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Divergent assembly mechanisms of the manganese/iron cofactors in R2lox and R2c proteins

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ABSTRACT

The Mn/Fe cofactor found in two classes of ferritin-like proteins, the small subunit (R2) of class Ic ribonucleotide reductase (R2c) and the R2-like ligand-binding oxidase (R2lox), performs multi-electron oxidative chemistry. It is unclear how a heterodimeric Mn/Fe metallocofactor is assembled in these two related proteins as opposed to a homodimeric Fe/Fe cofactor, especially considering the structural similarity and proximity of the two metal-binding sites in both protein scaffolds and the similar first coordination sphere ligand preferences of Mn^{II} and Fe^{II} . Using EPR and Mössbauer spectroscopies as well as X-ray anomalous dispersion, we examined metal loading and cofactor activation of both proteins *in vitro* (in solution). We find divergent cofactor assembly mechanisms for the two systems. In both cases, excess Mn^{II} promotes heterobimetallic cofactor assembly. In the absence of Fe^{II} , R2c cooperatively binds Mn^{II} at both metal sites, whereas R2lox does not readily bind Mn^{II} at either site. Heterometallic cofactor assembly is favored at sub-stoichiometric Fe^{II} concentrations in R2lox. Fe^{II} and Mn^{II} likely bind to the protein in a stepwise fashion, with Fe^{II} binding to site 2 initiating cofactor assembly. In R2c, however, heterometallic assembly is presumably achieved by the displacement of Mn^{II} by Fe^{II} at site 2. The divergent metal loading mechanisms are correlated with the putative *in vivo* function of R2c and R2lox, and most likely with the intracellular $\text{Mn}^{\text{II}}/\text{Fe}^{\text{II}}$ concentrations in the host organisms from which they were isolated.

1. INTRODUCTION

Bimetallic Mn/Fe cofactor-containing proteins represent a new metallocofactor paradigm, first identified in the purple acid phosphatase isolated from sweet potato [1-3]. Two types of proteins use a bimetallic Mn/Fe cofactor to perform chemically divergent oxidative reactions: the R2 subunit of class Ic ribonucleotide reductase (R2c), and R2-like ligand-binding oxidase (R2lox), which shares characteristics of R2 proteins and bacterial multicomponent monooxygenases (BMMs) [4-9]. In the case of R2c, the cofactor acts as a transient one-electron oxidant [10, 11], whereas R2lox is capable of performing a two-electron oxidation, generating a covalent cross-link between a tyrosine and a normally inert valine residue in the protein scaffold [8, 12].

Metallocofactor assembly in the R2c and R2lox proteins involves two discrete steps: the binding of labile Mn^{II} and Fe^{II} to the protein scaffold and the subsequent oxidation of the two metal sites upon reaction with molecular oxygen [7, 10, 12]. The mechanism of oxygen activation is thought to involve a transient bis- μ -oxo $\text{Mn}^{\text{IV}}/\text{Fe}^{\text{IV}}$ species in both proteins, but this intermediate has to date only been observed in R2c [13, 14]. This intermediate then undergoes either one- or two-electron reduction to yield the stable oxidized intermediate of the respective R2c/R2lox catalytic cycle. For R2c this is the active state which most likely represents a μ -oxo, μ -hydroxo bridged cofactor, poised at the EPR silent $\text{Mn}^{\text{IV}}/\text{Fe}^{\text{III}}$ level [10, 15-18], whereas the stable resting state of R2lox represents a μ -hydroxo, bis- μ -carboxylato bridged cofactor, poised at the EPR active $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ level [12, 16, 19].

An important debate in current literature is the means by which nature controls metallation or, more specifically, prevents mismetallation of iron and manganese proteins *in vivo* [7, 20-22]. In many systems, the availability of the metal in the discrete

cellular compartment in which the enzyme exists is thought to drive proper metal insertion [20, 23]. Metal binding in these instances is often described in terms of the Irving-Williams series of metal complex stability, where transition metals in the +II oxidation state have the affinities: $\text{Mn}^{\text{II}} < \text{Fe}^{\text{II}} < \text{Ni}^{\text{II}} < \text{Co}^{\text{II}} < \text{Cu}^{\text{II}} > \text{Zn}^{\text{II}}$ [24]. Metals late in this series, such as cobalt and copper, whose free intracellular concentrations are extremely low, often require metal-specific transporters/maturation factors for correct metallation [25-28], and even Fe^{II} requires metallochaperones for iron-sulfur cluster formation [29]. However, no chaperones have been identified to date for mono- or dinuclear iron centers or manganese cofactors [20]. Thus it remains unclear how heterodinuclear metal centers are selectively assembled in the absence of metallochaperones. More recently this puzzling question has been the topic of intense investigation using proteins such as R2c and R2lox [12, 30-32], as well as biomimetic model systems [33-35].

In the case of R2c and R2lox, it is not clear how a heterodimeric Mn/Fe metallocofactor is assembled in these two protein homologues as opposed to a homodimeric diiron cofactor. The two metal-binding sites of each protein are structurally very similar and in close proximity to each other (Figure 1). Nevertheless, in both R2c and R2lox each site shows high selectivity for a specific metal ion in the assembled, activated cofactor: site 1 preferentially contains Mn, while site 2 contains Fe, as demonstrated by X-ray anomalous dispersion [8, 12, 30-32]. For R2c this selectivity exists prior to oxygen activation [32]. Under anaerobic metal loading conditions, Mn^{II} associates with site 1, whereas Fe^{II} associates with site 2. Recent X-ray crystallography measurements have shown that in the absence of Fe^{II} , Mn^{II} can bind at both sites, suggesting it is Fe availability that initiates heterodimeric cofactor assembly, as the $\text{Mn}^{\text{II}}/\text{Mn}^{\text{II}}$ cofactor cannot be activated by oxygen [32]. In contrast, metal loading of R2lox is not exclusive

under anaerobic conditions [12]. While site 2 shows a high selectivity towards Fe^{II} , site 1 is non-specific, binding equal amounts of Mn^{II} and Fe^{II} [12]. Upon oxidation, however, the heterodimeric cofactor is accumulated, at the expense of the homodimeric Fe/Fe cofactor, as observed in the crystallized protein [12].

In this combined EPR, Mössbauer and X-ray anomalous dispersion study we further examine the binding of Mn^{II} and Fe^{II} to the R2c and R2lox protein scaffolds, under more physiological conditions, i.e. in aqueous solution as opposed to in apo-protein crystals that are soaked with high concentrations of metal ions. Our data show that while a Mn/Fe cofactor with the Mn ion in site 1 is preferentially assembled in both proteins, the same final outcome is achieved via distinctly different assembly pathways in the two systems.

2. MATERIALS AND METHODS

2.1. Protein production and purification. An N-terminally His-tagged full-length construct of the R2lox protein I from the thermophilic bacterium *Geobacillus kaustophilus* (accession number WP_011232245) was produced and purified in metal-free form as described previously [12]. The gene encoding the *Saccharopolyspora erythraea* class Ic R2 protein (accession number WP_009947973) was PCR-amplified from genomic DNA (DSM No. 40517) and inserted into a modified pET-28a vector (Novagen) in which the thrombin cleavage site following the N-terminal His₆ tag has been replaced by a TEV site. His-tagged full-length R2c protein was produced in metal-free form in *Escherichia coli* BL21(DE3) (Novagen) grown at 37°C in a bench-top bioreactor system (Harbinger) in M9 minimal media (ForMedium) supplemented with 50 µg/mL kanamycin, without addition of supplementary metals. As the cell culture reached an OD₆₀₀ of 0.8-1.0, 0.5 mM EDTA was added, followed by induction with

0.5 mM IPTG. Induction continued overnight at room temperature. The cells were harvested, resuspended in lysis buffer (25 mM HEPES-Na pH 7.0, 300 mM NaCl, 20 mM imidazole and 0.5 mM EDTA) and disrupted in a high-pressure homogenizer (EmulsiFlex-C3). The cell debris was cleared by centrifugation, and the supernatant was applied to a Ni-NTA agarose (Profinity) gravity flow column. Contaminants were removed with wash buffer (lysis buffer with 40 mM imidazole) followed by wash buffer 2 (lysis buffer with 40 mM imidazole and no EDTA). The protein was then eluted with elution buffer (lysis buffer containing 250 mM imidazole and no EDTA). The elution fraction was concentrated and loaded onto a size-exclusion column (HiLoad 16/60 Superdex 200 prep grade; GE Healthcare) equilibrated with SEC buffer (25 mM HEPES-Na pH 7.0, 50 mM NaCl). Fractions containing R2c were pooled and subjected to TEV protease treatment at a molar ratio of 1:50 TEV:R2c overnight at 4°C. TEV protease and any un-cleaved R2c was removed by passing the sample over a Ni-NTA gravity flow column equilibrated in SEC buffer. The flow-through containing pure cleaved R2c was concentrated to ~1 mM, aliquoted, flash-frozen in liquid nitrogen and stored at -80°C. The metal contents of purified apo-R2lox and R2c batches were quantified by TXRF (see below). The only metal that is generally present in significant amounts is Fe, occupying a fraction of $\leq 10\%$ of the cofactor sites.

2.2. EPR/Mössbauer sample preparation. To obtain the oxidized resting state of the Mn/Fe cofactor in R2lox or R2c, 100–200 μ M apo-protein (monomer concentration) was incubated with varying equivalents of MnCl_2 and $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ in 100 mM HEPES-Na pH 7.0, 50 mM NaCl for 1 h at room temperature under aerobic conditions. In order to assess cofactor assembly efficiency and mechanism, a number of different ratios of protein:Mn:Fe were used. The two metal salts were either added to the protein simultaneously, or one was added initially, followed by the second after 30 min

incubation, and a further 30 min incubation to mature the cofactor. Fe titration series were prepared by adding 4 equivalents (per monomer) of MnCl_2 to 100 μM apo-protein. Then, 0.05 equivalents of $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ were added from a 10 mM stock solution. After 10 min incubation at room temperature, an aliquot was removed, and another 0.05 eq. of $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ were added to the remaining solution. After another 10 min incubation, a sample was again removed, and another 0.1 equivalents of $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ were added. In this manner, 8 samples were prepared with final protein:Mn:Fe ratios of 1:4:0.05, 1:4:0.1, 1:4:0.2, 1:4:0.3, 1:4:0.4, 1:4:0.6, 1:4:0.8, 1:4:1. All samples were incubated at room temperature for 1 h after the last addition of $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, i.e. for a total of ~2 h. Following the reconstitution procedure, excess metal ions were removed by passing the samples through a HiTrap desalting column (GE Healthcare) equilibrated in 25 mM HEPES-Na pH 7.0, 50 mM NaCl. The reconstituted protein was concentrated to 0.1–0.3 mM, and 50% glycerol was added before transfer into sample holders and flash cooling in liquid nitrogen. Mn-only loaded samples were prepared analogously, both with and without removal of the excess Mn^{II} . Mössbauer samples of R2lox were prepared with $^{57}\text{FeSO}_4$ instead of natural abundance $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, using a protein:Mn:Fe ratio of 1:2.4:1.2 for Mn/Fe samples and 1:0:4 for Fe-only samples, and the reconstituted protein was concentrated to 2.5–3.5 mM before addition of 50% glycerol. Non-activated reduced Mn/Fe and Fe/Fe cofactors in R2lox were reconstituted in the same way in an anaerobic glove box with the addition of 1 mM sodium dithionite to all buffers to remove all traces of oxygen. Oxidized Mn/Fe-reconstituted R2c samples were reduced to the EPR-active $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ state by incubation with excess sodium dithionite in an anaerobic glove box for 10 – 15 min. Protein concentration was measured using experimentally determined extinction coefficients at 280 nm for the metal-free and metal-bound states of R2lox of 47757 M^{-1}

cm^{-1} and $50563 \text{ M}^{-1} \text{ cm}^{-1}$, respectively [16], and $72022 \text{ M}^{-1} \text{ cm}^{-1}$ and $79928 \text{ M}^{-1} \text{ cm}^{-1}$ for metal-free and metal-bound R2c, respectively.

2.3. CW-X band EPR measurements. X-Band CW-EPR measurements were performed between 5-20 K using a Bruker E500 spectrometer equipped with a Bruker ER 4116DM TE₁₀₂/TE₀₁₂ resonator, Oxford Instruments ESR 935 cryostat and ITC503 temperature controller. Microwave powers used were in the range of 10 μW to 20 mW and magnetic field modulation amplitude was 7.46 G. Quantifications of the X-band EPR signal were made by comparing the measured intensity to that of a sealed, 1.017 mM solution of Cu^{II} chloride dissolved in ethanol. Field modulated EPR spectra of R2c were integrated, corrected for baseline effects, and then integrated a second time using the Bruker XEPR and OriginLab software. R2lox spectra were fitted and the simulated EPR spectra were used for the double integration procedure (see Figures S1 and S2 in the Supplementary Information). The Cu^{II} standard was measured with 10 μW of microwave power at 10 K to avoid any possible saturation effects, while the R2lox and R2c samples were measured with 10 μW , 630 μW and 20 mW of microwave power at 10 K. The R2lox and Cu^{II} signals were normalized for power differences. The intensity of the $S = 1/2$ signal obtained was compared to the protein concentration to calculate the loading efficiency (see sections 2 and 3 of the Supplementary Information for details). Samples of R2c and R2lox were denatured by addition of 37% hydrochloric acid in order to release metals associated with the proteins. This procedure allows the Mn bound to the protein to be accurately determined by EPR, as following acid treatment all Mn ions resemble $\text{Mn}^{\text{II}}(\text{H}_2\text{O})_6$ and can thus easily be quantified by comparison to a Mn^{II} standard of known concentration [36]. The added volume of the acid was ~10% of the original volume of the samples. Upon the addition of the acid, the mixture was left at room temperature for ~20 min and then frozen in liquid nitrogen. The concentration

of free Mn^{II} was determined by comparison of the measured EPR intensity to that of a 1 mM solution of manganese(II) chloride tetrahydrate dissolved in a water-glycerol mixture (50% v/v).

2.4. Mössbauer measurements. Mössbauer spectra were recorded on an alternating constant acceleration spectrometer in an Oxford Variox cryostat (zero-field) or an Oxford Mössbauer-Spectromag (applied field) cryostat. The second cryostat is a split-pair conducting magnet system for applying fields of up to 8 T. The magnetic field at the sample is perpendicular to the γ -beam. The minimum experimental line width achieved was 0.24 mm/s (full width at half maximum). All isomer shifts (δ) are quoted relative to iron metal at 300 K.

2.5. Crystallization and data collection. Samples of R2lox aerobically reconstituted with Mn^{II} and Fe^{II} using a protein:Mn:Fe ratio of 1:2.4:1.2 or 1:2:1 were crystallized by vapor diffusion at 22°C in 20.0-22.5% (w/v) PEG 1500, 100 mM HEPES-Na pH 6.8-7.0. Crystals were cryoprotected by briefly soaking in 40% (w/v) PEG 1500, 100 mM HEPES-Na pH 6.8-7.0. Data were collected at 100 K at beamlines PX14.1/BESSY, Berlin, Germany, and P13/PETRA3, Hamburg, Germany. Data collection proceeded in the order Fe edge – Mn edge on the same crystal. A high resolution native dataset was collected on one crystal after the anomalous datasets.

2.6. Structure determination, model building and refinement. The high resolution dataset was integrated with XDS [37] (Table 1). The structure was solved by Fourier synthesis using a ligand-free model of oxidized state R2lox (derived from PDB ID 4HR0) [12] as a starting model. Refinement was carried out with phenix.refine [38, 39] and iterated with rebuilding in Coot [40]. Refinement included individual atomic coordinate and isotropic B factor refinement, occupancy refinement of the metal ions, and bulk solvent corrections. Metal-ligand bond lengths were restrained. Solvent

molecules were added with phenix.refine and manually. Hydrogens were added to the model in the later stages of refinement. Strong electron density connecting the phenolic oxygen of Tyr162 and the C β of Val72 was observed. This ether cross-link was restrained to an ideal distance of 1.45 ± 0.02 Å. The structure was validated using MolProbity [41]. Refinement statistics are given in Table 2. All figures were prepared with PyMOL (version 1.6.0.0, Schrodinger, LLC).

2.7. Analysis of anomalous diffraction data. All anomalous datasets were integrated over the same resolution range (50.0–3.0 Å) with XDS [37] (Table 1). The Fe and Mn edge datasets from one crystal were placed on a common scale with XSCALE [37]. Both scaled and unscaled datasets were analyzed. Anomalous difference maps were calculated with PHENIX [38] using the phases from a ligand-free model, and the relative amounts of Fe and Mn in each metal site were calculated using Mapman [42] as previously described [12].

2.8 Total-reflection X-ray fluorescence (TXRF). Metal contents of apo-protein preparations and selected EPR samples were quantified using TXRF analysis on a Bruker PicoFox instrument [43]. A gallium standard (Sigma) was added to the samples (v/v 1:1) prior to the measurements. TXRF spectra were analyzed using the routines provided with the spectrometer.

3. RESULTS

As described in the introduction, the R2lox and R2c protein scaffold can in principle accommodate multiple homodimeric and heterodimeric cofactors, the latter differing in the position of the second metal. In addition, each cofactor exists in two discrete stable states: the non-activated reduced M^{II}₂ state as well as the oxidized resting M^{III}₂ state in R2lox and the active M^{IV/III}₂ state in R2c, respectively. To characterize and quantify

each of these states multiple spectroscopic handles are therefore required. Here we use two independent techniques: EPR and Mössbauer spectroscopy. Multiple techniques are required because:

- 1) No single spectroscopic method can visualize all cofactor populations in isolation. EPR is only sensitive to paramagnetic states and thus cannot detect the diamagnetic $\text{Fe}^{\text{III}}/\text{Fe}^{\text{III}}$ homodimer, which arises due to a strong (exchange) interaction between the two metal sites. Similarly, Mössbauer spectroscopy is only sensitive to cofactors that contain ^{57}Fe (see Scheme 1).
- 2) EPR spectroscopy provides absolute cofactor concentrations, while Mössbauer spectroscopy typically provides relative concentrations.

These measurements are complemented by two different X-ray techniques: total reflection X-ray fluorescence (TRXF) and X-ray anomalous dispersion (XAD). With TRXF, all metal ions present in a sample can be quantified, but this method cannot distinguish whether metal ions are bound in the active site of the protein, loosely associated with the protein surface, or present in solution [43]. While XAD can detect all forms of the cofactor, like Mössbauer it addresses relative as opposed to absolute metal concentrations. The main advantage of XAD is that it provides positional information, so the relative amounts of different metal ions in each binding site can be quantified [12]. However, this method also requires the additional preparation step of crystallization post metal loading and cofactor activation. It cannot be excluded that only a subpopulation of the entire sample crystallizes selectively, and thus the results from this method may not correspond to the cofactor distribution in solution.

3.1. The EPR signatures of R2lox and R2c. Of key interest is the population of the oxidized heterometallic cofactor. The EPR signature for this state is a structured signal

centered at $g \approx 2$. It arises due to the strong antiferromagnetic coupling between the two metal sites in the +III oxidation state. The structure is indicative of a Mn cofactor which comes about due to the hyperfine interaction of the ^{55}Mn nucleus with the unpaired electron spin. For the R2lox cofactor, this redox level represents the resting state [12, 16, 19], whereas R2c instead is poised at the EPR silent $\text{Mn}^{\text{IV}}/\text{Fe}^{\text{III}}$ level [10, 15-18] (see Scheme 1). Thus, to observe the R2c cofactor it must first be reduced by 1 electron. This is readily achieved by treating the protein with an excess of sodium dithionite [10]. The CW-X-band EPR spectrum of the R2lox cofactor is shown in Figure 2A and B. It displays a characteristic seven-line structure with a total spectral breadth of 75 mT, as measured as the difference in field units between peak 1 and peak 7. This pattern represents the superposition of two six-line patterns, indicative of a Mn/Fe cofactor with axial g - and ^{55}Mn -hyperfine tensor [16]. The line shape of the metallocofactor spectrum is invariant under all metal loading conditions, but the intensity does vary. Additional low-intensity features can be seen for some R2lox samples between lines 1 and 2 which are due to a small amount of an underlying Mn^{II} signal. This signal is representative of free Mn^{II} ($\text{Mn}^{\text{II}}(\text{H}_2\text{O})_6$; see section 3.6). The desalting procedure used prior to loading samples in EPR tubes suppresses this contaminant signal. As discussed in [16], multifrequency EPR measurements, in combination with XAD data [12], demonstrate that the seven-line signal represents a single cofactor species, with the Mn^{III} ion bound selectively at site 1 and the Fe^{III} ion at site 2. The intensity of the EPR signal deviates from a linear dependence with reciprocal temperature, i.e. it shows non-Curie behavior, suggesting that the exchange interaction between the two metal sites is weak, as inferred from our earlier multifrequency study [16].

The CW-X-band EPR spectrum of the R2c cofactor is shown in Figure 2C and D. Notably, the EPR spectrum of the *S. erythraea* R2c protein used in this study is similar,

but not identical to that of the previously characterized *C. trachomatis* R2c [10, 11, 44]. Its spectrum displays a six-line pattern of similar width to the R2lox spectrum, with additional hyperfine structure resolved across the spectrum. As compared to the R2lox cofactor signal, the R2c cofactor relaxes more slowly and thus needs to be measured at lower microwave fluxes in order to avoid saturation effects. The structure of the R2c spectrum varies with microwave power (Figure 2C and D), and the order of loading with Mn^{II} and Fe^{II} (see below). This suggests that R2c spectra may not represent a single Mn/Fe cofactor but instead the variable superposition of two Mn/Fe cofactors, which differ in either the location of the two metals, the precise protein conformation and/or protonation state of the cofactor. In support of this hypothesis, we note that we were unable to simulate the EPR signal as a single species. As mentioned above for the R2lox cofactor, additional low-intensity features can be seen for R2c samples due to a small amount of underlying free Mn^{II} ($\text{Mn}^{\text{II}}(\text{H}_2\text{O})_6$; see section 3.6). After the desalting procedure this contaminant signal is still present, visibly stronger as compared to R2lox samples, and to some extent it disturbs our quantification of heterobimetallic metal loading. This also affects the power dependence behavior of the R2c EPR spectrum. Importantly, this Mn^{II} signal was present before and after dithionite addition, demonstrating it does not arise due to over-reduction of the cofactor.

Spin Hamiltonian simulations of the Mn/Fe cofactor spectra of R2lox and R2c are shown in Figure 3A and B. For the R2lox species, the raw spectrum was simulated (for details see section 1 of the Supplementary Information). For the R2c species, in order to suppress the Mn^{II} background signal, the spectrum of a sample where Fe^{II} was added to the apo-protein before Mn^{II} was subtracted from the spectrum of a sample where the metals were added simultaneously (see Figure 2D). The R2lox spectrum could be reproduced using the same values as reported by Shafaat et al. [16], while for the R2c

spectrum, the data was refit using the values reported in Voevodskaya et al. [44] as a starting point. As stated above, we were unable to simulate the Mn/Fe R2c signal as a single species, but we were able to simulate it as a 60:40 admixture of two different Mn/Fe cofactors. Spin Hamiltonian parameters for the two components fall within the range seen in the previous studies of the *C. trachomatis* R2c [10, 11, 44]. While the R2c simulation represents a satisfactory fit to the data, the spin Hamiltonian parameters and relative cofactor concentrations are unlikely to be unique. The spin Hamiltonian parameters used for the simulation shown in Figure 3B are: $g_x=2.0313$, $g_y=2.0225$, $g_z=2.0327$, $A_x=350.3886$, $A_y=278.6277$, $A_z=227.6641$ for Species 1, and $g_x=2.0111$, $g_y=2.0098$, $g_z=2.0303$, $A_x=346.5097$, $A_y=270.652$, $A_z=188.7994$ for Species 2. Further measurements at higher microwave frequencies are needed to characterize the two putative Mn/Fe populations, which forms ongoing work of our laboratories.

Using the two cofactor signatures described above, the efficiency of heterobimetallic cofactor assembly was assessed at different protein:Mn:Fe loading ratios. The concentration of the cofactor was estimated by double integration and comparison to a Cu^{II} standard of known concentration. This procedure was accompanied by: i) comparison of the peak-to-peak amplitudes of the EPR lines at different concentrations; and ii) acid denaturation of the proteins and comparison of the liberated Mn^{II} to a Mn^{II} standard of known concentration.

3.2. Fe^{II} addition in the absence of Mn^{II} inhibits assembly of a heterobimetallic cofactor for both R2lox and R2c. As a first experiment we examined if the order in which Mn^{II} and Fe^{II} are added to the protein scaffold affects heterobimetallic cofactor yields. Unless otherwise stated, the addition of the two metals was separated by a 30 min incubation period. The simultaneous addition of both metal ions at a concentration

of 2 metal ions per protein monomer yields a Mn/Fe cofactor signal for both proteins (Figure 2). For R2lox, the signal increases by ~13% if Mn^{II} is added before Fe^{II} , whereas the R2c cofactor yield is unchanged if Mn^{II} is added first. The most dramatic effect is seen if instead Fe^{II} is added before Mn^{II} . In R2lox, heterometallic cofactor assembly is completely suppressed, whereas in R2c, the cofactor signal decreases by at least 75%, suggesting that excess Fe^{II} inhibits Mn/Fe cofactor assembly. This is likely due to the fact that in the absence of Mn, and the presence of excess Fe, the protein can assemble the EPR silent stable $\text{Fe}^{\text{III}}/\text{Fe}^{\text{III}}$ homodimer, lowering the heterodimer yield when Mn is added. We note that in the Fe^{II} -first R2lox samples a ‘radical-like’ EPR signal was also observed, which may also contribute to the Mn/Fe cofactor spectrum. This radical signal, which is most readily seen at lower microwave power levels, changes the relative intensities of lines 3 and 4 compared to the rest of the R2lox EPR spectrum, and thus these peaks were excluded when estimating the cofactor concentration by peak-to-peak amplitudes. In principle this species could represent an R2-like tyrosyl radical ($\text{Y}^{\bullet}\text{Fe}^{\text{III}}/\text{Fe}^{\text{III}}$) cofactor state.

3.3. High Mn^{II} concentrations promote the assembly of a heterobimetallic cofactor for both R2lox and R2c. As a next test we examined the dependence of heterometallic cofactor assembly on the relative concentration of added Mn^{II} to Fe^{II} . As high Fe^{II} concentrations are seen to inhibit heterometallic cofactor assembly, the Fe^{II} level was fixed at either 1 or 2 Fe per protein monomer, and an excess of Mn^{II} was added. Figure 3C shows the concentration of the Mn/Fe cofactor obtained in R2lox and R2c as a function of the concentration of Mn^{II} added.

For R2lox, the EPR simulations were used for double integration. For R2c, due to the inadequate accuracy of the fitting results, experimental EPR spectra of the Mn/Fe

cofactors were integrated. Since the EPR line shape does not change significantly from sample to sample, trends observed using the double integration procedure were double-checked against peak-to-peak amplitudes. The two methods give similar Mn dependence curves for both R2c and R2lox. The absolute values of the loading efficiency, however, can be affected by possible systematic errors in the double integration procedures. Therefore, cofactor quantification estimates were supplemented with acid denaturation experiments. R2c and R2lox samples were denatured by adding concentrated hydrochloric acid, and the concentration of liberated free Mn^{II} was determined by comparison to a Mn^{II} standard. Taking into account the initial free Mn^{II} concentration in the samples and assuming all the liberated Mn^{II} was released from the Mn/Fe cofactor, concentrations of the cofactors were calculated. For R2lox, results of the acid denaturation agreed with the quantification based on double integration to within 6%. For R2c, acid denaturation instead yielded values ~1.5 times higher than the results based on double integration. We cannot immediately rationalize why our double integration procedure systematically underestimates cofactor concentration. One possibility is that the reduction step with excess dithionite, which renders the cofactor EPR active, is not complete in our samples. We also cannot exclude the possibility that the R2c cofactor could be partially over-reduced by the dithionite treatment, although we think this scenario less likely (see above). In any case, as the acid denaturation procedure is robust (see also section 3.6) and not subject to data processing errors, the R2c loading efficiency curves shown in Figure 3C were normalized using the acid denaturation values.

As seen in Figure 3C, full Mn/Fe loading could not be achieved in either protein, but the maximal Mn/Fe loading in R2c was typically higher than that of R2lox, in the data shown 65% vs. 35%. We note though that the maximal Mn/Fe loading in R2lox is

highly batch dependent and that loading of up to 60% could be achieved (see section 3.7).

It is readily seen that the heterometallic cofactor content in both proteins increases with the concentration of Mn^{II} and saturates at approximately the same concentration for both systems, >6 Mn per protein. We note, however, that this saturation point depends on the relative concentration of Fe^{II} present. We explored two regimes: i) an Fe^{II} level stoichiometric with the protein (monomer) concentration; and ii) an Fe^{II} level of twice the protein concentration, i.e. equivalent to the number of metal-binding sites. The lower Fe^{II} condition was seen to decrease the efficiency of R2c cofactor assembly by 45% relative to the same Mn titration points with 2 Fe^{II} per monomer present. The opposite tendency was seen for R2lox, in which cofactor assembly increases by about 75% at the lower Fe^{II} concentration compared to 2 Fe^{II} per monomer. This result agrees with the R2lox data presented in Figure 2, demonstrating that high Fe^{II} concentrations suppress Mn/Fe assembly in R2lox. For R2c however, these results instead show that Fe addition does not inhibit cofactor assembly if Mn is present and that low Fe concentrations actually suppress cofactor assembly, suggesting divergent assembly mechanisms for the two proteins.

3.4. Can efficient Fe/Fe homodimer assembly explain the lower yield of the oxidized Mn/Fe cofactor in R2lox? As the overall yield of the heterobimetallic cofactor is typically lower for R2lox vs. R2c, we performed further Mössbauer spectroscopy measurements to test whether R2lox more efficiently assembles the EPR silent $\text{Fe}^{\text{III}}/\text{Fe}^{\text{III}}$ cofactor as opposed to the $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ cofactor (Scheme 1) when an excess of Mn is present, or if instead R2lox is simply a poorer metal chelate in our solution loading conditions, leaving a large unmetallated population after

metallation/activation. Figure 4A shows the zero-field Mössbauer spectra of R2lox anaerobically loaded at a protein:Mn:Fe ratio of 1:2.4:1.2 or 1:0:4. The spectra are essentially identical, resolving a single doublet centered at 1.26 mms^{-1} with quadrupole splitting of 2.98 mms^{-1} , characteristic of high spin Fe^{II} (d^6 , $S = 2$). The sample loaded with both Mn^{II} and Fe^{II} has a ~50% smaller signal, suggesting Mn^{II} occupies half of the total protein sites.

In the aerobically reconstituted samples (Figure 4B), the zero-field Mössbauer spectra shift such that the doublet is now centered at 0.51 mms^{-1} with quadrupole splitting of 0.81 mms^{-1} , characteristic of high spin Fe^{III} (d^5 , $S = 5/2$). Consistent with the reduced samples, in the sample loaded with both Mn^{II} and Fe^{II} the Mössbauer signal is >50% smaller as compared to the sample only loaded with Fe^{II} . Applied field Mössbauer spectra recorded at 4.2 K with 0.1 T field aligned perpendicular to the incident γ -radiation are shown in Figure 4C. These spectra demonstrate that the doublet seen at zero-field (Figure 4B) for samples loaded with both Mn^{II} and Fe^{II} is composed of two signals: i) a doublet identical to that seen at zero applied magnetic field corresponding to a diamagnetic cofactor population; and ii) a structured signal spread across $\pm 5 \text{ mms}^{-1}$, corresponding to a paramagnetic cofactor population. The latter signal represents the EPR active $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ ($S = 1/2$) cofactor described in section 3.1, whereas the former represents the EPR inactive homobimetallic $\text{Fe}^{\text{III}}/\text{Fe}^{\text{III}}$ cofactor, an antiferromagnetically coupled ($S = 0$) dimer, typical of ferritin proteins [45]. A spin Hamiltonian simulation of the paramagnetic population, using cluster spin $S = 1/2$ with the parameters derived from EPR simulation, is also shown in Figure 4C, in agreement with the earlier EPR analysis of Shafaat et al. [16]. (For fitting and simulation parameters see Table 3.) Heterogeneity of the iron sites in the Fe/Fe homodimers could

not be resolved in the zero-field spectra of oxidized or reduced samples, nor the mixture of Mn/Fe and Fe/Fe dimers in the samples prepared with Mn and Fe.

Quantifying the contribution of the two cofactor populations based on the relative spectral intensity, and assuming equal Lamb-Mössbauer factors, the diamagnetic homodimer accounts for 55% of the total iron. This requires that the Mn/Fe and Fe/Fe centers constitute 62% and 38% of the total cofactors, respectively. Thus, heterodimer assembly is preferred, and a large Fe/Fe cofactor population cannot explain the lower Mn/Fe loading seen in R2lox when an excess of Mn is present. This result implies that a substantial fraction of the R2lox protein remains in the apo form, in contrast to R2c which approaches complete metallation.

3.5. X-ray anomalous dispersion further confirms that heterodimer formation is favored in solution and that site 2 is selectively occupied by Fe in R2lox. To assess the cofactor distribution in the solution-loaded protein and be able to unambiguously locate the metal ions in the two binding sites, R2lox aerobically reconstituted with Mn^{II} and Fe^{II} at a protein:Mn:Fe ratio of 1:2.4:1.2 or 1:2:1 was crystallized, and the relative amounts of Mn and Fe in the two metal sites were quantified in 2 crystals each by XAD as described in Griese et al. [12]. The results are consistent with the EPR and Mössbauer data described above (Table 4, Figure 5). In the sample reconstituted at protein:Mn:Fe = 1:2.4:1.2, site 2 is exclusively occupied by Fe, and the Mn:Fe ratio in site 1 is ~3:2. The ratio of Mn/Fe to Fe/Fe cofactors in this sample is therefore identical to that of the Mössbauer sample that was reconstituted at the same protein:Mn:Fe ratio. In the sample that was reconstituted at protein:Mn:Fe = 1:2:1, a small Mn signal above noise is observed in site 2 in one crystal, but not the other, whereas site 1 contains ~80% Mn. Small differences in the protein:Fe ratio can therefore have a large influence on the

efficiency of Mn/Fe cofactor assembly, further suggesting that excess Fe inhibits assembly of heterobinuclear clusters. However, heterodimer formation is favored in the presence of excess Mn^{II}.

The apo-protein population inferred from the EPR/Mössbauer measurements cannot be reliably determined from this experiment because apo-protein may not crystallize together with the metallated protein. If it did, the apo-protein population, which represents only a slightly different protein conformation [12], would probably manifest as disorder, i.e. high *B* factors, in the crystal structures. A high resolution structure of one of the crystals reconstituted at protein:Mn:Fe = 1:2:1 does indeed display overall very high *B* factors, although it should be noted that high *B* factors are always observed with this protein. Both metal ions refine to an occupancy of 0.79 with *B* factors in the range of the surrounding ligands (Table 2), suggesting that ~20% of the protein molecules in the crystal are not metal-bound. Consequently, ~60% of the total protein contains a Mn/Fe cofactor, in line with the maximum Mn/Fe loading observed by EPR (see section 3.7).

No significant structural differences are observed between solution-loaded R2lox and protein produced and purified in metal-bound form or aerobically reconstituted by soaking of apo-protein crystals with metal ions [12, 19]. The tyrosine-valine ether cross-link refines to a bond length of 1.54 Å (ideal distance 1.45 Å, coordinate error 0.33 Å) with good density fit (not shown). In contrast, electron density for the cross-link is, though present, generally too weak for the link to refine to a reasonable bond length and good density fit in structures of soaked crystals [19], suggesting that cross-link formation is more efficient in solution.

3.6. Divergent cofactor assembly in R2lox and R2c correlates with differing Mn^{II}

binding affinities in the reduced state. The relatively large population of apo-protein inferred for reconstituted R2lox samples prompted us to examine if the metal affinity of the two proteins, R2lox and R2c, is different in the reduced state. This is most easily achieved by simply adding Mn^{II} alone. Since the Mn homodimer cannot be activated by oxygen, this metal-binding assay allows the affinity of the metal-binding sites to be assessed in the absence of cofactor maturation.

CW-X-band EPR spectra of Mn-only loaded R2lox and R2c are shown in Figure 6. In these experiments Mn^{II} was added to the protein scaffold at a concentration of 1 or 2 Mn^{II} per monomer. No desalting procedure was applied post metal loading, and thus the measurement shows a protein-bound fraction, a surface-associated fraction, as well as a non-protein associated ‘free’ Mn^{II} hexaaquo species. We note that in spectra of R2lox loaded with Mn^{II} reported in [12] only a very small Mn^{II} signal was observed. These samples were prepared with a desalting step to remove excess metal ions. In samples prepared in the same way in this study, no Mn^{II} signal was observed at all.

In R2c samples in which 1 Mn^{II} per monomer was added, a structured signal is observed, which is indicative of a $\text{Mn}^{\text{II}}/\text{Mn}^{\text{II}}$ dimer (see Figure 6A) [46-50]. It originates from the weak antiferromagnetic interaction of the two Mn ions bound at site 1 and site 2 of the protein scaffold and is similar to that seen for purple acid phosphatase loaded only with Mn [51]. The ground state of such a spin system is EPR silent ($S = 0$). But if the exchange coupling is small, excited spin states of the manifold, which do display characteristic EPR signals, can be thermally accessed at cryogenic temperatures. The most prominent of these is the signal that arises from the $S = 3$ excited spin state. It appears as a multiline signal centered at $g = 2.2$, with a characteristic ^{55}Mn -hyperfine spacing of 4.5 mT [46-50]. All other excited spin states produce EPR spectra that are

broad and typically unstructured. The $S = 3$ EPR signal increases approximately 3-fold in samples where 2 Mn^{II} per monomer were added.

In addition to the $\text{Mn}^{\text{II}}/\text{Mn}^{\text{II}}$ dimer, a free Mn^{II} signal is also observed. In the sample where two Mn^{II} per monomer were added, this signal also increases by approximately a factor of 3. No other species is clearly observed. The concentration of this non-chelated Mn^{II} population was readily assessed by comparison to a 1 mM Mn^{II} hexaaquo standard. Its concentration was ~20% of the added Mn^{II} , requiring that the remainder (80%) binds exclusively as a dimer, which suggests that R2c has a high affinity for Mn^{II} and that metal binding is cooperative. The concentration of total Mn^{II} added was verified by subsequent acid denaturation of the protein, which abolished the Mn^{II} dimer signal, owing to the release of Mn^{II} back into solution.

In stark contrast, the addition of 1 or 2 Mn^{II} per monomer to the R2lox protein resulted in a very small $\text{Mn}^{\text{II}}/\text{Mn}^{\text{II}}$ dimer population (see inset in Figure 6B), with a concomitantly larger apparent free Mn^{II} population. However, based on comparison to the Mn^{II} standard, the free Mn^{II} concentration did not quantitatively match the concentration of added Mn^{II} . The amount of free Mn^{II} was seen to vary from batch to batch in the range of 30-80% of the added Mn^{II} . In addition, the protein has a broad underlying signal reminiscent of EPR signals seen for chelated Mn^{II} , i.e. Mn^{II} -EDTA ([52] and references therein), suspected to be the ‘missing’ Mn^{II} population. In principle this signal could also represent a Mn^{II} dimer signal. We note though that for this to be the case, the exchange interaction between the two Mn sites would need to be much larger in comparison to R2c, as the $S = 3$ excited state contribution is strongly suppressed. This broad signal and the $S = 3$ signal would then have to be strongly temperature dependent over the 5-20 K range, which is not the case. The line shape of the broad Mn signal is invariant at all temperatures measured, and both signals have

approximately Curie temperature dependence, although the microwave saturation properties of the two signals complicate this analysis. Thus, the broad signal is instead assigned to non-specifically, presumably surface-bound Mn^{II} . This assignment was confirmed by acid denaturation, which resulted in the complete loss of the broad, unstructured signal and the emergence of a Mn^{II} signal identical to the Mn^{II} standard of concentration equivalent to the amount of Mn^{II} added to the protein. Thus, in contrast to R2c, R2lox is a poor chelator of Mn^{II} in the absence of Fe^{II} , and does not spontaneously assemble a Mn/Mn cofactor under solution loading conditions.

These observations further demonstrate that metal binding proceeds via very different pathways in these two highly similar proteins, at least in terms of their regulation of Mn^{II} binding. As only the dimer signal is observed for R2c even at substoichiometric loading, the protein must preferentially bind two metals per active site rather than one. This suggests that R2c inserts metals via a cooperative binding mechanism, i.e. the binding of the first Mn^{II} increases the metal-binding affinity of the second site. The opposite appears to be the case for R2lox, which, if anything, prevents or excludes the binding of Mn^{II} to the apo-protein scaffold.

3.7. Optimizing Mn/Fe cofactor assembly in R2lox: efficient heterometallic cofactor assembly at substoichiometric Fe^{II} concentrations. The above information shows that while high Mn^{II} concentrations promote heterometallic cofactor assembly in R2lox, this effect is not due to Mn^{II} association with the protein prior to Fe^{II} binding, as is the case with R2c [32] (Figure 3, Figure 6). It can therefore be supposed that metal binding to the R2lox scaffold is triggered by Fe^{II} binding to site 2, its preferred location as shown by XAD (section 3.5).

Overall, the homo- or heterobinuclear metallocofactor assembly reaction involves four reactants: the apo-protein, Mn^{II} , Fe^{II} , and O_2 . Cofactor maturation is a multi-step process involving the 4-electron reduction of O_2 to water and subsequent oxidation of an aliphatic protein sidechain. This reaction likely involves many elementary steps, and discrete intermediates and rate constants will not necessarily be the same for the two different metallocofactors. In the context of this work, however, the effects of limiting metal concentrations are expected to dominate the assembly process. Ergo, at Fe^{II} concentrations that are low relative to those of Mn^{II} , Mn/Fe assembly should be the dominant reaction pathway in R2lox, because low Fe^{II} concentrations should mitigate Fe/Fe assembly. Starting from this hypothesis, titration experiments were conducted using substoichiometric Fe concentrations. In this experiment, 0.05 eq. of Fe was added from a concentrated stock solution to a sample pre-incubated with 4 eq. Mn under aerobic conditions. After a 10 min incubation, an aliquot was taken. A second 0.05 eq. Fe was then added and the entire process repeated (with varying step sizes) until 1 eq. of Fe per protein was added. All samples were incubated at room temperature for a total of 1 h after the last addition of Fe to allow for cofactor maturation to complete before excess metal ions were removed by desalting. This yielded a total of 8 samples with final protein:Mn:Fe ratios of 1:4:0.05, 1:4:0.1, 1:4:0.2, 1:4:0.3, 1:4:0.4, 1:4:0.6, 1:4:0.8 and 1:4:1. Two batches of samples (sets B and C) were prepared using this procedure. A third set (set A, prepared from the same apo-R2lox batch as C) consisted of 5 samples with the protein:Mn:Fe ratios of 1:4:0.2, 1:4:0.4, 1:4:0.6, 1:4:0.8 and 1:4:1.

Enrichment of the heterobimetallic cofactor was studied as a function of the total Fe concentration added. Figure 7 shows the resulting titration curves obtained for the three sets of samples using CW-X-band EPR. Ideal loading, which is represented in Figure 7 by the straight line with a 45° slope, refers to the hypothetical situation where every Fe^{II}

ion added to the solution goes on to assemble a Mn/Fe cofactor, i.e. 1 Fe/protein. The initial phase for all samples (0-to-0.4 Fe added) approaches the ideal loading scenario, showing a linear increase in cofactor assembly as a function of the Fe concentration, in line with the above arguments. The second half of the titration curve demonstrates incomplete heterobimetallic loading. While results at stoichiometric metal ion concentrations were always reproducible for any particular protein batch, variation between batches is large. In this instance, sets A and C reach a Mn/Fe cofactor level of approximately 30%, whereas the Mn/Fe cofactor yield in set B is approximately two-fold higher.

An equivalent Fe titration series was prepared of R2c. However, because $\text{Mn}^{\text{II}}/\text{Mn}^{\text{II}}$ homodimers are assembled in R2c when Mn^{II} is in excess, the samples reconstituted at substoichiometric Fe^{II} levels represent a mixture of $\text{Mn}^{\text{II}}/\text{Mn}^{\text{II}}$ and $\text{Mn}^{\text{IV}}/\text{Fe}^{\text{III}}$ centers (reduced to $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ by dithionite treatment) that are impossible to quantify by EPR. We therefore used TXRF to quantify the metal contents of these and other selected EPR samples.

3.8 TXRF data confirm the EPR, Mössbauer and XAD results. Within the error of the method, similar loading trends were observed by TXRF as by EPR, Mössbauer and XAD (Figure 8). The TXRF data were interpreted assuming that all metals are bound in binuclear clusters, and that the formation of heterobinuclear clusters is preferred, in line with the results presented above. Following this interpretation, a higher fraction of Mn/Fe centers are assembled in R2c than in R2lox under the same conditions. Moreover, complete metallation is more easily achieved in R2c. Excess Mn^{II} is sufficient for efficient assembly of Mn/Fe cofactors in R2c, whereas in R2lox Mn^{II} must be in excess and Fe^{II} must be substoichiometric to achieve a high yield of Mn/Fe

cofactors. In R2c a Mn/Mn homodimer is assembled when Mn^{II} is in excess and Fe^{II} is at substoichiometric concentrations, but the subsequent addition of Fe^{II} displaces the Mn^{II} ion from one site, such that the amount of Mn/Fe clusters then increases linearly with the added Fe^{II} concentration. In contrast, a Mn/Mn homodimer does not form in R2lox, and a larger fraction of Fe/Fe homodimers is obtained in R2lox than in R2c.

TXRF data also reveal a possible origin of the large batch-to-batch variability in Mn/Fe cofactor yields in R2lox. An obvious cause of batch variability might be the partial (mis)metallation of the proteins in the heterologous expression system. Although metallation of the proteins is suppressed by addition of EDTA to the growth media, the EPR silent diiron cofactor can accumulate in a fraction of centers [19], and this fraction varies from batch to batch. TXRF measurements were performed on the apo-protein preparations used for all EPR samples (Figure 8). The apo-R2c batch used for the Mn titration in Figure 3 contained 0.26 Fe/protein prior to metal loading, while the batch used for the Fe titration contained only about half as much Fe. We cannot ascertain whether this Fe is in the form of mononuclear or homobinuclear centers. A similar percentage of pre-existing Fe content was seen in the R2lox batches, whereas the concentration of all other transition metals (Mn, Ni, Zn and Cu) was negligible. This was also the case for R2c apo preparations. The conditions used to produce unmetallated protein also illustrate that R2lox has a much lower metal-binding affinity than R2c. While for R2lox production in rich media (terrific broth) with addition of 0.5 mM EDTA is sufficient to suppress metallation to the levels shown here (i.e. $\leq 10\%$ Fe/Fe-bound protein), much more stringent conditions are required to achieve the same low level of contamination in R2c: minimal media without addition of supplementary metals, but with addition of 0.5 mM EDTA.

The effect of the Fe content of the apo-protein on Mn/Fe loading in R2lox is most clearly demonstrated by the Fe titration series shown in Figure 7. TXRF data show that the batch from which sets A and C were prepared had a larger Fe content in the apo-protein than the batch used to prepare set B, providing a potential explanation why set B showed a much higher final Mn/Fe cofactor yield. The apo-R2lox batch used for the Mn titration shown in Figure 3 had a similar Fe content as the batch used for the Fe titration sets A and C, and a similar maximum yield of Mn/Fe cofactors, although the reconstitution conditions used for the Mn titration also led to formation of a higher fraction of Fe/Fe centers (Figure 8).

Notably, however, the Fe-preloaded fraction in R2lox does not correspond to the entire fraction of protein that does not contain a Mn/Fe cofactor after reconstitution at substoichiometric Fe levels. Thus, TXRF measurements demonstrate that, as inferred from Mössbauer and XAD data, a large fraction of unmetallated protein always remains when R2lox is reconstituted with ≤ 1 equivalent of Fe (Figure 8), which in fact has to be the case if a significant proportion of the added Fe is assembled into Fe/Fe centers.

4. DISCUSSION

This study probes a possible physiological assembly mechanism of the Mn/Fe cofactor of R2lox. Interestingly, completely different strategies are used for cofactor assembly in R2lox and its structural homologue, R2c. The data for the *S. erythraea* R2c shown here agree with the mechanism developed from an earlier X-ray crystallography study of *C. trachomatis* R2c [32]: in R2c Mn^{II} can bind to both metal-binding sites and subsequently be displaced from site 2 by Fe^{II} (Figure 9). This mechanism enriches Mn/Fe cofactors because Mn/Mn centers cannot reduce oxygen and therefore remain labile [14, 53], whereas oxygen activation by the Mn/Fe center ‘fixes’ the metal ions in

the active site [54]. Correct metallation in this system is therefore presumably promoted by different affinities of site 1 and site 2 for Fe^{II} . Importantly, in order for the heterobimetallic cofactor to be formed in R2c, Mn^{II} has to bind before Fe^{II} , as diiron centers will preferentially form if Fe^{II} binds first because metal binding is cooperative. However, formation of diiron cofactors in R2c appears to be unfavorable, at least more so than in R2lox, given that incubation with Fe^{II} did not completely suppress assembly of Mn/Fe cofactors upon subsequent addition of Mn^{II} in R2c, whereas it did so in R2lox. Therefore higher Fe^{II} concentrations promote higher Mn/Fe yields in R2c when Mn^{II} and Fe^{II} are added simultaneously, but have the opposite effect on R2lox. But even if Mn^{II} binds first, mismetallation of R2c can still occur in the form of a Fe/Mn cofactor with the Mn ion in site 2. Our EPR data suggest that mismetallation may indeed occur, although as noted above, further work is needed to characterize the different populations of Mn/Fe cofactors observed in R2c and their relative concentration (see Figure 3). Notably, this cofactor assembly mechanism makes it possible to achieve near-complete Mn/Fe loading *in vitro* by simply adding Mn^{II} to R2c first.

In contrast, Mn^{II} does not bind to either binding site of R2lox on its own. Instead, cofactor assembly in R2lox is triggered by binding of Fe^{II} at site 2, followed by binding of either Fe^{II} or Mn^{II} in site 1 (Figure 9). Therefore heterobimetallic cofactor assembly in R2lox is achieved by keeping the concentration of Fe^{II} that the protein is exposed to at a substoichiometric level. Elevated concentrations of Mn^{II} lead to enrichment of the heterobinuclear cofactor. As is the case for R2c, different affinities of site 1 and site 2 for Fe^{II} are presumably responsible for formation of the heterobimetallic cofactor, albeit via a different assembly pathway. Thus the R2lox scaffold has been tuned to break Irving-Williams behavior, at least to the extent that it shows no preference at site 1 for either Mn or Fe, as pointed out in our earlier study [12]. Unfortunately, coupled with the

apparently low metal affinity of the R2lox scaffold, this mechanism makes it impossible to achieve more than ~60% Mn/Fe loading *in vitro*: the more Fe^{II} is added, the more diiron cofactors are formed; the less Fe^{II} is added, the more unmetallated protein remains. R2c appears to have a much higher metal affinity than R2lox, which, coupled with greater selectivity of the binding sites, leads to the formation of almost exclusively Mn/Fe cofactors.

Notably, the mechanism that can be deduced for Mn/Fe cofactor assembly in R2lox from the data presented here differs partially from the hypothesis put forward in our previous study [12]. XAD measurements on crystals of metal-free R2lox that had been soaked with a large excess of Mn^{II} and Fe^{II} at equal concentrations under anoxic conditions showed that site 2 prefers Fe, whereas site 1 is non-specific. This observation agrees with the results of the present study. However, when the same crystal soaking experiments were performed in the presence of oxygen, site 1 was found to contain only Mn, while site 2 still showed a preference for Fe. Based on computational data suggesting that oxygen activation is several orders of magnitude faster with a Mn/Fe than a diiron cluster [14], we then proposed that fast exchange of Mn^{II} and Fe^{II} at the non-specific site 1 coupled with the faster activation rate of the Mn/Fe cluster may lead to its enrichment [12]. The conditions in our solution reconstitution experiments are drastically different: it is not possible to perform these at the same large excess of metal ions because the protein precipitates at Fe^{II} concentrations above 1 mM, whereas crystals are not affected by concentrations of as much as 5 mM. The solution experiments were therefore performed at close to stoichiometric concentrations of metal ions, and consequently fast metal exchange is not possible under these conditions. At the large excess of metal ions over protein used for crystal soaking, Mn can be ‘pushed’ into both binding sites [12, 19], whereas it does not bind to either site in solution,

illustrating that the crystal soaking experiments cannot be directly compared to the experiments shown here. Hence, based on the data presented here, we cannot exclude that the enrichment mechanism we proposed does not operate in crystals.

What is the structural basis for the differences between cofactor assembly in R2c and R2lox? More specifically, do the structures reveal why R2c readily forms dimanganese clusters, whereas R2lox does not bind Mn^{II} on its own at all? When considering the ligating atoms only, R2c and R2lox appear to be very similar, as both have a $M(O)_5(N)$ coordination sphere around each metal center (Figure 1) [8, 9, 12, 19, 32]. However, the precise structures of the oxygenic ligands are quite distinct. R2lox features a bis(μ, κ^2 -carboxylato-), mono(μ, κ^1 -carboxylato) bridging motif in the reduced state, while R2c contains a (μ, κ^2 -carboxylato-), (μ, κ^1 -carboxylato), (μ -O($H_{(2)}$)) bridge. As one possible explanation, the monodentate bridge coupled with an open/aquo coordination site should confer increased flexibility to R2c relative to R2lox, which may contribute to its metal-binding plasticity and allow for Mn^{II} coordination at both sites.

R2lox and R2c also display significantly different tendencies for binding divalent metals, with the latter showing much higher affinities for both Mn^{II} and Fe^{II} . This may be related to differences in the structures of the apo-proteins. In R2c, both metal-binding sites are preformed in the absence of metal ions, whereas in R2lox only site 2 is largely preformed, while site 1 is completely disordered [12, 32]. This observation in fact first led us to propose that binding of Fe^{II} in site 2 induces ordering of site 1 in R2lox, and consequently that Fe^{II} has to bind to site 2 before any metal ion can bind in site 1 [12]. Another possible contributor to metal selectivity relates to the level of distortion in the unmetallated active site. As noted previously [7], both sites in both R2c and R2lox have distorted octahedral coordination spheres, with the distortion being more pronounced at site 2. Due to its symmetric orbital occupancy, it is more favorable

for Mn^{II} to adopt a perfect octahedral coordination geometry, whereas Fe^{II} requires minor Jahn–Teller distortion [55, 56]. Thus, the geometry of site 2 is perhaps slightly more favorable for Fe^{II} binding, as suggested by computational data [12]. The binding geometry of site 2 in the disordered R2lox apo-protein may be much more distorted than in apo-R2c, excluding Mn^{II} binding to site 2 in the former, while allowing binding in the latter protein, though the fact that it can easily be displaced from site 2 by Fe^{II} suggests that it is weakly bound even in R2c. We consider it possible that Mn^{II} can bind in the R2lox active site as well, but, as per the Irving-Williams series, does so with sufficiently weaker affinity than Fe^{II} that it is not trapped, since it cannot activate oxygen [14, 53], and is thus easily replaced. Detailed isothermal titration calorimetry experiments, which form the basis of ongoing work in our laboratories, will provide quantitative information on the thermodynamics of divalent metal binding to both R2c and R2lox.

Structural differences also offer a potential explanation for why Mn is preferentially found in site 1 in the oxidized cofactor. A primary distinction between the two metal binding sites within both R2c and R2lox is that site 1 has an open coordination site for water, while site 2 is constrained by protein-derived ligands (Figure 1) [12, 19, 32]. Upon oxidation, Mn^{III} must undergo Jahn-Teller distortion to stabilize the singly occupied, doubly degenerate $d_{x^2-y^2}$ and d_{z^2} orbitals. An external water ligand allows this distortion to readily occur without requiring structural changes in the protein, as would be required for a Mn^{III} ion in site 2. The anisotropy of the Mn fine structure and quadrupole tensor as well as the Mn-water distance in the crystal structures reflect this strong distortion [12, 16, 19]. The preferred asymmetry in the oxidized cofactor would thus be under kinetic control, as the energetic penalty for ligand rearrangement of a Mn-occupied site 2 upon oxygen exposure renders this configuration mostly inaccessible.

Weak binding affinities of both Mn^{II} and Fe^{II} might enable rapid metal exchange in the active site until oxidation traps the cofactor in the preferred configuration, similar to our previous hypothesis [12]. Notably, this mechanism may be at play in both R2lox and R2c.

Interestingly, a recent study of a biomimetic model system for the R2c/R2lox cofactor found that while replacement of Mn^{II} with Fe^{II} in both sites was favorable, the mixed-metal state was slightly more stable than expected [34]. Although it is not clear how this stabilization is achieved, it suggests that there is a favorable interaction between the metal ions in the heterodinuclear complex. These data thus indicate another avenue that should be investigated in the future to understand how exactly Mn/Fe cofactors are assembled in these scaffolds.

5. CONCLUSIONS

The data presented here indicate that Mn/Fe cofactor assembly is driven largely by a combination of metal availability and the differential metal affinities of the two binding sites in both R2lox and R2c. However, our results do not exclude the possibility that fast metal exchange coupled with the kinetics of oxygen binding or activation affect cofactor assembly in R2lox and R2c *in vivo*, where the proteins might be exposed to high local concentrations of metal ions and/or low oxygen availability. Because in R2lox Fe^{II} is required to bind first, the final outcome of the metallation process is dictated by the relative concentrations of Mn^{II} and Fe^{II} . This assembly pathway suggests that R2lox proteins may be cambialistic enzymes, utilizing either diiron or Mn/Fe cofactors *in vivo* depending on the cellular metal status, a possibility that was also raised by the observation that Mn/Fe and Fe/Fe centers in R2lox are structurally nearly identical and both active, at least as far as oxygen activation and tyrosine-valine ether

cross-link formation are concerned [19]. R2c on the other hand is not catalytically active with a diiron cofactor [10, 11], and it is therefore perhaps not surprising that a different mechanism is employed in this system that strongly favors Mn/Fe cluster assembly by allowing Mn^{II} to bind first. Notably, both R2c and R2lox proteins are predominantly found in extremophiles and pathogens [21], i.e. organisms that generally live under iron-limiting conditions and replace iron with manganese in protein cofactors as much as possible [57]. Hence, intracellular manganese concentrations are likely to be much higher relative to iron levels in these organisms than in well-studied model organisms such as *E. coli* that depend much more heavily on iron [58, 59]. The relative availabilities of manganese and iron might therefore favor Mn/Fe cofactor assembly in both R2c and R2lox proteins *in vivo*.

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TABLES

Table 1. Crystallographic data statistics

protein:Mn:Fe loading ratio	1:2.4:1.2				1:2:1				
Crystal	1		2		1			2	
PDB ID					5EKB				
Beamline	PX14.1/ BESSY	PX14.1/ BESSY	PX14.1/ BESSY	PX14.1/ BESSY	P13/ PETRA3	P13/ PETRA3	P13/ PETRA3	P13/ PETRA3	P13/ PETRA3
Dataset	Fe peak	Mn peak	Fe peak	Mn peak	Fe peak	Mn peak	native	Fe peak	Mn peak
Wavelength (Å)	1.73	1.88	1.73	1.88	1.73	1.88	0.98	1.73	1.88
Resolution range (Å)	50.00-2.98 (3.16-2.98)	50.00-2.99 (3.17-2.99)	50.00-2.98 (3.16-2.98)	50.00-2.98 (31.6-2.98)	50.00-2.99 (3.17-2.99)	50.00-2.98 (3.16-2.98)	50.00-2.07 (2.20-2.07)	50.00-2.97 (3.15-2.97)	50.00-2.98 (3.16-2.98)
Space group	I222	I222	I222	I222	I222	I222	I222	I222	I222
Unit cell dimensions a, b, c (Å)	55.72, 97.32, 128.84	55.74, 97.17, 128.96	55.29, 96.87, 127.91	55.47, 96.95, 128.27	55.97, 97.18, 128.27	55.98, 97.21, 128.38	56.04, 97.22, 128.28	56.68, 97.90, 128.86	56.65, 97.76, 128.73
Unique reflections	13758 (2224)	13640 (2132)	13504 (2114)	13620 (2151)	13614 (2148)	13686 (2288)	21369 (3166)	14015 (2071)	13973 (2146)
Multiplicity	6.7	6.7	6.6	6.5	6.8	6.7	6.4	6.7	6.7
Completeness (%)	99.6 (98.0)	99.1 (95.0)	99.5 (97.3)	99.6 (97.8)	99.3 (96.9)	98.5 (92.0)	98.8 (92.8)	98.4 (90.4)	98.9 (94.0)
I/σ(I)	17.49 (2.74)	16.40 (2.15)	17.78 (3.09)	16.09 (2.16)	19.59 (6.42)	16.45 (4.86)	11.80 (0.95)	10.37 (1.70)	11.82 (1.88)
R _{merge}	6.9 (51.3)	7.6 (70.0)	7.9 (50.2)	7.8 (63.5)	7.0 (23.4)	8.5 (31.7)	9.1 (157.6)	12.6 (81.3)	10.5 (81.1)
R _{meas}	7.5 (55.7)	8.2 (76.0)	8.6 (54.7)	8.4 (69.1)	7.6 (25.5)	9.2 (34.7)	9.9 (172.9)	13.6 (88.9)	11.3 (88.6)
CC _{1/2} [§]	99.9 (85.8)	99.9 (77.1)	99.9 (87.5)	99.9 (80.4)	99.9 (96.9)	99.8 (94.6)	99.9 (25.6)	99.8 (70.5)	99.8 (77.0)
Anomalous signal	1.02 (0.65)	0.85 (0.66)	1.05 (0.73)	0.86 (0.63)	0.99 (0.84)	0.86 (0.85)	-	0.88 (0.72)	0.83 (0.69)

Values in parentheses are for the highest resolution shell. Friedel pairs were merged for the native dataset only. [§]Percentage of correlation between intensities from random half-datasets [60]. The correlation is significant at the 0.1% level in all resolution shells in all datasets.

Table 2. Refinement statistics

protein:Mn:Fe loading ratio	1:2:1
Crystal	1
PDB ID	5EKB
reflections used	21362
$R_{\text{work}}/R_{\text{free}}$ (%) [†]	19.0/24.4
coordinate error (Å)	0.33
non-H atoms	2400
Protein residues [‡]	285 (2-286)
Water molecules	23
Ligand molecules	1
Metal ions	2
rmsd bonds (Å) [¶]	0.017
rmsd angles (°) [¶]	1.174
Ramachandran	96.1/3.9/0.0
favoured/allowed/ outliers (%)	
Clashscore	5.53
Wilson B (Å ²)	50.94
Isotropic B (Å ²) ^{**}	
all atoms	67.3
protein main and side chains	67.3
Mn (site 1)	45.9
Fe (site 2)	47.2
fatty acid	69.4
water	50.10
occupancies <1.0	
Mn (site 1)	0.79
Fe (site 2)	0.79

[†] R_{free} is calculated from a randomly selected 5% subset of reflections excluded from refinement.

[‡]Residues out of the 302 residue full-length protein included in the final model are given in parentheses.

[¶]Root-mean-square deviation from ideal geometry. ^{||}Geometry statistics were calculated with MolProbity [41]. ^{**} Average B factors were calculated with Baverage in the CCP4 suite [61].

Table 3. Mössbauer isomer shift (δ), quadrupole splitting (ΔE_Q), and full line width (Γ) of Fe/Fe and Mn/Fe loaded R2lox samples, measured at 80 K.

sample	δ / mms ⁻¹	ΔE_Q / mms ⁻¹	Γ / mms ⁻¹
Mn/Fe, reduced	1.26	2.98	0.56
Fe/Fe, reduced	1.27	2.97	0.55
Mn/Fe, oxidized ^a	0.51	0.81	0.55
Fe/Fe, oxidized ^b	0.51	0.81	0.48

^aAt 4.2 K the values are $\delta = 0.56$ mms⁻¹; $\Delta E_Q = -0.81$ mms⁻¹; asymmetry parameter of the electric field gradient (efg), $\eta = 0.5$, with an Euler rotation of the efg according to $\beta = 92^\circ$, $\gamma = 45^\circ$. Hyperfine tensor components $A/g_N\mu_N = (-45.13, -37.26, -60.59)$ Tesla and g-values, $g = (1.96, 1.97, 2.93)$ used for the simulation of the magnetic spectrum of Mn/Fe clusters as shown in Figure 4C (green trace) have been taken from EPR. ^bAt 4.2 K the values are $\delta = 0.52$ mms⁻¹; $\Delta E_Q = 0.81$ mms⁻¹.

Table 4. Relative amounts of Mn and Fe in the two metal sites of R2lox reconstituted with Mn and Fe in aerobic solution, as quantified by XAD. The error of the quantification is in the range of 10%.

		site 1		site 2	
protein:Mn:Fe loading ratio	crystal	Mn	Fe	Mn	Fe
1:2.4:1.2	1	0.60	0.40	0.00	1.00
	2	0.58	0.42	0.00	1.00
1:2:1	1	0.78	0.22	0.16	0.84
	2	0.81	0.19	0.00	1.00

FIGURE AND SCHEME CAPTIONS

Figure 1. Crystal structures of (A) *Chlamydia trachomatis* R2c (4M1I [32]) and (B) *Geobacillus kaustophilus* R2lox in the reduced state (PDB ID 4HR4 [12]). The site 1 metal ion in R2lox represents an approximately equal mixture of Mn and Fe.

Scheme 1. Possible metal loading combinations for R2lox and R2c and their corresponding EPR and/or Mössbauer activity. The main assembly pathway, as proposed in current literature [7, 10, 12, 32], is shown on top, with secondary/side pathways shown below. The net oxidation state of the as-isolated R2c cofactor is at the EPR silent $\text{Mn}^{\text{IV}}/\text{Fe}^{\text{III}}$ level, whereas for the R2lox cofactor it is at the EPR active $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ level. Hence to observe R2c by EPR, the cofactor must first be chemically reduced. In all oxidized states of the cofactor, the magnetic interaction between the two metals is large and thus the EPR and applied Mössbauer signal of these states is representative of both metals. In the reduced state however, the interaction is small, comparable to magnetic relaxation, leading to single ion-like signals for the mixed valence and Fe-only cofactor. Only the reduced, Mn-only cofactor shows a coupled metal signal. Note that while Fe^{II} is paramagnetic (high spin d^6 ion), its intrinsically large fine structure splitting renders it EPR silent at low microwave frequency bands such as X-band.

Figure 2. CW-X-band EPR spectra of the $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ cofactors in R2lox (A and B) and R2c (C and D) recorded at $T = 10$ K for two microwave power levels: 20 mW (A and C) and 10 μW (B and D). The three curves in each subplot correspond to the assembly conditions, where Mn^{II} and Fe^{II} were added to the apo-protein simultaneously (upper), Mn^{II} before Fe^{II} (middle), and Fe^{II} before Mn^{II} (lower).

Figure 3. A) CW-X-band EPR spectrum of the $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ R2lox cofactor. B) CW-X-band EPR spectrum of the $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ R2c cofactor (after correction for a Mn^{II} contribution). C) Heterobimetallic cofactor enrichment of R2lox (blue) and R2c (pink) as a function of the concentration of Mn^{II} during assembly. The concentration of Fe^{II} was fixed to either 1 or 2 Fe per protein monomer.

Figure 4. Zero-field Mössbauer spectra of R2lox loaded with $^{57}\text{Fe}^{\text{II}}$ only or Mn^{II} and $^{57}\text{Fe}^{\text{II}}$ under (A) anaerobic or (B) aerobic conditions, recorded at 80 K, and (C) magnetic Mössbauer spectra of the aerobically loaded samples recorded at 4.2 K with 0.1 T field applied perpendicular to the γ -rays. The solid red lines in the zero-field spectra and the 4.2 K spectrum of the oxidized Fe/Fe sample (bottom trace) are the result of fits with Lorentzian doublets. The magnetic spectrum of the oxidized Mn/Fe sample was simulated with the spin Hamiltonian for the total spin $S = 1/2$ of the $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ heterodimer (green trace), superimposed with a simulation for the $S = 0$ ground state of $\text{Fe}^{\text{III}}/\text{Fe}^{\text{III}}$ homodimers (blue trace). The latter fraction of diamagnetic homodimers accounts for 55% of the total iron (based on the relative spectral intensity, assuming equal Lamb-Mössbauer factors), which reveals a ratio of 62:38 of Mn/Fe and Fe/Fe clusters in that sample.

Figure 5. Anomalous difference density at the Fe (grey) and Mn (pink) edges from crystals of R2lox reconstituted with Mn and Fe under aerobic conditions at a protein:Mn:Fe ratio of 1:2.4:1.2 or 1:2:1, contoured at 5 electrons/ $\text{\AA}^3$. At the Fe edge, both Fe and Mn display an anomalous signal. The Mn contribution is subtracted from the signal measured at the Fe edge to obtain the relative amounts of Mn and Fe in each site [12]. The active site structure of oxidized Mn/Fe-bound R2lox is shown for clarity

with Mn (purple sphere) in site 1 and Fe (orange sphere) in site 2. The anomalous maps are shown for each crystal 1 (see Table 4).

Figure 6. A) CW-X-band EPR spectrum of R2c loaded with 1 (red) or 2 (black) Mn^{II} per monomer, $T = 10$ K. Here a $\text{Mn}^{\text{II}}/\text{Mn}^{\text{II}}$ dimer signal is observed. B) CW-X-band EPR spectra of R2lox loaded with 1 (red) or 2 (black) Mn^{II} per monomer, $T = 10$ K. Non-bound Mn^{II} is predominantly observed. C) CW-X-band spectra of R2c and R2lox loaded with 1 Mn^{II} per monomer before and after acid denaturation. The spectrum of a 1 mM Mn^{II} solution that was used as a standard is shown for comparison.

Figure 7. Heterobimetallic cofactor enrichment of R2lox under substoichiometric Fe^{II} loading conditions. Each data point represents the cumulative addition of Fe^{II} during assembly. The concentration of Mn^{II} was fixed to 4 Mn per monomer.

Figure 8. (A) Mn and Fe contents of R2c and R2lox samples before and after reconstitution with Mn^{II} and Fe^{II} in solution, as determined by TXRF. The error is ~ 0.1 metals/protein (standard deviation from at least 4 independent measurements on each sample). The amounts of other transition metals (Ni, Zn and Cu) were negligible in all samples. Protein concentrations were ~ 1 mM (apo samples) and $0.10 - 0.14$ mM (reconstituted samples). (B) The amounts of binuclear cluster species were calculated assuming that all metals are bound in binuclear clusters, and that the formation of heterobinuclear clusters is preferred. The category axis labels denote the protein:Mn:Fe reconstitution ratios of the samples.

Figure 9. Model for cofactor assembly in R2c and R2lox. Mn^{II} binding occurs at different points during the assembly process in the two systems. In R2c, a $\text{Mn}^{\text{II}}/\text{Mn}^{\text{II}}$ homodimer is assembled efficiently. Mn^{II} is then displaced by Fe^{II} at one site,

preferentially site 2, to initiate cofactor activation by O₂. The different metal binding affinities of site 1 and 2 drive correct metallation. It is unclear whether the inverse configuration of the R2c cofactor is catalytically active [31]. In R2lox, Mn^{II} is excluded from the protein in the absence of Fe^{II}. Binding of Fe^{II} at site 2 promotes metal binding at site 1. Under conditions where Mn^{II} is in excess, heterobimetallic cofactor assembly is favored. In R2lox, both the Mn/Fe and the Fe/Fe cofactor activate oxygen and support formation of the tyrosine-valine ether crosslink, suggesting they may both be active [19].

Scheme 1

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Graphical abstract

The heterobinuclear Mn/Fe cofactor in two ferritin-like proteins, the small subunit (R2) of class Ic ribonucleotide reductase (R2c) and the R2-like ligand-binding oxidase (R2lox), is assembled via divergent pathways.

Highlights

- Two classes of ferritin-like di-metal carboxylate proteins bind a Mn/Fe cofactor.
- The heterometallic cofactor assembles via different mechanisms in the two classes.
- Differential metal availabilities likely control Mn/Fe cofactor assembly *in vivo*.