Mechanistic Implications for the Formation of the Diiron Cluster in Ribonucleotide Reductase Provided by Quantitative EPR Spectroscopy

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Abstract: The small subunit of Escherichia coli ribonucleotide reductase (R2) is a homodimeric (ββ) protein, in which each β-peptide contains a diiron cluster composed of two inequivalent iron sites. R2 is capable of reductively activating O2 to produce a stable tyrosine radical (Y122•), which is essential for production of deoxyribonucleotides on the larger R1 subunit. In this work, the paramagnetic MnII ion is used as a spectroscopic probe to characterize the assembly of the R2 site with EPR spectroscopy. Upon titration of MnII into samples of apoR2, we have been able to quantitatively follow three species (aquamnII, mononuclear MnII-R2, and dinuclear MnII-R2) and fit each to a sequential two binding site model. As previously observed for FeII binding within apoR2, one of the sites has a greater binding affinity relative to the other, Ki = (5.5 ± 1.1) × 103 M−1 and K2 = (3.9 ± 0.6) × 104 M−1, which are assigned to the B and A sites, respectively. In multiple titrations, only one dinuclear MnII-R2 site was created per homodimer of R2, indicating that only one of the two β-peptides of R2 is capable of binding MnII following addition of MnII to apoR2. Under anaerobic conditions, addition of only 2 equiv of FeII to R2 (Fe2II-R2) completely prevented the formation of any bound MnR2 species. Upon reaction of this sample with O2 in the presence of MnII, both Y122• and MnII-R2 were produced in equal amounts. Previous stopped-flow absorption spectroscopy studies have indicated that apoR2 undergoes a protein conformational change upon binding of metal (Tong et al. J. Am. Chem. Soc. 1996, 118, 2107–2108). On the basis of these observations, we propose a model for R2 metal incorporation that invokes an allosteric interaction between the two β-peptides of R2. Upon binding the first equiv of metal to a β-peptide (βI), the aforementioned protein conformational change prevents metal binding in the adjacent β-peptide (βII) approximately 25 Å away. Furthermore, we show that metal incorporation into βII occurs only during the O2 activation chemistry of the β-peptide. This is the first direct evidence of an allosteric interaction between the two β-peptides of R2. Furthermore, this model can explain the generally observed low Fe occupancy of R2. We also demonstrate that metal uptake and this newly observed allosteric effect are buffer dependent. Higher levels of glycerol cause loss of the allosteric effect. Reductive cycling of samples in the presence of MnII produced a novel mixed metal FeIII-MnIII-R2 species within the active site of R2. The magnitude of the exchange coupling (J) determined for both the MnII-R2 and FeIII-MnIII-R2 species was determined to be −1.8 ± 0.3 and −18 ± 3 cm−1, respectively. Quantitative spectral simulations for the FeIII-MnIII-R2 and mononuclear MnIII-R2 species are provided. This work represents the first instance where both X- and Q-band simulations of perpendicular and parallel mode spectra were used to quantitatively predict the concentration of a protein bound mononuclear MnII species.

Ribonucleotide reductase (RNR) catalyzes the reduction of ribonucleotides to deoxyribonucleotides necessary for DNA synthesis. As isolated from Escherichia coli, RNR is composed of two nonidentical homodimeric subunits (α2β2). The α2 subunit (R1) is the larger of the two homodimers and contains the substrate binding site and two allosteric sites. The active site of the R1 subunit is composed of 5 redox-active dithiols derived from cysteines. These cysteines are ultimately responsible for the reduction of the ribonucleotide within the R1 active site.

Each polypeptide within the smaller β2 subunit (R2) contains a binuclear non-heme iron site. This subunit belongs to a diverse group of proteins that are capable of reductively activating O2 to perform a variety of biological reactions. 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. The radical is essential for the catalytic activity of RNR.3–8

Although the above reaction implies that each Schematic representation of the diferrous R2 active site adapted from refs 7, 17.

The complete reduction of O2 to H2O by R2 requires four electrons, three of which are derived from the diiron cluster and Y122•. In the absence of an additional reductant, FeII can be oxidized by O2 to form a cysteinyl radical in the R1 subunit active site.4,15,24 During the O2 activation of R2, the radical generating reaction is shown below

\[
3\text{Fe}^{II} + \text{Y122-OH} + \text{O}_2 + H^+ \rightarrow \text{Fe}^{III}(\text{O}_2)^{\text{II}}\text{Fe}^{III} + \text{Y122-O•} + \text{Fe}^{III} + H_2O
\]

The use of FeA and FeB to designate the crystallographic iron sites Fe1 (5.3 Å from Y122) and Fe2 (8.3 Å from Y122) is independent of FeII and O2 concentration, formation of X involves in apoR2. Therefore, neither FeII nor O2 can be involved in the rate-limiting step. Additionally, preloading apoR2 with 2 equivalents of FeII prior to introduction of O2 increases the rate of X formation by an order of magnitude (8 s1 vs 60–80 s1).21,24 Therefore, it was proposed that initial binding of the first FeII is followed by a protein conformational change prior to binding the second FeII.29,30 Moreover, it has been reported that during the assembly of the diferrous active site, shown in Figure 1, the two iron binding sites within a R2 monomer have different binding affinities. Mössbauer and MCD studies have shown that the FeB site (or Fe2, 8.3 Å from Y122) exhibits approximately a 5-fold greater binding affinity for FeII than the FeA site (or Fe1, 5.3 Å from Y122).17,18,32,33 The current model for assembly of the diferrous site of R2 involves noncooperative binding of FeII by both β-peptides independently of each other; sequentially occupying the FeA site of each β-peptide prior to the FeB sites.21,32,33

Protein-bound FeII is spectroscopically difficult to differentiate from free FeII in solution and to quantify accurately in low concentrations. This and the high oxygen sensitivity of R2 make characterization of the active site assembly experimentally difficult. For these reasons, Mn is an attractive spectroscopic probe for metal incorporation into R2. Manganese(II) has been shown to bind tightly within the active site of R2 with the same ligation as FeII.32,34,35 The MnII binding affinity must be comparable to FeII because addition of FeII to MnII incorporated R2 does not displace detectable amounts of MnII.34 In addition, MnII can serve as a probe for studying the assembly of the initial FeIR2 complex without the complication of further oxygen chemistry taking place. Finally, the various species of MnII are readily identifiable and quantified with EPR spectroscopy.

In the present study, MnII is used to probe the metal binding properties of R2. The formation of three spectroscopically distinct paramagnetic species were quantitatively monitored with EPR spectroscopy: mononuclear MnII-R2, dinuclear MnII-R2, and aquaMnII. The results will show that only 2 MnII ions are taken up sequentially (noncooperative binding) within a single

Figure 1. Schematic representation of the diferrous R2 active site adapted from refs 7, 17.
β-peptide of the R2 homodimer (ββ). This indicates that the two β-peptides of R2 do not act independently during metal incorporation. Therefore, we introduce nomenclature to differentiate the β-peptide forming a bimetallic cluster first as P1 and the adjacent second β-peptide as P2. The two β-peptides of R2 exhibit negative homotropic allosteric interaction during metal binding at the active sites. Incorporation of MnIII into the βP1-peptide only occurs during or after the activation of R2 with O2. We will propose this turnover-dependent metal binding explains the low Fe occupation of the protein that is generally observed.15,22,23,28,32

Quantitative X-band (perpendicular and parallel mode) and Q-band (perpendicular mode) simulations are provided for the mononuclear MnIII-R2 species, which is novel for MnIII sites with larger zero-field splitting. Reductive cycling samples of FeII-R2 in the presence of MnIII produced an additional minority species, which could be assigned to a new antiferromagnetically coupled FeIII-MnIII-R2 center and quantitatively simulated. Furthermore, because this signal can be reductively quenched only in the presence of the mediator methyl viologen, we conclude that it must originate from a cluster within the protein and not adventitiously bound.

Materials and Methods

Protein Purification. R2 was isolated from an overproducing strain of E. coli N4045/pSPS2 as previously described.16,37 During purifica tion proteolytic degradation of R2 was decreased by addition of a general purpose cocktail of protease inhibitors (Sigma P 2714). The integrity of R2 β-peptides was confirmed by a previously outlined anion exchange FPLC technique.23,38 Samples were eluted on a POROS HQ/H 4.6 mmD/100 mmL FPLC column (PerSeptive Biosystems) by NaCl gradient (0–700 mM) in 25 mM Hepes, pH 7.6. Peak detection was performed spectrophotometrically at 280 nm. The apoR2 was prepared as previously described.16,20,37 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 HEPES, 100 mM KCl, 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)].37

Sample Preparation. Stock MnIII 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 MnIII-stock solution was confirmed by EPR. The FeII-stock solutions were assayed spectrophotometrically as described below. These stock solutions (typically 10 to 20 mM in metal) were prepared fresh prior to addition to apoR2. For each titration point, 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.

Anaerobic Protocols. All anaerobic work was carried out on a gas train, in which analytical grade argon was passed through a copper catalyst (BASF) to remove trace O2 impurities within the gas. The argon was then sparged through water to hydrate the gas.

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.

Iron Analysis. The colorimetric determination of iron content by bathophenanthroline disulfonic acid was adapted from several published techniques.39–41 For each assay, 100 μL of approximately 0.1 mM R2 was added to a microfuge tube. The protein solution was acid hydrolyzed by addition of 50 μL of each of concentrated sulfuric acid and concentrated nitric acid. The resulting suspension was heated to 95 °C for 15 min. After heating, 50 μL of 30% hydrogen peroxide was added to the solution, and the sample was heated for an additional 15 min. This effectively bleached the brown color produced during acid hydrolysis and the end solution was transparent. After digestion of the sample, 1.0 mL of 3 M acetic buffer pH 7.5, 0.2 mL of 1% NH4OH, and 0.2 mL of 0.3% bathophenanthroline disulfonic acid were added to the sample. The resulting solution (pH 4.5) was allowed to equilibrate overnight at ambient temperature to ensure complete reduction of all ferric iron to ferrous. Iron quantitation was performed spectrophotometrically (λ = 535 nm, ε = 22.1 mM–1 cm–1).41,42 Two standard solutions were prepared using ferrous ammonium sulfate and ferric chloride. Results of the colorimetric assay were independently verified by quantitative EPR analysis of acid hydrolyzed samples.

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. Q-band (35 GHz) EPR spectra were recorded on a Bruker 200 spectrometer equipped with a locally built low-temperature microwave probe and a cryogenic system.43 For both instruments, the microwave frequency was calibrated by a frequency counter and the magnetic field with a NMR gaussmeter. The temperature of both instruments was calibrated using devices from Lake Shore Cryometrics. For X- and Q-band EPR, the modulation was 100 and 3 kHz, respectively. All experimental data were collected under nonsaturating conditions.

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

$$H_s = D\left(\frac{S_z}{3} - \frac{S(S + 1)}{3}\right) + E\left(S_x^2 - S_y^2\right) + \beta B \cdot g \cdot S + S \cdot A \cdot I$$

where D and E describe the zero-field splitting (zfs), and g is the g-tensor.44 Nuclear hyperfine interactions (A) are treated with second-order perturbation theory. Simulations of the EPR spectra are calculated from diagonalization of this equation with software created by the authors. The powder pattern is generated for a uniform spherical distribution of the magnetic field vector B. The transition intensities are calculated from the square of the transition moment. The spectral line width is dominated by D-strain and simulations use distributions of the D and E/D to give the correct line width, specified as σD and σE/D. Least squares and deconvolution analysis of the spectra are combined to allow relevant parameters to vary, while maintaining a sum of multiple species which best fits the experimental data. The simulations are generated with consideration of all intensity factors both theoretical and experimental to allow concentration determination of species.45 This allows direct comparison of simulated spectra to the absolute intensity scale of the experimental spectrum having a known

sample concentration. The only unknown factor relating the spin concentration to signal intensity is an instrumental factor that depends on the microwave detection system. However, this factor is determined by the spin standard, CuEDTA, for which the copper concentration was accurately determined from plasma emission spectroscopy.

Results

Titration of apoR2 with Manganese. Using the iron analysis protocol described in the Experimental Section, preparations of apoR2 were found to contain < 0.1 equiv of iron per R2 homodimer (\(\beta\beta\)). Reactivation of the apoR2 by addition of excess Fe II and ascorbate followed by gel filtration gave an optical spectrum similar to that published previously.\(^\text{16}\) The concentration of tyrosine radical was 1.2 per R2 homodimer as determined from EPR spectroscopy. Iron analysis of the reactivated R2 indicated the presence of 3.5 Fe atoms per R2 homodimer. Both of these values are comparable to the best reported values to date,\(^\text{15,22,28,32}\) thus, the preparations of apoR2 were essentially free of iron and viable to reactivation.

Representative X-band EPR spectra of apoR2 as a function of added Mn II are shown in Figure 2 for microwave fields (\(B_i\)) perpendicular (A) and parallel (B) to the static magnetic field (B). As the concentration of added MnII approaches 1.0 equiv per \(\beta\beta\), EPR signals from a MnII species are observed to grow in simultaneously at \(g = 15.4, 5.3, 3.0, \text{ and } 2.0\) (\(B_i \perp B\)) and a broad signal with a valley at \(g = 4.8\) (\(B_i \parallel B\)). In addition, a sharper signal grows in from a second species at \(g = 2\) with a sharp 6-line hyperfine pattern which is characteristic of aqua-MnII. AquaMnII (or free MnII) refers to Mn with predominantly water coordination which is not bound to interior protein sites. Above 1 equiv, the signals from the first species decrease and are virtually absent at 2 equiv of MnII added. However, the 6-line signal from aquaMnII continues to increase.

Figure 3 shows X- and Q-band EPR spectra of the MnII titration at 1 equiv of MnII. Overlaid on these spectra are quantitative simulations of a \(S = 5/2\) species with \(I = 5/2\). The spectra from both frequencies are fit with zero-field parameters \(D = 0.10 \text{ cm}^{-1}\) and \(E/D = 0.21\). The simulations unambiguously identify the signals as originating from a mononuclear MnII species. The large \(D\)-value is indicative of Mn binding to a protein site, and thus, we label this species MnII R2. As mentioned in the Method section, the fitting protocol links the signal intensity to the sample concentrations. The amount of aquaMnII in this signal is less than 0.02 equiv, and the amount of MnII R2 determined from the simulations is within 12% of 1 equiv. Thus, nearly all added MnII is observed as the MnII R2 species. The X-band features at \(g = 5.3\) (\(B_i \perp B\)) and \(g = 4.8\) (\(B_i \parallel B\)) originate from the \(\Delta m_s = \pm 2\) transitions, whereas all other signal originate from \(\Delta m_s = \pm 1\) transitions. The simulations of the parallel mode signals deviate from the data (45) Hendrich, M. P.; Petasis, D.; Arciero, D. M.; Hooper, A. B. J. Am. Chem. Soc. 2001, 123, 2997–3005.
Distributions in $D$ at low field due to nonlinear line shape distribution at $B = 0$. Distributions in $D$ and $E/D$ were found to be the dominant contribution to the line shape. The observed line widths are reasonably well matched for Gaussian distributions of contribution to the line shape. The observed line widths are for complexes with both display an inverse temperature dependence, as expected for complexes with $D \propto kT$.

As the $Mn^{II}$ is titrated above 1 equiv, the $Mn^{II}R2$ signal losses intensity and a new signals appear in both perpendicular and parallel mode. The temperature dependence of the new signals is shown in Figure 4 for a sample with 2 equiv of $Mn^{II}$. At the lowest temperature (2 K), the dominant signals are from small amounts of the mononuclear $Mn^{II}R2$ and aqua$Mn^{II}$ species ($\approx 0.1$ eq. each). As the temperature is raised, the new signals grow in at $g = 2.8, 2.4, 2.0$ and 1.5 ($B_1 \perp B$) and $g = 11.7, 7.7, and 4.6$ ($B_1 \parallel B$). The spectra of Figure 4 are scaled in intensity as signal times temperature ($S \times T$). Instrumental conditions: microwave frequency, 9.62 GHz ($B_1 \perp B$), 9.26 GHz ($B_1 \parallel B$); microwave power, 2–63 $\mu$W ($B_1 \perp B$), 0.2–6.3 $mW$ ($B_1 \parallel B$).

The spectra of Figure 4 are scaled in intensity as signal times temperature ($S \times T$). Instrumental conditions: microwave frequency, 9.62 GHz ($B_1 \perp B$), 9.26 GHz ($B_1 \parallel B$); microwave power, 2–63 $\mu$W ($B_1 \perp B$), 0.2–6.3 $mW$ ($B_1 \parallel B$).

Figure 4. Perpendicular (A) and parallel mode (B) $Mn^{II}R2$ signals at increasing temperatures; $T = 2, 5, 11, 18,$ and 23 K. The spectra are plotted as signal times temperature ($S \times T$). Instrumental conditions: microwave frequency, 9.62 GHz ($B_1 \perp B$), 9.26 GHz ($B_1 \parallel B$); microwave power, 2–63 $\mu$W ($B_1 \perp B$), 0.2–6.3 $mW$ ($B_1 \parallel B$).

Figure 5. Expanded parallel mode $Mn^{II}R2$ showing a multiline hyperfine pattern with splitting of 4.5 $\pm 0.5$ mT. Temperature, 11 K.

Figure 6. Signal times temperature versus temperature dependence of the $g = 11.7$ (●) and $g = 4.6$ (△) signals. The theoretical curves are the fractional populations of the $S = 1$ and $S = 2$ manifolds for $J = -1.8 \pm 0.3$ cm$^{-1}$ from $\alpha 2$.

an antiferromagnetically coupled $Mn^{II}$ site. In addition, the signals at $g = 7.7$ and 4.6 ($B_1 \parallel B$) shown in Figure 5 in expanded scale, exhibit a multiline hyperfine pattern with an average splitting of 4.5 mT. The intensity of the signals from the $Mn^{II}R2$ species is $90\%$ of maximum after addition of 2 equiv of manganese per $R2$, and reaches maximum at 2.3 equiv. Additional $Mn^{II}$ above 2.3 equiv. results in an increase in the aqua$Mn^{II}$ signal only, with no further changes to either $Mn^{II}R2$ or $Mn^{II}R2$ signals. The growth of $Mn^{II}R2$ species is concomitant with the loss of the $Mn^{II}R2$ species, indicating that the second $Mn^{II}$ ion is occupying a protein site adjacent to the site of the $Mn^{II}R2$ species. Thus, there are only two distinct $Mn$ protein binding sites for Mn, and we associate these with the normal Fe binding sites of $R2$.

Figure 6 shows the temperature dependence of the $g = 11.7$ and 4.6 ($B_1 \parallel B$) resonances plotted as signal intensity times temperature versus temperature. The two signals have different temperature dependences indicating that they originate from different spin manifolds of the antiferromagnetically coupled $Mn^{II}R2$ site. The decrease in the signal intensity of the $g = 11.7$ resonance prior to the $g = 4.6$ signal indicates that the $g = 11.7$ signal originates from a lower lying manifold. The isotropic exchange coupling term ($J$) from the Heisenberg Exchange Hamiltonian ($H = -2JS_1S_2$) can be determined from Figure 6. The data are fitted to theoretical curves using the

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following equation for the fractional population of a spin manifold ($n_s$)

$$\text{intensity} \times T \approx n_s = \frac{(2S + 1)e^{-JS(S+1)/kT}}{\sum_s (2S' + 1)e^{-JS'(S'+1)/kT}} \quad (2)$$

As shown in Figure 6, for $J = -1.8 \pm 0.3$ cm\(^{-1}\), the temperature dependencies of the $g = 11.7$ and $4.6$ signals can be simultaneously fit to transitions within the $S = 1$ and $2$ manifolds, respectively. The $g = 2.8$ and $1.5$ ($B_1 \perp B$) resonances have the same temperature dependence as the $g = 4.6$ ($B_1 \parallel B$) resonance, indicating that these signals are also derived from the $S = 2$ manifold.

The concentration of aquaMn\textsuperscript{II} was determined by double integration of the signal at $g = 2.0$ and by measurement of the 6-line hyperfine intensity. The monomeric Mn\textsuperscript{II}R2 concentration was determined by calibration of its signal intensity at low concentrations of Mn\textsuperscript{II} (<0.5 equiv), where the added Mn\textsuperscript{II} titrates linearly with the signals from the Mn\textsuperscript{II}R2 species. In addition, the simulations of Figure 3 confirmed the expected amount of Mn\textsuperscript{II}R2 species. The amount of Mn\textsuperscript{II}R2 formed during the titration was measured from the intensity of the $g = 4.6$ ($B_1 \parallel B$) signal at a temperature of 15 K. At 2 equiv of Mn\textsuperscript{II}, the amount of Mn\textsuperscript{II}R2 was determined by subtracting the amount of Mn\textsuperscript{II}R2 and aquaMn\textsuperscript{II} species observed from the total Mn\textsuperscript{II} added. This value was used to establish a response factor for the $g = 4.6$ signal at this temperature. The concentration of Mn\textsuperscript{II}R2 at other titration points is then determined by multiplying the measured signal intensity by the response factor.

At all titration points, the concentrations of Mn\textsuperscript{II}R2 obtained from this procedure were within 4% of the value predicted from subtracting the Mn\textsuperscript{II}R2 and aquaMn\textsuperscript{II} species from the total Mn\textsuperscript{II} added. These data indicate that the three Mn\textsuperscript{II} species account for all the Mn added to the sample, and there are no Mn species which are otherwise not observable by EPR. All three Mn\textsuperscript{II}-containing species can be accurately measured over the course of the titration. Table 1 gives representative concentrations from the full titration series. The concentrations of each of these three species per [R2] versus [Mn\textsuperscript{II}] added per [R2] are plotted in Figure 7. For all points on the titration curve ($n = 12$), the sum of all three species observed by EPR quantitatively agrees with the total Mn added to the sample. The titration was continued to 9 equiv (data not shown) which showed only a linear increase in the aquaMn\textsuperscript{II} species.

The addition of 100 mM KCl to the protein buffer was necessary for observation of bound Mn\textsuperscript{II} within the active site of R2. Figure S1 (see the Supporting Information) shows representative EPR signals with the addition of 3 equiv of Mn\textsuperscript{II} to apoR2 in the presence and absence of KCl. In the absence of KCl all Mn\textsuperscript{II} was quantitatively accounted for as aquaMn\textsuperscript{II}.

In the presence of KCl, the characteristic signals from Mn\textsuperscript{II}R2 are observed at $g = 4.6$ and 11.7 (with correct temperature dependence), and the aquaMn\textsuperscript{II} signal is significantly weaker. Concentration determinations of these species from the signals found 1 equiv of Mn\textsuperscript{II}R2 and 1 eq of aquaMn\textsuperscript{II}.

### Table 1. Distribution of Manganese\textsuperscript{a} within R2 Homodimer at Representative Points along the Titration Curve

<table>
<thead>
<tr>
<th>Mn\textsuperscript{a} added</th>
<th>Mn\textsuperscript{II}R2</th>
<th>Mn\textsuperscript{II}R2</th>
<th>aquaMn\textsuperscript{II}</th>
<th>% mass balance\textsuperscript{b}</th>
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</tr>
<tr>
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<td>0.00</td>
<td>1.00</td>
<td>2.52</td>
<td>96</td>
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</table>

\textsuperscript{a} Equivalents of each species per apoR2 (βββ). \textsuperscript{b} Percent mass balance is defined as the amount of Mn\textsuperscript{II} from each species divided by the amount of Mn\textsuperscript{II} added.

### Figure 7. Equivalents of observed paramagnetic species as a function of added Mn\textsuperscript{II}, and theoretical curves for a two site sequential binding model with $K_1 = (5.5 \pm 1.1) \times 10^4$ M\textsuperscript{-1} and $K_2 = (3.9 \pm 0.6) \times 10^4$ M\textsuperscript{-1}. The species are Mn\textsuperscript{II}R2 (●), Mn\textsuperscript{II}R2 (●), and aquaMn\textsuperscript{II} (○).
protein, and after formation of the Mn$^{II}$R2 site, it is not observed again. Therefore, upon titration of up to 9 equiv of Mn$^{II}$, only one $\beta$-peptide of the R2 homodimer (\(\beta\beta\)) is completely occupied by Mn$^{II}$. Thus producing a Mn$^{II}$ cluster in a single $\beta$-peptide and leaving the adjacent $\beta$-peptide unoccupied. This indicates that the two $\beta$-peptides of R2 do not behave identically during metal incorporation. Therefore, as mentioned in the Introduction, we introduce nomenclature to differentiate the $\beta$-peptide forming the first bimetallic cluster as $\beta_{1}$, and the adjacent second $\beta$-peptide as $\beta_{2}$. This distinction comes about only after binding the first equivalent of metal. Hence, addition of up to 9 equiv of Mn$^{II}$ to apoR2 resulted in an Mn$^{II}$ occupied $\beta$-peptide and a vacant $\beta_{2}$-peptide, i.e., (Mn$^{II}_{1}\beta_{1})(\beta_{2})$.

**Titration of apoR2 with Manganese (20% Glycerol).**

Previously published results from other laboratories\textsuperscript{32,34} have demonstrated greater than 2 equiv of Mn$^{II}$ or Fe$^{II}$ can bind within apoR2 upon simple titration. These previous reports of metal titration into apoR2 used buffers with significantly higher concentrations of glycerol. Thus, we have considered the possibility that the buffer conditions could influence incorporation of metal into apoR2. We performed a limited titration of Mn$^{II}$ under the conditions published by Atta et al.\textsuperscript{100 mM Tris, pH 7.5, 20% glycerol).\textsuperscript{34} Under these conditions, as shown in Figure S2 (Supporting Information), we observe that the Mn$^{II}$R2 species is initially formed upon addition of the up to 2 equiv Mn$^{II}$, after which this species decreases concomitantly with the formation of the Mn$^{III}$R2 species. Addition of 3.8 equiv of Mn$^{II}$ to apoR2 generates 1.7 equiv of Mn$_{1}^{II}$R2 (3.4 Mn$^{II}$ per R2). Thus, in agreement with previous reports, both $\beta$-peptides of R2 appear to be accessible to metal binding under these conditions. With the exception of glycerol content, all other buffer conditions of our previous titration are similar to typical published conditions.\textsuperscript{15,22,23,28,32}

Following the same methodology as above for determination of the association constants, we observe for 20% glycerol both $K_{1}$ and $K_{2}$ decrease to approximately $1.0 \times 10^{5}$ M$^{-1}$ and $1.4 \times 10^{4}$ M$^{-1}$, respectively. The observed EPR signals of both the protein bound Mn$^{II}$R2 and Mn$_{2}^{II}$R2 species were identical to what was observed during our previous titration. Thus, this different buffer condition does not change the ligand coordination to the metal centers in R2. In a separate experiment, we have also added 4 equiv of Mn$^{II}$ to a sample of apoR2 using our normal HEPES buffer conditions, except with no glycerol. For this sample, we observed only 2 equiv of Mn$^{II}$ bound to the protein as the Mn$_{1}^{II}$R2 species.

**Manganese Binding to Fe$^{II}$R2 as a function of O$_{2}$ Turnover.**

Using the common procedures for iron incorporation, our preparations of apoR2 bind 3.5 Fe atoms per $\beta\beta$. Iron occupancy $<4$ per R2 is typical for this protein.\textsuperscript{15,22,23,28,32} Our data demonstrates that only one Mn$^{II}$ site is formed within homodimeric R2 (Mn$_{1}^{II}_{1}\beta_{1})(\beta_{2})$) by simple titration of Mn$^{II}$. Although the differential binding affinity observed here for Mn$^{II}$ agrees with previous findings for Fe$^{II}$, the reason only one $\beta$-peptide incorporates Mn$^{II}$ was unclear. We hypothesized that perhaps the state of the $\beta_{1}$-peptide metals affects metal binding in the $\beta_{2}$-peptide sites. Assuming for the moment that Fe$^{II}$ (like Mn$^{II}$) binds to a single $\beta$-peptide of R2 only, then apoR2 pre-loaded with 2 equiv of Fe$^{II}$ should result in (Fe$^{II}_{1}\beta_{1})(\beta_{2})$. This can then react with O$_{2}$ to access different oxidation states of the metal in the $\beta_{1}$ sites. To test this hypothesis, Mn$^{II}$ was added to samples of apoR2 pre-loaded with 2 equiv of Fe$^{II}$, before (Protocol 1) and after (Protocol 2) turnover with O$_{2}$. These samples were also reacted with O$_{2}$ in the presence of Mn$^{II}$ (Protocol 3). The results for the three experimental protocols for metal incorporation, P1, P2, and P3 are provided in Table 2.

**Table 2. Equivalents of Observed Species during Manganese Binding Protocols**

<table>
<thead>
<tr>
<th>Sample Condition</th>
<th>aquaMn$^{II}$</th>
<th>Mn$^{II}$/R2</th>
<th>Y122$^{a}$</th>
<th>Fe$^{II}$/Mn$^{II}$/R2</th>
<th>% Mn$^{II}$ Observed$^{d}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn$^{II}$ present before and during O$_{2}$ exposure (P1 &amp; P3)</td>
<td>before O$_{2}$</td>
<td>2.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>after O$_{2}$</td>
<td>1.0</td>
<td>0.6</td>
<td>0.6</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>d.t. + m.v.$^{b}$</td>
<td>1.4</td>
<td>0.6</td>
<td>-</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>after O$_{2}$$^{c}$</td>
<td>1.4</td>
<td>0.6</td>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td>Mn$^{II}$ added after O$_{2}$ exposure (P2)</td>
<td>Mn$^{II}$ after O$_{2}$</td>
<td>0.4</td>
<td>0.0</td>
<td>0.6</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>d.t. + m.v.$^{b}$</td>
<td>0.4</td>
<td>0.0</td>
<td>-</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>after O$_{2}$$^{c}$</td>
<td>0.4</td>
<td>0.0</td>
<td>-</td>
<td>0.1</td>
</tr>
</tbody>
</table>

$^{a}$ Equivalents of each species per R2 homodimer. $^{b}$ d.t. + m.v.: anaerobic sample reduced by dithionite and methyl viologen addition. $^{c}$ Air-oxidation of d.t. + m.v. sample. $^{d}$ Mn accounted for within the observed species divided by the total amount added.

**Figure 8.** Perpendicular and parallel mode X-band EPR signals observed before (dashed lines) and after (solid lines) exposure to O$_{2}$. (A) Inset showing the Y122$^{a}$ signal. (B) Difference spectrum showing the $g = 4.6$ signal (B$_{1} || B$) from the Mn$_{2}^{II}$R2 species produced upon O$_{2}$ exposure. (C) Signal from aquaMn$^{II}$. For clarity, the Y122$^{a}$ signal was subtracted out of (C). Instrumental conditions: temperature, 87 K (A), 11 K (B), and 2 K (C); frequency, 9.62 GHz (B$_{1} \perp B$) (A and C) 9.26 GHz (B$_{1} || B$) (B); modulation amplitude, 0.3 mT (A) and 1.0 mT (B and C); microwave power, 0.2 $\mu$W (A and C) and 2.0 mW (B).
lack of any bound Mn$^{II}$ species indicates that the 2 equiv of Fe$^{III}$ added are not occupying both $\beta$-peptides of R2 equally. If this were the case, an Fe$^{III}$Mn$^{III}$R2 species$^{32}$ would be produced resulting in a significant decrease in the aquaMn$^{III}$ signal, but this decrease is not observed. Thus, in agreement with the Mn$^{II}$ titration, 2 equiv of Fe$^{III}$ result in occupation of only one $\beta$-peptide to give (Fe$^{III}$)$(\beta_{I})$. Furthermore, because all of the added Mn$^{III}$ could be observed as aquaMn$^{III}$, no detectable amount of Fe$^{III}$ was displaced by Mn$^{III}$ addition.

**Protocol 2: Mn$^{II}$ Added after O$_2$ Turnover (P2).** To determine if the R2$_{act}$ state is responsible for Mn$^{II}$ binding, a sample of (Fe$_{2}^{II}$)$(\beta_{I})$ was prepared anaerobically and subsequently allowed to turnover with O$_2$ in the absence of Mn$^{II}$. Addition of 2 equiv of Fe$^{III}$ to apoR2 produced 0.6 equiv of Y122*, as observed by EPR, upon air oxidation of (Fe$_{2}^{II}$)$(\beta_{I})$-$\beta_{II}$. This represents half of the Y122* typically observed from holoR2$_{act}$ and is consistent with the commonly observed 3 Fe$^{III}$ to 1 Y122* ratio.$^{15,22,23,28,32}$ To this oxidized sample, 0.5 equiv of Mn$^{II}$ was added to the aerobic protein solution. The EPR spectrum of this sample showed an aquaMn$^{II}$ signal at $g = 2$ which quantified to 0.4 equiv of Mn$^{II}$. No Mn$^{III}$R2 or Mn$^{II}$R2 signals were observed. A fraction of the added Mn (0.1 equiv) is not observed as aquaMn$^{II}$. Later, we will show that this can be attributed to a minority mixed metal species.

**Protocol 3: Mn$^{III}$ Present during O$_2$ Turnover (P3).** Figure 8 illustrates the change in the EPR signals of the sample in P1 following exposure to air by aerobic thawing and gently mixing for approximately 5 min prior to refreezing. Upon oxidation of (Fe$_{2}^{II}$)$(\beta_{I})$$(\beta_{II})$ in the presence of 2.7 equiv Mn$^{IV}$, 1.7 equiv of aquaMn$^{IV}$ was lost as indicated by reduction of the 6-line signal at $g = 2$. Figure 8C (solid line). The dashed line in Figure 8C is prior to O$_2$ addition. Furthermore, two additional species are now observed: the characteristic Y122* signal of R2$_{act}$ at $g = 2.005$, and signals characteristic of the Mn$^{III}$R2 species. The inset of Figure 8 shows EPR spectra before (dashed line) and after (solid line) exposure to O$_2$. Prior to air exposure, only trace levels of Y122* were observed (<0.1 equiv). However, following air exposure, the characteristic Y122* signal of R2$_{act}$ is observed. Double integration of the Y122* signal after O$_2$ exposure gives 0.6 equiv per R2 $\beta$. In addition to the Y122* signal observed in perpendicular mode, another signal is observed in parallel mode upon O$_2$ exposure. Figure 8B shows the difference in the parallel mode EPR spectrum after oxidation, which shows a $g = 4.6$ signal similar to the (Mn$^{II}$)$\beta_{I}$$(\beta_{II})$ species of Figure 4B. The Mn$^{II}$ signal observed in Figure 8B has the same $g$-value and temperature dependence as the species in Figure 4B, indicating that an antiferromagnetically coupled Mn$^{II}$R2 species is formed upon turnover of (Fe$_{2}^{II}$)$(\beta_{I})$$(\beta_{II})$ with O$_2$ in the presence of Mn$^{II}$. Using the known concentrations of (Mn$^{II}$)$\beta_{I}$$(\beta_{II})$ from the titration samples allows an estimate of the concentration of the Mn$^{II}$R2 species formed in the (Fe$_{2}^{II}$)$(\beta_{I})$+Mn$^{II}$+O$_2$ sample, from which we find 0.6 equiv of Mn$^{II}$R2. Thus, upon turnover of the (Fe$_{2}^{II}$)$(\beta_{II})$ site with O$_2$, Mn$^{II}$ is loaded into the $\beta_{II}$-peptide to produce the differential $\beta$-peptide occupation (Fe$_{2}^{II}$)$(\beta_{I})$(Mn$^{II}$) for the majority of the protein.

The amount of Mn$_{2}^{II}$ observed accounts for only 1.2 equiv of the total Mn$^{II}$ lost (1.7 equiv) upon air oxidation of (Fe$_{2}^{II}$)$(\beta_{II})$$(\beta_{II})$. However, in this experiment, we have not added reductant such as ascorbate or dithionite to supply the fourth electron necessary in the catalytic cycle of R2 during Y122* generation. This fourth electron could come from either Fe$^{III}$ or Mn$^{III}$ in this sample. EPR detection of Mn$^{III}$ ($S = 2$) is less sensitive and more complicated than Mn$^{II}$ because it is a non-Kramer’s ion. Typically, Mn$^{III}$ species exhibit a 6-line hyperfine signal near $g = 8$ split by ~10 mT in parallel mode EPR.$^{49}$ The signal intensity would be fairly low and may be overwhelmed by the half-field transitions of aquaMn$^{III}$, which also have signals in this region. Therefore, a slight excess of the reductant dithionite with catalytic amounts of methyl viologen was added to the sample to reduce any Mn$^{III}$ produced. Upon reduction of the sample, the 6-line signal at $g = 2$ associated with aquaMn$^{III}$ increased in intensity to account for an additional 0.4 equiv of aquaMn$^{III}$ lost upon air oxidation of (Fe$_{2}^{II}$)$(\beta_{II})$ in the presence of Mn$^{III}$. This indicates that the fourth electron necessary for Y122* formation by (Fe$_{2}^{II}$)$(\beta_{II})$ oxidation was, in part, provided by Mn$^{III}$ during turnover with O$_2$. The amount of Mn$^{III}$ recovered after reduction plus the amount of Mn$^{III}$R2 produced accounts for 1.6 equiv of the Mn$^{III}$ lost (1.7 equiv) after oxidation, as given in Table 2. Typically, Mn$^{III}$ ions disproportionate to Mn$^{II}$ and Mn$^{IV}$ in aqueous solutions.$^{30}$ However, we suspect that the Mn$^{III}$ produced is adventitiously bound to the protein. Mn$^{III}$ is expected to have low rates of ligand exchange, and thus disproportionation of Mn$^{III}$ due to a bimolecular reaction would be inhibited.

**Detection of a Mixed Metal Fe$^{III}$Mn$^{III}$R2 Species.** In samples of (Fe$_{2}^{II}$)$(\beta_{II})$$(\beta_{II})$ where Mn$^{III}$ was present during O$_2$ turnover (P3) and where Mn$^{III}$ was added after turnover (P2), a minor fraction of the added manganese (0.3 equiv P3, 0.1 equiv P2) was not observed as aquaMn$^{III}$, Mn$^{II}$R2, or Mn$^{III}$R2. However, reductive cycling of these samples in the presence of O$_2$ with excess dithionite (5x) and catalytic amounts of methyl viologen resulted in the generation of a new signal shown in Figure 9. This new signal has a 6-line hyperfine pattern centered at $g = 2.0$ ($\mathbf{B}_{1} \perp \mathbf{B}$). The observed hyperfine splitting of this signal ($a = 11$ mT) is significantly larger than expected for Mn$^{II}$ signals ($a = 9.5$)

![Figure 9](image-url)

**Figure 9.** Perpendicular mode X-band EPR spectra (solid line) and simulations (dashed lines) of the mixed metal Fe$^{III}$Mn$^{III}$R2 signal. For clarity, the Y122* signal was cut out. The least-squares fit on the data is the sum of two species (0.62 species I + 0.38 species II). Instrumental conditions: temperature, 11 K; frequency, 9.62 GHz; (B1, $\perp$ B); modulation amplitude, 0.1 mT; microwave power, 0.2 mW. Simulation parameters for species I and II are given in Table 3.


n(T). This signal is observed at \( g = 2 \), suggesting it originates from an \( S = 1/2 \) doublet. Possible species containing Mn exhibiting a \( S = 1/2 \) state low in energy are the antiferromagnetically coupled dinuclear clusters of Mn II Mn III, Mn III Mn IV, Mn III Mn IV, S from an addition of dithionite, no effect was observed on the signal mediator was added to a sample containing this species. Upon 

\[ \text{Figure 10. Signal time-temperature versus temperature dependence of the } g = 2 \text{ signal from the Fe}^{II}\text{Mn}^{III}\text{R}_2 \text{species shown in Figure 9. The theoretical curve is the fractional population of the } S = 1/2 \text{ ground state with } J = -18 \pm 3 \text{ cm}^{-1}, (H = -2JS_1S_2). \]

at which point the signal vanished. Because the mediator methyl viologin is necessary to reductively quench the signal, the mixed metal Fe III Mn III cluster must reside within R2, rather than adventitiously bound to the protein.

For samples of (Fe II Mn II R2) with Mn III added during and after O2 turnover, the Fe III Mn III R2 signal represents 0.3 and 0.1 equiv per \( \beta \beta \), respectively. Table 2 summarizes the distribution of manganese species created upon initial exposure of (Fe II Mn II R2) with Mn III added during (P3) and after O2 turnover (P2).

The formation of this Fe III Mn III R2 species likely originates from the oxidation of an Fe III Mn III R2 species present during turnover with O2. The reduced mixed metal cluster could be formed either by competition for binding sites under anaerobic conditions or by metal mixing during turnover with O2. However, because all of the Mn III added to (Fe II Mn II R2) species was observed as aquaMn III, no significant amount of Fe III Mn III R2 species could have been present prior to turnover. Therefore, the Fe III Mn III R2 species is more likely produced during reductive cycling with mediator, reductant, and O2.

**Discussion**

**Mechanism of apoR2 Metal Incorporation.** The titration of apoR2 presented here unequivocally demonstrates that once one \( \beta \)-peptide of R2 (\( \beta_1 \)) binds the first Mn III ion, the adjacent binding site on \( \beta_1 \) is occupied next, producing a dinuclear Mn II cluster and an unoccupied second \( \beta \)-peptide (\( \beta_2 \)) to give (Mn II \( \beta_1 \) \( \beta_2 \)). After formation of this cluster, no further mono or dinuclear Mn III species are formed with up to 9 equiv of Mn III added. To our knowledge, this is the first indication that the two \( \beta \)-peptides of R2 do not act independently of each other. We also show that these results extend to the physiological relevant metal, iron. The anaerobic addition of 2 equiv of Fe III to apoR2, followed by 2.7 equiv of Mn III exhibited no bound Mn III species. Because all of the added Mn III is observed as aquaMn III, no mixed metal Fe III Mn III R2 species was formed. This species has been observed previously,15 therefore, there is no reason to suggest that Fe III binding to apoR2 prevents Mn III from binding within the same \( \beta \)-peptide. This suggests that both equivalents of Fe III are bound within a single \( \beta \)-peptide to give (Fe III \( \beta_1 \) \( \beta_2 \)) and that Mn III binding within the opposite \( \beta \)-peptide is prevented.

On the basis of these results and a body of previous work to be discussed next, we suggest Scheme 1 for metal uptake in the absence of R2 activation.

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**Table 3. Spectral Parameters Used for Simulation of the Fe III Mn III R2 Signal**

<table>
<thead>
<tr>
<th>species</th>
<th>( g )-values ( \frac{1}{2} )</th>
<th>( A )-values ( \frac{1}{2} )</th>
<th>fraction of signal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>((g_x, g_y, g_z))</td>
<td>((A_x, A_y, A_z)) MHz</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>(2.0396, 2.0200, 2.0134)</td>
<td>(250, 371, 267)</td>
<td>62</td>
</tr>
<tr>
<td>II</td>
<td>(2.0527, 2.0132, 2.0357)</td>
<td>(184, 309, 314)</td>
<td>38</td>
</tr>
<tr>
<td>Fe III Mn III/TACN</td>
<td></td>
<td>2.037</td>
<td>314</td>
</tr>
</tbody>
</table>

\( ^a \) Fe III Mn III(\( \mu\)-O)(\( \mu\)-MeCO 2 ) TACN complex from refs 52 and 53, isotropic values only. \( ^b \) \( g \) and \( A \)-values for the coupled basis set.

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We have also considered the possibility of noncoaxial \( g \) and \( A \)-tensors. However, allowing for noncoaxial coordinate systems did not improve the accuracy of the fit or account for the observed triplet splittings observed at \( g = 2.22 \) and 1.86.
The first metal equivalent binds to only one β-peptide to give (M\(\beta_1\)(\(\beta_\text{H}\)). This first metal alters the conformation of the whole protein homodimer to restrict metal binding at the second β-peptide (\(\beta_\text{H}\)). This conformational change occurs after binding of the first metal, since the mononuclear Mn\(\text{II}\)R2 species does not exceed 1 equiv. The second equivalent of metal does not bind to \(\beta_1\) as expected, rather the metal binds adjacent to the first metal on \(\beta_\text{H}\), resulting in (M\(\beta_1\)(M\(\beta_\text{H}\)). No further metal uptake is observed beyond the addition of 2 equiv.

Stubbe and co-workers first suggested the concept of a protein conformational change as an explanation for a kinetic lag phase observed during the formation of active R2 upon mixing apoR2, Fe\(\text{II}\), and O\(_2\). Moreover, our observation that metal binding within \(\beta_1\) is dependent on O\(_2\) activation on the \(\beta_1\) cluster, indicates that the two β-peptides do indeed exhibit some type of allosteric interaction.

The Mössbauer spectroscopy data of Huynh et al. indicated that the Fe\(_2\) site has a 5-fold higher binding affinity than the Fe\(_1\) site. Our results with Mn\(_{\text{II}}\) also indicate a significant difference in binding affinity between the two sites (K\(_1\)/K\(_2\) = 14). However, we suggest that the binding affinities measured for Fe\(_B\) and Fe\(_A\) sites in this previous work are for \(\beta_1\) only and not for both monomers, \(\beta_1\) and \(\beta_\text{H}\). Because crystal structures of Mn\(_{\text{III}}\)R2\(_{\text{II}}\) show the same protein ligation as reduced Fe\(_{\text{II}}\)R2, binding of Mn\(_{\text{II}}\) by R2 should be similar to that observed for Fe\(_{\text{II}}\). Furthermore, the MCD spectroscopic data of Solomon et al. indicates that as with Fe\(_{\text{II}}\), Mn\(_{\text{II}}\) preferentially binds within the B-site. Therefore, the Mn\(_{\text{II}}\) species observed during titration are assigned as (Mn\(_{\text{III}}\)\(\beta_1\)(Mn\(_{\text{II}}\)β\(_\text{H}\)) and (Mn\(_{\text{III}}\)Mn\(_{\text{II}}\)-\(\beta_1\)), with association constant of K\(_1\) and K\(_2\), respectively, stated in the results. The Mn\(_{\text{II}}\) binding affinities determined here for R2 sites A and B are consistent with other protein bound Mn\(_{\text{II}}\) centers of similar coordination.

The aforementioned MCD titration finds saturation of both the Fe\(_B\) and Fe\(_A\) sites of \(\beta_1\) and \(\beta_\text{H}\) at approximately 3 equiv of Fe\(_{\text{II}}\). Furthermore, previous EPR titrations of apoR2 with Mn\(_{\text{II}}\) performed by Fontecave et al. indicated that 4 equiv of Mn\(_{\text{II}}\) could be incorporated by apoR2. Also, the crystal structure of Mn\(_{\text{II}}\)R2 shows incorporation of 4 Mn ions. We have demonstrated here that under identical conditions as reported by the Fontecave et al. (20% glycerol) apoR2 is capable of binding nearly 4 equiv of Mn\(_{\text{III}}\) per R2. However, under our buffer conditions of 5% glycerol or less, we clearly observe only 2 equiv or Mn\(_{\text{II}}\) binding per R2. We conclude that high levels of glycerol result in a loss of the allosteric effect between the β-monomers of R2. Although high levels of glycerol are not native for the protein, our experiments are in the absence of the R1 subunit of RNR. Thus, experiments in the presence of the R1 subunit are needed to determine if this newly observed allosteric effect is present. Nevertheless, the isolated R2 subunit of RNR is the subject of much previously published work, and our findings are certainly relevant for that body of work. We have shown that other buffer conditions are also important for metal binding. Lower concentrations of KCl resulted in no Mn\(_{\text{II}}\) binding internal to R2. Because R2 is an acidic protein, we suspect that the addition of cations to the buffer inhibits binding to external protein sites.

Our results for Mn incorporation indicate that only two metal ions are incorporated during a titration in buffers with less than 5% glycerol. We also believe that the conformational change proposed by Stubbe et al. occurs after binding a single metal atom to the R2 homodimer. However, these results do raise the important question as to how full metal incorporation is achieved. Interestingly, the answer involves the O\(_2\) turnover chemistry of R2. Scheme 2 summarizes the results, which demonstrate that R2 activation chemistry is required for metal incorporation into the \(\beta_1\)-peptide. Our rational for using both Fe and Mn in these experiments was that we can easily quantify the partitioning of Mn species in R2 separately from the Fe species, into aquaMn\(_{\text{III}}\), mononuclear Mn\(_{\text{II}}\)R2, or dinuclear Mn\(_{\text{III}}\)R2 species.

The discussion starts on the left side of Scheme 2 with the protein in the (Fe\(_{\text{II}}\)(\(\beta_1\))(\(\beta_\text{H}\)) state. The addition of Mn\(_{\text{II}}\) to (Fe\(_{\text{II}}\)(\(\beta_1\))-\(\beta_\text{H}\)) (upper pathway) has no effect. Nearly all of the added Mn\(_{\text{II}}\) is observed as aquaMn\(_{\text{III}}\). Next, the (Fe\(_{\text{II}}\)(\(\beta_1\))(\(\beta_\text{H}\)) state is exposed to O\(_2\), (lower pathway) resulting in the formation of active \(\beta_1\) sites (Fe\(_{\text{III}}\)(\(\beta_1\)) Y122-)(\(\beta_\text{H}\)). Addition of Mn\(_{\text{II}}\) to this active state also shows no effect. Again, nearly all of the added Mn\(_{\text{II}}\) is observed as aquaMn\(_{\text{III}}\).

The key observation is the second step of the upper pathway. With the (Fe\(_{\text{II}}\)(\(\beta_1\))(\(\beta_\text{H}\)) site poised for turnover in the presence of Mn\(_{\text{II}}\), upon O\(_2\) addition we observe the formation of Mn\(_{\text{II}}\) clusters and Y122• in equal amounts. The amount of Y122• produced is as expected based on the accepted 3 Fe\(_{\text{III}}\) to 1 Y122• stoichiometry of R2. However, it is interesting to note that the ‘extra’ electron necessary for generation of Y122• is supplied in part by Mn\(_{\text{II}}\). As with previous turnover experiments, even when enough Fe\(_{\text{III}}\) and electrons are present for complete Y122• generation, only 2/3 of the Fe\(_{\text{II}}\)R2 sites produce radical. The outcome of the remaining Fe\(_{\text{III}}\)R2 remains unclear. The amount of Y122• generated and Mn\(_{\text{II}}\) sites formed requires that the Mn predominately binds within the vacant \(\beta_\text{H}\) peptide, because Y122• radicals are not generated by the Mn\(_{\text{III}}\)R2 site. It is possible that the less than full occupation of \(\beta_1\) under these conditions can in part be attributed to competition between Fe\(_{\text{II}}\) and Mn\(_{\text{II}}\) for binding sites under anaerobic conditions. Formation of inactive mixed metal FeMn clusters within \(\beta_1\) would detract from the amount of cycled R2, and thus Y122• generated.
The incorporation of Mn_{II} into \beta_{II} only during O_{2} turnover at amounts equal to the Y122• concentration suggests that there is a state of the protein in R2 activation chemistry which facilitates metal binding within the \beta_{II} peptide sites. The current models for the reductive activation of O_{2} and generation of Y122• are based on the observation of an intermediate X in the reaction shown below.\(^{15,13,21,28,29,61}\)

Another intermediate, U, which contains a protonated tryptophan radical is neglected in the above reaction due to its short lifetime (k > 20 s\(^{-1}\)).\(^{15,13,62}\) Compound X is observable because its rate of consumption is significantly slower than its rate of creation.\(^{25-27}\) Because X exists for a significant period of time, we speculate that when X is present, the protein is in a conformation that favors \beta_{II} metal binding.

In light of these results, we propose that addition of excess Fe_{II} to apoR2 produces a single O_{2} reactive diffrerous cluster within only one \beta-peptide of R2, to give (Fe_{II}β_{II})(β_{II}). Then, during turnover with O_{2}, a second diffrerous cluster is generated on the adjacent \beta_{II}-peptide and capable of Y122• formation, (Fe_{II}(O_{2})•; Y122•β_{II})(Fe_{II}β_{II}). This model could explain why the theoretical complement of 4 Fe per R2 is not observed. As previously mentioned, the average experimentally observed ratios for Y122• production and Fe incorporation per R2 are 1.2 and 3.2–3.6, respectively.\(^{15,22,23,28,32}\) If only Fe_{II}R2 sites, which produce Y122• are capable of full Fe incorporation, generation of 1.2 Y122• per R2 corresponds to 0.6 equiv of R2 capable of binding 4 Fe per R2. Therefore, the Y122• producing fraction corresponds to 2.4 equiv Fe per R2. The remaining 0.8 equiv of un-oxidized Y122•OH corresponds to 0.4 equiv of R2, which did not participate in the O_{2} activation chemistry, and therefore can only bind 2 Fe per R2. Thus, corresponding to an additional 0.8 equiv Fe per R2. The sum of both fractions (2.4 + 0.8 equiv) yields 3.2 Fe per R2, which is consistent with experimental observations of low Fe occupancy in R2.

**Electronic Characteristics of Mn species.** A quantitative interpretation of EPR spectra from mononuclear Mn_{II} proteins has generally been limited to Mn_{II} sites with low anisotropy, D < 0.03 cm\(^{-1}\). For such proteins, the spectra show hyperfine lines with forbidden transitions and the analysis of this spectra is based on these forbidden transitions.\(^{51}\) For Mn sites in proteins such as R2 with high anisotropy (D = 0.1 cm\(^{-1}\)), the spectra are complicated and interpretation requires a different approach, which is demonstrated here. The simulations provided for the Mn_{II}R2 species are novel in that they represent the first published quantitative analysis for such proteins utilizing both perpendicular and parallel mode spectra at two different frequencies (X- and Q-band). The intensity scale of our simulations is determined by the species concentration. We find that both the X- and Q-band simulations quantitatively agree to within 12% of the Mn_{II} concentration.

The larger D-value is consistent with a protein site that has substantial protein ligation and low water ligation.\(^{63}\) More detailed assessment would benefit from the addition of structurally characterized complexes with larger D-values. Given the greater binding affinity observed from the Fe_{II} site relative to the Fe_{A} site, the Mn_{II}R2 signal is assigned to Mn_{II} binding in the B site of a single \beta-peptide within apoR2, (Mn_{II}β_{II})(β_{II}).

The crystal structure of the Mn_{II}R2 protein shows two Mn ions bridged by two carboxylato ligands. In agreement, the EPR spectra of the Mn_{II}R2 species show similarities to previous Mn_{II}-complexes with carboxylato bridging ligands and similar metal–metal distances.\(^{64}\) Furthermore, the measured coupling.
$J = -1.8 \text{ cm}^{-1}$ is comparable to values reported for such complexes.\textsuperscript{55–67} The multiline splitting of 4.5 mT is also comparable to that observed for other spin-coupled Mn\textsuperscript{II}-centers.

In samples containing both Fe\textsuperscript{III} and Mn\textsuperscript{II} for the \(O_2\) turnover experiments, a new paramagnetic species is observed which is unambiguously assigned to a mixed metal Fe\textsuperscript{II}Mn\textsuperscript{III} cluster within the protein. This minor species likely results from competition between Fe\textsuperscript{III} and Mn\textsuperscript{II} for R2 sites, whereas in the reduced state or during reductive cycling. The reduced mixed-metal species Fe\textsuperscript{II}Mn\textsuperscript{II}R2 has been reported previously.\textsuperscript{32} Although we did not see evidence by EPR that this species was formed, \(g\)-values for Fe\textsuperscript{II} can be highly anisotropic compared to Mn\textsuperscript{II} or Fe\textsuperscript{III}, which would significantly broaden the \(S = 1/2\) signal of Fe\textsuperscript{III}Mn\textsuperscript{II}R2, and thereby make it difficult to detect under the intense aquaMn\textsuperscript{II} signal. Under anaerobic conditions, all Mn\textsuperscript{II} added to (Fe\textsuperscript{II}(\(\beta\))Mn\textsuperscript{II}(\(\beta\))KCl) is accounted for as aquaMn\textsuperscript{II}. Therefore, displacement of Fe\textsuperscript{II} for Mn\textsuperscript{II} in the absence of turnover does not appear to occur. Consequently, we believe that this Fe\textsuperscript{II}Mn\textsuperscript{II}R2 species is generated by metal mixing during reductive cycling with mediator, reductant, and \(O_2\). The observed Fe\textsuperscript{II}Mn\textsuperscript{II} species is presumably produced by the 2-electron oxidation of the Fe\textsuperscript{III}Mn\textsuperscript{II}R2 species during reductive cycling. The Fe\textsuperscript{III}Mn\textsuperscript{III}R2 species can only be reduced in the presence of a mediator like methyl viologen, the same mediator commonly used for reduction of the native iron containing protein. Therefore, the Fe\textsuperscript{III}Mn\textsuperscript{III} site must reside within protein, rather than adventitiously bound. We suggest that species I and II used for simulation of the Fe\textsuperscript{III}Mn\textsuperscript{III}R2 signal originates from different Fe\textsuperscript{II} containing sites, Fe\textsuperscript{A} or Fe\textsuperscript{B}, displaced by Mn\textsuperscript{II}. Thus, the observed signal is a combination of Mn\textsuperscript{II}Fe\textsuperscript{A} II and Fe\textsuperscript{A} III Mn\textsuperscript{II} species within R2. The lower binding affinity observed for Fe\textsuperscript{II} 32 in site A makes displacement of the Fe\textsuperscript{A} more favorable than Fe\textsuperscript{B}. Therefore, species I, which comprises the greater fraction of the Fe\textsuperscript{III}Mn\textsuperscript{III}R2 signal, is believed to originate from Mn\textsuperscript{II}Fe\textsuperscript{A} III R2. Consequently, species II assigned to Fe\textsuperscript{A} III Mn\textsuperscript{II}R2.

The EPR signal of the Fe\textsuperscript{III}Mn\textsuperscript{II}R2 species reported here is comparable to that of Fe\textsuperscript{III}Mn\textsuperscript{II}TACN.\textsuperscript{52,53} Both the hyperfine splittings and \(g\)-values for the \(S = 1/2\) state are similar. However, the exchange coupling observed for Fe\textsuperscript{III}Mn\textsuperscript{II}R2 (\(-18\) cm\(^{-1}\)) is significantly lower than Fe\textsuperscript{III}Mn\textsuperscript{II}TACN (\(-73\) cm\(^{-1}\)). The crystal structure of the Fe\textsuperscript{III}Mn\textsuperscript{II}TACN complex shows an oxo-bridge between the two metals. The magnitude for \(J\) for the Fe\textsuperscript{III}Mn\textsuperscript{II}R2 species is too large to be attributed to carboxylato bridging alone, but low compared to the TACN complex. Thus, we suggest the presence of a substituted single atom bridging ligand such as a \(\mu\)-hydroxo or \(\mu\)-carboxylato group within the Fe\textsuperscript{III}Mn\textsuperscript{III}R2 site.

**Conclusion**

We provide compelling evidence of an allosteric effect between the two \(\beta\)-peptides during the uptake of metal into the R2 subunit of ribonucleotide reductase. We suggest that upon binding of the first metal into a single \(\beta\)-peptide (\(\beta\)I, \(\beta\)II of apoR2), a conformational change occurs which prevents metal from binding to the \(\beta\)II peptide. However, metal incorporation in the \(\beta\)II-peptide occurs upon (Fe\textsuperscript{II}(\(\beta\))I Mn\textsuperscript{II}(\(\beta\))II) catalyzed \(O_2\) activation. This model for R2 metal incorporation can explain the generally observed low Fe occupation of R2.

We also demonstrate that metal uptake and the allosteric effect are sensitive to buffer conditions. Higher levels of glycerol result in loss of the newly observed allosteric effect. Although high levels of glycerol are not native for the protein, the effect of the R1 subunit of RNR on this allosteric effect is unknown. Nevertheless, the isolated R2 subunit of RNR is the subject of much previously published work, and our findings are certainly relevant for that body of work.

**Abbreviations:** RNR, ribonucleotide reductase; R2, small subunit of ribonucleotide reductase R2; R2act, fully reduced dimeric R2; \(R_2\)act\(,\) active form of R2 [Fe\textsuperscript{III}R2; Tyr\(\bullet\), \(\beta\)I, \(\beta\)II homodimer; \(\beta\)I, first single \(\beta\)-strand of R2 to bind metal; \(\beta\)II, second single \(\beta\)-strand of R2 to bind metal; Fe\textsuperscript{II}(\(\beta\))I Mn\textsuperscript{II}(\(\beta\))II apo R2 with 2 equivalents of Fe\textsuperscript{II} per R2 homodimer; Mn\textsuperscript{II}R2, mononuclear Mn\textsuperscript{II} substituted R2; Mn\textsuperscript{II}R2, binuclear Mn\textsuperscript{II} substituted R2; Mn\textsuperscript{II}(\(\beta\))I and Mn\textsuperscript{II}(\(\beta\))II, binuclear Mn\textsuperscript{II} cluster on first and second \(\beta\)-strand, respectively; Fe\textsuperscript{III}Mn\textsuperscript{II}R2, mixed metal cluster within R2; MnCat, manganese catalase; TACN, N,N',N"-trimethyl-1,4,7-triazacyclononane; EPR, electron paramagnetic resonance; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid.

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**Supporting Information Available:** Representative EPR signals with the addition of 3 equiv of Mn\textsuperscript{II} to apoR2 in the presence and absence of KCl, cubic equation expression of [Mn\textsuperscript{II}R2] in terms of \(K_1, K_2, [R_2]\text{total},\) and [Mn\textsuperscript{II}]\text{total}. Periodic and parallel mode X-band EPR spectra for 3 equiv Mn\textsuperscript{II} added to apoR2 in the presence of 100 mM KCl (dashed line) and absence of any KCl (solid line) (Figure S1), and equivalents of observed paramagnetic species as a function of added Mn\textsuperscript{II} in 20% glycerol (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.


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