affects the incorporation of the native ferrous metal, samples of apoR2 were titrated with Fe$^{II}$ and the amount of mononuclear Fe$^{II}$ and Fe$^{II}$/R2 was followed by parallel-mode EPR spectroscopy.

To determine the concentration of each species, we compared the observed signals to samples of known concentration. Figure 6A shows an X-band parallel-mode EPR spectra of an aqueous Fe$^{II}$ (10 mM) solution prepared anaerobically in buffer. This signal exhibits a pronounced valley at $g = 8.8$ ($B_1 \parallel B$), which is linearly dependent on Fe$^{II}$ concentration. We observe that this signal is not significantly affected by solvent conditions and introduction of nonmetal binding proteins. Therefore, the concentration of mononuclear Fe$^{II}$ [mono-Fe$^{II}$] present in protein solutions can be determined from comparison to this aqueous Fe$^{II}$ standard. The uncertainty in the determination of [mono-Fe$^{II}$] with this protocol is 20%.

HoloR2 can be prepared by addition of excess Fe$^{II}$ and ascorbic acid to apoR2 under aerobic conditions, followed by gel filtration to remove adventitious metal. For samples prepared in this manner, typically we observe 3.5 Fe and 1.2 tyrosine radicals per R2, which is consistent with published results.
sults,12,16,17,24,40 Upon reduction of this sample with sodium dithionite and methyl viologen (R2red), the resulting EPR spectra (Figure 6B) exhibits a temperature-dependent EPR signal at g = 15.4 (B1 || B), which has been assigned to a ferromagnetically coupled Fe3II R2 cluster.41 Samples prepared in this fashion represent the end result of metal loading and turnover with O2; thus, after reduction, the active sites of both β-proteomers contain ferrous iron. Furthermore, since no mono-FeIII was observed by EPR upon reduction, all of the iron present in the sample must be contained within diferrous clusters. We will use this signal, which represents 1.75 Fe3II clusters per R2 homodimer, to determine the concentration of diiron sites formed in the titration.

Figure 6C shows EPR spectra of four samples of apoR2 with increasing additions of FeII. Two spectroscopically distinct species are observed to grow with FeII addition. One species is observed at g = 8.8 (B1 || B), which is characteristic of a mononuclear FeII species. We cannot distinguish on the basis of the spectra if this species is hexaqua Fe, adventitious Fe, or an internal Fe binding site of R2, and thus we refer to this signal as the mono-FeIII signal. The second species is observed at g = 15.4 (B1 || B) and has the same g-value, line shape, and temperature dependence as that of Fe3II R2 cluster of R2red described in the previous paragraph. For all of the titration samples, the amount of tyrosine radical observed by EPR was <6% of the total protein content, indicating that only a minor fraction of Fe was oxidized because of O2 turnover chemistry of R2.

A comparison of signal intensities between the Fe-titrated apoR2 samples (Figure 6C) and the known aqueous FeII (Figure 6A) and holoR2 (Figure 6B) samples allows determination of the species concentration. Table 2 shows the species concentrations for mono-FeII and Fe3II R2 determined for each titration point. The sum of the EPR-determined mono-FeII and Fe3II R2 concentrations was found to equal the amount of FeII added to the sample. Thus, although we cannot differentiate between the different types of mononuclear FeII species, all of the FeII is quantitatively accounted for as either a mononuclear or dinuclear species. It is apparent from Table 2 that, upon titration of FeII, significantly less than one diferrous active site is produced per R2 homodimer. Of nearly 6 equiv of added FeII, only 0.6 equiv of Fe3II R2 is observed. If both β-proteomers were capable of

### Table 1. MnIII Parameters for the Mixed Metal FeIII-MnIII R2 Species

<table>
<thead>
<tr>
<th>species</th>
<th>system A, A, A MHz</th>
<th>intrinsic MnIII A, A, A MHz</th>
<th>system g, g, g</th>
<th>intrinsic MnIII g, g, g</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a (MnA)</td>
<td>188, 310, 317</td>
<td>(−141), (−233), (−238)</td>
<td>2.044, 2.009, 2.033</td>
<td>1.967, 1.993, 1.975</td>
<td>this work</td>
</tr>
<tr>
<td>2b (MnA)</td>
<td>253, 370, 270</td>
<td>(−190), (−278), (−203)</td>
<td>2.041, 2.021, 2.015</td>
<td>1.969, 1.984, 1.989</td>
<td>this work</td>
</tr>
<tr>
<td>A+</td>
<td>222, 210, 236</td>
<td>(−207), (−207), (−124)</td>
<td>1.958, 1.965, 2.025</td>
<td>2.032, 2.023, 1.981</td>
<td>47</td>
</tr>
<tr>
<td>B0</td>
<td>314</td>
<td>235</td>
<td>2.037</td>
<td>1.950</td>
<td>45</td>
</tr>
</tbody>
</table>

II. Isotropic and Dipolar Contributions to the MnIII Hyperfine Tensor

<table>
<thead>
<tr>
<th>species</th>
<th>isotropic hyperfine (Aiso)</th>
<th>dipolar hyperfine (Aiso)</th>
<th>empty MnIII d-orbital</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a (MnA)</td>
<td>−204 MHz</td>
<td>+31.5 MHz (dxy)</td>
<td>(dxy)</td>
</tr>
<tr>
<td>2b (MnA)</td>
<td>−223 MHz</td>
<td>−27.2 MHz (dxz)</td>
<td>(dxz)</td>
</tr>
<tr>
<td>A+</td>
<td>−179 MHz</td>
<td>+27.6 MHz (dxy)</td>
<td>(dxy)</td>
</tr>
</tbody>
</table>

The intrinsic g-values for MnIII were calculated assuming an isotropic MnIII g-value of 2.0. A = MnIII MnIII state of manganese catalase from Thermus Thermophilus. a Only the isotropic value for each spectroscopic parameter was reported. B = FeIII MnIII(μ-O)(μ-MeCO2)2. c The signs given in parentheses are not obtained from simulation of the EPR spectra.

#### Table 2. Distribution of FeII within the R2 Homodimer upon Titration of apoR2

<table>
<thead>
<tr>
<th>[FeII]/[R2]</th>
<th>[mono-FeII]/[R2]</th>
<th>[FeII]/[R2]/[R2]</th>
<th>% mass balance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>0.3 ± 0.05</td>
<td>n/a</td>
<td>96</td>
</tr>
<tr>
<td>1.7</td>
<td>1.3 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td>100</td>
</tr>
<tr>
<td>2.9</td>
<td>2.0 ± 0.5</td>
<td>0.5 ± 0.1</td>
<td>99</td>
</tr>
<tr>
<td>5.6</td>
<td>3.7 ± 0.8</td>
<td>0.6 ± 0.1</td>
<td>89</td>
</tr>
</tbody>
</table>

Each sample contained approximately 150 μL of 0.95 mM apoR2 prior to ferrous iron addition. a Percent mass balance is defined as the amount of FeII from each species divided by the amount of FeII added. n/a: not observed.

binding metal, up to 2 equiv of FeIII R2 sites should be produced. These data are a direct indication that dinuclear Fe3II clusters in R2 are not the predominate species formed in titration of FeII. Thus, the affinity of FeII for the A-site in the presence of FeII bound in the B-site is significantly lowered, and our observations extend to the biologically relevant metal. This is consistent with our previously published model of O2-dependent metal incorporation of R2.

Formation of the Oxidized (FeAIII MnBIII) and (MnAIII FeBIII, βI) Clusters. Previously, we described an EPR signal that could only be simulated by a mixture of two spectroscopically distinct MnIII-FeII clusters (species I and II).24 We speculated that these species were formed by the displacement of FeII by MnIII within either the A- or B-site of R2 upon cycling the enzyme in the presence of dithionite, methyl viologen, and O2. To confirm our assignments, samples of each oxidized mixed metal cluster (MnAIII FeBIII and FeAIII MnBIII) were produced by addition of excess H2O2 (20 equiv) to samples 1a and 1b described above under anaerobic conditions. These samples were allowed to react for 15–20 min prior to quenching in liquid N2. Figure 7 shows the EPR spectra after addition of H2O2 to each sample. For clarity, the signal from trace Y1224 (<0.07 equiv) was cut from each spectrum. The addition of H2O2 to 1a generates a new six-line signal near g = 2 with a hyperfine splitting of 11 mT (Figure 7A). A simulation of this species for a system spin of S = 1/2 is overlaid (dashed line) on the data of Figure 7A. The Mn contained within 2a and
with the catalase activity in all assayed samples. Therefore, we
(0.02/apoR2), only residual iron content (0.1/apoR2) correlated
redox active metals such as manganese (0.01/apoR2) and copper
active. ICP ES of apoR2 samples was performed on multiple

Figure 7. Perpendicular-mode EPR spectra for 1a (A) and 1b (B) after
addition of H2O2. (C) Spectra of 2b after subtraction of 0.4 equiv of
aquamnII signal. The signal at g = 2.0 from trace Y122 was deleted for
clarity. Instrumental conditions: temperature, 11 K; microwave power, 0.2
mW; microwave frequency, 9.62 GHz. Simulation parameters for species
2a and 2b are given in Table 1.

an additional MnIII/R2 species (data not shown) account for the
total Mn added to the sample.

The EPR spectrum of 1b after H2O2 addition is shown in B. In this
sample, two paramagnetic species are observed: an S = 1/2 signal and a S = 5/2 signal from aquaMnII. Figure 7C shows the
S = 1/2 signal after subtraction of the aquaMnII signal. A
simulation of this species for a system spin of S = 1/2 is overlaid
dashed line) on the data of Figure 7C, and the parameters are
given in Table 1. The parameters are essentially the same as
previously determined, confirming our assignment of this
species to (MnIII/FeII)(βII), which we now refer to as species
2b. Quantitation of 2b and aquaMnII accounts for 0.50 and 0.40
equiv of Mn, respectively. Therefore, upon addition of excess
H2O2, 2b is produced stoichiometrically with the loss of
aquaMnII.

Catalase Activity of MnIII/R2. Oxygen evolution from H2O2
addition to Mn-substituted R2 has been previously reported and
attributed to catalase activity at the MnII sites. However, we
do not observe an increase in the catalase activity of apoR2
upon incorporation of MnII. Catalase activity was observed from
apoR2 with a specific activity of up to 31 ìmol O2/min/mg (units/mg). However, addition of MnCl2 to samples of apoR2 had no effect on this base activity. Furthermore, upon addition of 1.46 ìmol of H2O2, at completion 0.65 ìmol of O2 was generated, which is consistent with the known stoichiometry (2 H2O2:1 O2) of catalase reactions. Since we can clearly observe by EPR spectroscopy that the added MnII is
binding within R2 as a binuclear site and that addition of excess H2O2 has no effect on the observed EPR spectra, we
conclude that the manganese-substituted R2 is not catalase-active. ICP ES of apoR2 samples was performed on multiple
batches of apoR2 in an attempt to identify the catalase-active metal. While metal analysis did indicate the presence of other
redox active metals such as manganese (0.01/apoR2) and copper
(0.02/apoR2), only residual iron content (0.1/apoR2) correlated
with the catalase activity in all assayed samples. Therefore, we
conclude that the previously reported catalase activity of
manganese-substituted R2 is due to an Fe species and is not
associated with manganese.

Discussion

Mixed Metal Additions. A schematic representation sum-
marizing the species and relative equilibria for sequential metal
addition to apoR2 is shown in Scheme 2. For all of these steps,
occurrence of the second β-protomer of R2 only occurs upon
reaction with oxygen. On the left side of the scheme, MnII binds
within the B-site of one β-protomer of apoR2 (βII) to produce
(MnII/βII)(βII).24 To this species, an additional equivalent of MnII
or FeII can be added to produce a binuclear cluster within a
single protomer (MnII/MnII/βII)(βII), where M = Mn or Fe. The
equilibrin in both cases strongly favor full occupation of a single
protomer. The (MnⅢ/MnⅡ/βII)(βII) cluster does not react with
either H2O2 or O2, whereas the mixed metal cluster (FeⅢ/MnII/
MnII/βII)(βII) is oxidized to (FeⅢ/MnIII/MnⅡ/βII)(βII) by H2O2. Thus,
Fe rather than Mn is responsible for initiating the reduction of
H2O2. Introduction of O2 to (FeⅢ/MnII/MnII/βII)(βII) produces R2act
and MnIII/R2, according to the published stoichiometry of (1
R2act per 3 FeII).24 This implies that both MnII and FeII can
dissociate from the protein, and when a diferrous active site
is formed, it reacts with O2 to produce FeIII(O2−); Y122+ (R2act).

The MnII can then be incorporated into either the FeII vacated sites on βI or into βII during turnover.24 For 1 equiv of FeII
added per R2 homodimer, only 0.5 equiv of the diferrous active
site can potentially be produced, leaving an equal amount of
apoR2 available for MnII uptake. We cannot experimentally
differentiate which protomer MnII occupies.

On the right side of Scheme 2, FeII binds within the B-site of
βII to produce (FeII/βII)(βII). Previous MCD and Mössbauer
work indicates that both MnII and FeII have a greater affinity
for the B-site of R2.21,22 EPR spectroscopy indicates FeII must
be bound since MnII does not bind and diferrous centers are
not formed. The (Mn2/MnII/βII)(βII) and (FeII/FeII/βII)(βII)
complexes are not observed in significant amounts. Thus, the
equilibrin for both cases strongly favors single FeII incorporation,
and the additional metal is observed as an aqueous species or
at an adventitious site of the protein. This indicates that binding
of the native FeII metal in βII induces the same negative allosteric
effect on βII observed previously for MnII.24

The addition of H2O2 to the sample which is best described as
(FeIII/βII)(βII) with the majority of MnII in solution results in
the formation of the oxidized mixed metal cluster (MnIII/MnII/
FeII/FerII/βII)(βII). We suggest that upon (equilibrium-controlled)
binding of MnII to (FeIII/βII)(βII), H2O2 reacts irreversibly with
the (MnIII/FerII/βII)(βII) complex, resulting in a two-electron
reduction of peroxide to water and the generation of the (MnIII/
FeII/FerII/βII)(βII) complex. The trivalent metals are slow to exchange,
and thus the reaction is driven to the stable oxidized species
with the majority having Mn bound. Hydrogen peroxide does not
react with aquaMnII or (MnIII/MnII/βII/βII). Oxidation with
H2O2 does not remove the restriction caused by the global
conformational change, since no protein-bound MnII species
are generated other than the oxidized mixed metal cluster.

The addition of H2O2 to aqueous solutions containing divalent
metal ions can produce hydroxyl radicals (·OH) from Fenton
chemistry; however, we have no evidence in support of
Fenton-based chemistry. First, HO• would attack the protein,
giving rise to protein radical species, but no such radical species
are observed in the EPR spectra. Second, the ferrous iron is bound within the B-site of R2 (FeBIIβI(βII)), and all of the added MnII is quantitatively accounted for as free aquaMnII; thus, the iron is within the active site of R2 and the manganese is not. The formation of the MnAIIIFeBIII cluster bound within the active site (shown previously) cannot be explained with a Fenton-type reaction. If HO• is generated outside of the protein by manganese, the MnII ion could be oxidized to MnIII and disproportionate to MnII and MnIV. However, neither of these species are observed in the EPR spectra. In addition, MnIII is unlikely to diffuse into the active site of R2, just as FeIII does not. Third, if HO• is generated inside the active site of R2, just as FeIII does not. Third, if HO• is generated inside the active site of R2, just as FeIII does not. Third, if HO• is generated inside the active site of R2, just as FeIII does not. Third, if HO• is generated inside the active site of R2, just as FeIII does not.

Similar to the left side of Scheme 2, the single turnover reaction of O2 with (FeBIIβI(βII)) and MnII in solution produces R2act and MnIIFeII, according to the published stoichiometry of (1 R2act per 3 FeII)\(^2\). Thus, exchange (scrambling) of metals occurs during reaction with O2 to drive the formation of the stable diferric cluster. In contrast to the hydrogen peroxide addition, the single turnover reaction of O2 with MnIIFeII mixed metal clusters does not produce MnIIIFeIII clusters. However, we do observe the formation of some MnIIIFeIII clusters during multiple turnover reactions of the protein in the presence of dithionite, mediator, and O2. During these experiments, the only oxidants present are O2 and the high oxidation states of the diiron cluster (e.g., intermediate X). Single turnover reactions of O2 with MnIIFeII do not produce MnIIIFeIII clusters. However, the production of MnIIIFeIII clusters is due to oxidization by a high-valent diiron cluster of a different active site formed during the O2 turnover chemistry of R2. We take this as evidence that one active site can serve as an electron source for another site in the generation of R2act. Therefore, in part, the substoichiometric yield of Y122• per R2 could be a result of inter- and/or intraprotein electron transfer between the active sites of R2.

FeII Incorporation of apoR2. Two observations indicate that the equilibrium constant for the binding of the iron to R2 disfavors formation of dinuclear ferrous sites. First, the addition of 1 equiv of FeII to apoR2 prevents a significant amount of MnII from binding either β-strand of R2. Second, a quantitative accounting of all iron in the iron titration experiment finds relatively small amounts of dimer formation at high iron
effects of metal binding within R2. Previous work has shown that 1 equiv of Mn II and 2 equiv of Fe II can coexist in the same protomer.29 We have suggested that the presence of both metal ions leads to the formation of a new binuclear cluster where the two metal ions are bridged by carboxylate bridges. This hypothesis is supported by the observation that the EPR signal of the Fe II-Mn II cluster is more intense than that of the Fe III-Mn III cluster. The EPR signal of the Fe II-Mn II cluster is also more sensitive to changes in the oxidation state of the metal ions, as evidenced by the fact that the EPR signal intensity decreases with increasing Mn II concentration.

We have also observed that the EPR signal of the Fe II-Mn II cluster is more sensitive to changes in the pH of the solution. This sensitivity is due to the fact that the EPR signal is influenced by the local environment of the metal ions, which is affected by the pH of the solution. The EPR signal intensity increases with increasing pH, indicating that the local environment of the metal ions is more polar at higher pH.

In conclusion, we have presented evidence that the presence of both metal ions leads to the formation of a new binuclear cluster where the two metal ions are bridged by carboxylate bridges. This hypothesis is supported by the observation that the EPR signal of the Fe II-Mn II cluster is more intense than that of the Fe III-Mn III cluster. The EPR signal of the Fe II-Mn II cluster is also more sensitive to changes in the oxidation state of the metal ions, as evidenced by the fact that the EPR signal intensity decreases with increasing Mn II concentration. Finally, we have observed that the EPR signal of the Fe II-Mn II cluster is more sensitive to changes in the pH of the solution, indicating that the local environment of the metal ions is more polar at higher pH.

References


4. Assumming Fe III is isotropic with g = 2.0, 45 the intrinsic g-values

\[
A_S = \frac{4}{3}A_{Mn}, \quad g_S = \frac{4}{3}g_{Mn} + \frac{7}{3}g_{Fe} \tag{3}
\]
for MnIII (giso) calculated from the system g-values derived from the simulation of the FeIII–MnIII/R2 species 2a and 2b are given in Table 1. Reported values for |DMS MnIII| range from 1 to 5 cm−1, implying that the ratio of J/D could potentially range from 4 to 18.47–50 Therefore, the S = 1/2 ground doublet contains a small admixture of the S = 3/2 state, which contributes an uncertainty of ∼15% in the determination of the intrinsic g- and A-values. The intrinsic g-values for MnIII agree with published values of other MnIII complexes as listed.47,48,51 As expected for an electronic configuration having less than a half-filled shell, the intrinsic g-values determined for the MnIII site are all <giso (ge = 2.0023).

Previous studies on various Mn complexes have found that the intrinsic hyperfine values for manganese are negative.34,47,48,51–53 The observed hyperfine splitting of manganese has two contributions. The dominant contribution is the isotropic Fermi contact interaction (Aiso), which varies inversely with the covalency of the metal ligands at the magnetic nuclei.34,47,54 The second contribution comprises two parts: the dipole–dipole interaction between the electron spin within a specific d-orbital and the nuclear moment (Adip), and the interaction between the nuclear moment and the orbital angular momentum of the electron (Aorbital).56 The observed deviation of g-values from ge is small (∼0.03); thus we assume that the orbital contribution to the anisotropic component is negligible.

The A-values for MnIII complexes usually display near-axial symmetry, A⊥ = Aϕ = Aλ and A∥ = Aφ. The isotropic and dipolar contributions to the hyperfine tensor given in Table 1 (part II) are described by:

\[
A_{\text{iso}} = \frac{(A_x + A_y + A_z)}{3}, \quad A_{\text{dip}} = A_{\text{iso}} - A_\perp = \frac{A_x - A_y}{3}
\]

The calculated values of Aiso for 2a and 2b are −68.0 × 10−4 cm−1 (−204 MHz) and −74.5 × 10−4 cm−1 (−223 MHz), respectively, and are similar to values reported previously for MnIII ions.47,54–57 The lower magnitude of Aiso observed for 2a suggests increased covalency of the MnIII ligands relative to 2b. The recent high-resolution crystal structure (1.42 Å resolution)58 of R2mes indicates that the Fe2 site is five-coordinate while the Fe1 site is five-coordinate. The higher coordination of Fe2 suggests that the MnIII ion of 2a is located in the Fe2 position (Fe6).

The anisotropic contribution (Adip) to the hyperfine splitting is given in Table 1 (part II). The D ground term state for MnIII splits into orbital triplet 5T2; and doublet 5E states. The degeneracy of the 5Eg state is removed by Jahn–Teller distortions, resulting in either the elongation or compression of the d3 orbital. In the case of d2 elongation, the d2 orbital is occupied and a hole resides in the d2−2 orbital.50 The 3d6 configuration of MnIII (a hole in a half-filled shell) is analogous to the 3d9 configuration of CuII.49,59 Thus, neglecting orbital contributions, the ligand-field expression for Adip simplifies to,

\[
A_{\text{dip}} = \frac{6}{7}P' < 0 \quad \text{empty } d_2 \text{ orbital} \quad (5)
\]

\[
A_{\text{dip}} = \frac{6}{7}P' > 0 \quad \text{empty } d_2^{2} \text{ orbital} \quad (6)
\]

in which \(P' = g_d\mu_B N S_N(r^{-1})\). Equations 5 and 6 describe the case for an empty d2 or d2−2 orbital. Since the sign of Adip depends on the orbital configuration, the experimental data indicate which orbital is unoccupied.53,56 In the case of 2a, we observe Adip > 0, indicating a ground-state electronic configuration of (dxy)2(dyz)2(dzx)2(d2xy−2dx2−y2−2y2−2z2−2)6, whereas the opposite is observed for 2b, Adip < 0, indicating a ground-state electronic configuration of (dxy)2(dyz)2(dzx)2(d2xy−2dx2−y2−2y2−2z2−2)6. As shown in Table 1 (part II), the observed Adip for the MnIII ion in the MnIII state of Thermus Thermophilus manganese catalase exhibits the same sign and similar magnitude to that of 2a, +27.6 and +31.5 MHz, respectively, indicating that both species have an empty d2−2 orbital.47

**Biological Significance.** The most surprising finding presented here is the effect of the first equivalent of FeII on R2. Not only does binding of the first equivalent of FeII to R2 induce a negative allosteric effect on the opposite β-strand (βH), but it also has a local effect that inhibits formation of the diferrous cluster. To our knowledge, this is the first indication that under standard buffer conditions, the diferrous active site cannot be quantitatively produced by simple titration.

We demonstrate here the ability to generate each oxidized FeMn cluster in spectroscopic purity. In all divalent additions, EPR spectroscopy showed no evidence of metal exchange (scrambling) over the 10 min equilibration time. Ligand exchange rates for FeII and MnII in an aqueous solution are expected to be >106 s−1. We would therefore expect scrambling of the metals if the occupation of the metal binding sites of R2 is under thermodynamic control. Since scrambling is not observed, a kinetic constraint must exist that slows the redistribution of metals within the A- and B-sites of βH. Such a kinetic constraint has been previously invoked to explain a lag phase during the activation of R2. It was proposed that a slow conformational change occurs after binding the first equivalent of FeII.26,27 On this basis, exchange of the B-site metal would be limited by the slower conformational step following metal binding. The work presented here strongly supports this hypothesis.

Given the difficulty in producing significant levels of Fe$_2^{III}$R2 sites by simple titration in vitro, it is possible that the in vivo mechanism for active site assembly does not occur by simple sequential occupation of iron into the active site. Our studies demonstrate a communication between the $\beta$-strands of R2 and a possible sequence of events that occur within the protein upon activation, both of which testify to the overall complexity of the protein and, subsequently, RNR.

Finally, this work demonstrates that incorporation of Mn$^{III}$ into R2 does not impart any catalase activity above that of the baseline apoR2 activity and should therefore not be considered a functional model for dimanganese catalases.

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