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Electronic structural flexibility of heterobimetallic Mn/Fe cofactors: R2lox and R2c, a case study

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ABSTRACT: The electronic structure of the Mn/Fe cofactor identified in a new class of oxidases (R2lox) described by Andersson and Högbom [*Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 5633] is reported. The R2lox protein is homologous to the small subunit of class Ic ribonucleotide reductase (R2c) but has a completely different *in vivo* function. Using multifrequency EPR and related pulse techniques, it is shown that the cofactor of R2lox represents an antiferromagnetically coupled Mn^{III}/Fe^{III} dimer linked by a μ -hydroxo/bis- μ -carboxylato bridging network. The Mn^{III} ion is coordinated by a single water ligand. The R2lox cofactor is photoactive, converting into a second form (R2lox_{photo}) upon visible illumination at cryogenic temperatures (77 K) that completely decays upon warming. This second, unstable form of the cofactor more closely resembles the Mn^{III}/Fe^{III} cofactor seen in R2c. It is shown that the two forms of the R2lox cofactor differ primarily in terms of the local site geometry and electronic state of the Mn^{III} ion, as best evidenced by a reorientation of its unique ⁵⁵Mn hyperfine axis. Analysis of the metal hyperfine tensors in combination with density functional theory (DFT) calculations suggests that this change is triggered by deprotonation of the μ -hydroxo bridge. These results have important consequences for the mixed-metal R2c cofactor and the divergent chemistry R2lox and R2c perform.

1. INTRODUCTION

Heterobimetallic cofactors are found in an increasing number of proteins and perform a diverse range of chemical reactions. Examples include the Ni/Fe cofactor of the [NiFe]-hydrogenase, which produces and oxidizes hydrogen,¹ the heme a₃/CuB arrangement in cytochrome c oxidase, which reduces dioxygen,² and the Cu/Zn binding motif of eukaryotic SOD, which detoxifies (dismutates) the superoxide radical.³ Recently this set has been extended to Mn/Fe-containing proteins, which are activated by molecular oxygen. The Mn/Fe cofactor was first identified in a variant of purple acid phosphatase⁴ and has subsequently been proposed as the native cofactor of the small subunit (R2) of class I ribonucleotide reductase (RNR) from *Chlamydia trachomatis* (CtR2c),^{5,6} and possibly an N-oxygenase from yeast.⁷⁻⁹

The R2 subunit of class I RNR acts as a single electron oxidant, initiating the catalytic conversion of ribonucleotides to deoxyribonucleotides.¹⁰⁻¹² In most subclasses of R2, the one-electron oxidant is stored in the form of a tyrosyl radical in close proximity to a dinuclear metal cofactor (Fe/Fe in class Ia¹³⁻¹⁵ or Mn/Mn in class Ib¹⁶⁻¹⁸). It is the metallocofactor that initially oxidizes the tyrosine via its reaction with oxygen or a reactive oxygen species (ROS).¹⁹ However, the R2 subclass that contains a Mn/Fe cofactor (R2 class Ic, R2c) adopts a different strategy. While the active catalytic site is also generated via oxygen activation, the redox-active tyrosine residue is replaced by a redox-inert phenylalanine,²⁰ and the oxidizing

equivalent is instead stored at the metal cofactor itself in the form of the high valent Mn^{IV}/Fe^{III} state.^{5,6} A crystal structure of the activated, mixed-metallated R2c with full occupancy has yet to be reported. However, a structure of the presumably non-physiological di-iron cofactor in CtR2c has been determined in the resting (Fe^{III}/Fe^{III}) state,²⁰ and more recently a structure of the preactivated Mn^{II}/Fe^{II} state²¹ (**Figure 1A, B**) was reported. It is unclear to what extent the activated Mn/Fe cofactor differs from the activated di-iron state.

A homologous Mn/Fe cofactor to that seen in R2c was recently discovered and structurally characterized in two R2-like proteins from the pathogenic bacterium *Mycobacterium tuberculosis* (Rv0233, MtR2lox)²² and the thermophilic bacterium *Geobacillus kaustophilus* (GkR2loxI)²³ (**Figure 1C, D**). Owing to the structural similarity this new class of proteins has with R2c, they are referred to as ‘R2lox’, as in ribonucleotide reductase R2-like ligand-binding oxidase. Although an *in vivo* function for the R2lox protein has yet to be identified, it is anticipated that the chemistry it performs resembles that of the Mn/Fe cofactor of R2c, i.e., that the cofactor acts as an oxidant. Under anaerobic conditions, the protein is inactive, binding metals in the +II oxidation state. In the presence of oxygen, an oxo/hydroxo bridge cofactor is formed with the concomitant two-electron oxidation of a nearby protein side-chain, generating a covalent ether cross-link between a tyrosine and a normally inert valine residue.^{22,23}

Figure 1. Crystal structures of R2c and R2lox proteins. **(A)** The oxidized Fe/Fe-cofactor of *CrR2c* (PDB ID 1SYY)²⁰ at 1.7 Å resolution. **(B)** The reduced Mn/Fe cofactor of *CrR2c* (PDB ID 4M11)²¹ at 1.8 Å resolution. **(C)** Superposition of the oxidized Mn/Fe-bound active sites of *Gkr2loxI* (cyan, metal-coordinating residues; blue, fatty acid ligand; PDB ID 4HR0)²³ and *Mtr2lox* (gray; PDB ID 3EE4),²² both at 1.9 Å resolution. Residue numbering is for *Gkr2loxI*. **(D)** The reduced Mn/Fe-bound active site of *Gkr2loxI* (PDB ID 4HR4)²³ at 1.9 Å resolution.

Both R2lox proteins were isolated and crystallized with an exogenous lipid bound, and in both cases the carboxylate head group provided an oxygen ligand to each of the metal sites (**Figure 1C, D**).^{22,23} As a consequence, it was speculated that its biological function is that of a lipid oxidase or possibly a desaturase.²²⁻²⁴ Both in R2c and in R2lox, the Mn/Fe cofactor is assembled with the Mn ion located in site 1.^{21-23,25,26} Structurally, the two cofactors appear to be almost identical, with the only difference being the fatty acid ligand in R2lox which takes the place of an oxo/hydroxo bridge in R2c (**Figure 1**). It is unclear therefore how the Mn/Fe cofactor is directed towards 1e⁻ redox chemistry in R2c vs. 2e⁻ redox chemistry in R2lox.

Because of the complexity of large metalloprotein systems, site-specific spectroscopies such as electron paramagnetic resonance (EPR) are invaluable tools, providing details on the structure of the metal site. EPR results complement those obtained with X-ray crystallography as the protein can be measured under dilute conditions in a near-native, solvated environment, representing the active, unperturbed conformation. Furthermore, because high-valent, oxidized Mn and Fe cofactors are highly susceptible to photoreduction during X-ray data collection,²⁷⁻³⁰ EPR has the added advantage of being a non-destructive technique. In terms of manganese metallocofactors, EPR studies have provided direct information on the oxidation state of the metal ion(s)^{16,31,32} and the identity and protonation state of first coordination sphere ligands (for recent examples see refs.³³⁻³⁵). In systems performing multi-electron catalysis, EPR can also be used to monitor the cofactor reaction cycle, providing information

on structural rearrangements, substrate binding, and reactivity.

In this work, a complete description of the electronic structure of the R2lox metallocofactor is assessed using a comprehensive set of EPR techniques, including CW- and pulse experiments at X-, Q-, and W-band frequencies in conjunction with ENDOR and ESEEM methods^{34,36,37} to probe nuclear transitions coupled to the active site. It is shown that the oxidized, Mn^{III}/Fe^{III} cofactor is similar to synthetic Mn^{III}/Fe^{III} and isoelectronic Mn^{II}/Mn^{III} complexes such as those reported by Wiegardt and coworkers.^{38,39} The discrete metallation of each site could be assessed via the first coordination sphere ligands (¹⁴N-histidine), which serve as local spin reporters on metal identity. This novel technique allows a semi-quantitative assessment of mismetallation in heterobimetallic cofactors, with broad applicability. The oxidation state, electronic structure, and ligand arrangement of the cofactor are discussed in the context of site selectivity, cofactor assembly, and its *in vivo* activity as compared to R2c.

2. EXPERIMENTAL SECTION

2.1 EPR sample preparation. The R2lox protein from *Mycobacterium tuberculosis* (Rv0233, *Mtr2lox*) was prepared in the oxidized Mn/Fe-bound state as described previously by Andersson and Högbom.²² The R2lox protein homologue I from the thermophilic bacterium *Geobacillus kaustophilus* (accession number yp_148624, *Gkr2loxI*)²³ was used for most CW- and all pulse- EPR measurements. The *Gkr2loxI* apoprotein was produced and purified as

previously described by Griese et al.²³ Protein concentrations were determined using an extinction coefficient at 280 nm of 47.76 mM⁻¹ cm⁻¹ and 50.56 mM⁻¹ cm⁻¹ for metal-free and metal-bound GkR2loxI, respectively. These extinction coefficients were obtained by measuring the absorbance at 280 nm of metal-free and metal-bound protein before and after unfolding with 5.4 M guanidine hydrochloride and using the calculated extinction coefficient at 280 nm for the unfolded GkR2loxI monomer of 45.87 mM⁻¹ cm⁻¹. To reconstitute the oxidized Mn/Fe cofactor, 200 μM apoprotein was incubated with 2 equivalents of MnCl₂ and 1 or 2 equivalents of (NH₄)₂Fe(SO₄)₂ in 100 mM HEPES-Na pH 7.0, 50 mM NaCl for 1 h at room temperature. Excess metal ions were removed by passing the sample 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 0.4–1.4 mM, and 50% glycerol was added before transfer into X-, Q- and W-band EPR tubes and flash freezing in liquid nitrogen. To label the oxidized cofactor with ⁵⁷Fe, ⁵⁷FeSO₄ was used instead of natural abundance (NH₄)₂Fe(SO₄)₂. Mn^{III}/Fe^{III}-reconstituted samples were exchanged into ²H₂O-based buffer (25 mM HEPES-Na pD 7.0, 50 mM NaCl) by four rounds of dilution and concentration. Photoexcitation of the Mn^{III}/Fe^{III} sample was carried out using a broadband halogen lamp (12 V, 50 W) through a UV filter at 77 K.

2.2 Multifrequency EPR measurements. X-Band EPR measurements were performed at 5–20 K using a Bruker E580 spectrometer, equipped with an Oxford Instruments ESR 935 cryostat and ITC4 temperature controller. Q-band pulse-EPR measurements were performed at 5 K using a Bruker ELEXSYS E580 Q-band pulse-EPR spectrometer, equipped with a homebuilt TE₀₁₁ microwave cavity,⁴⁰ Oxford-CF935 liquid helium cryostat and an ITC-503 temperature controller. High-field EPR experiments were performed at 4.8 K using a W-band EPR spectrometer (Bruker ELEXSYS E680) operating at about 94 GHz, as described in Rapatskiy et al.³⁴ Electron spin echo-detected (ESE) field-swept spectra were measured using the pulse sequence: $t_p - \tau - 2t_p - \tau$ -echo. The length of the $\pi/2$ microwave pulse was generally set to $t_p = 8$ ns (X-band), 12 ns (Q-band) and 24 ns (W-band). The interpulse distance was varied in the range $\tau = 140$ –200 ns (X-band), 200–500 ns (Q-band). Pseudomodulated EPR spectra were generated by convoluting the original absorption spectra with a Bessel function of the 1st kind. The peak-to-peak amplitude used was 2–3 mT. Three pulse X-band ¹H-ESEEM and Q/W-band ¹⁴N-ESEEM spectra were measured using the pulse sequence: $t_p - \tau - t_p - T - t_p - \tau$ -echo. The same pulse length and interpulse distance (τ) as for the ESE spectra were used. The interpulse distance T was sampled in the range of 100–16500 ns (X-band), 100–10000 ns (Q-band). The time base increment (ΔT) was 32 ns (X-band), 16 ns (Q-band). Q-band ²H-HYSCORE spectra were measured using the pulse sequence: $t_p - \tau - t_p - t_1 - 2t_p - t_2 - t_p - \tau$ -echo. A pulse length of $t_p = 12$ ns and interpulse spacing of $\tau = 240$ ns were used. The interpulse distances t_1 and t_2 were sampled in the range of 100–2000 ns. The time base increment (Δt_1 , Δt_2) was 32 ns. A shot repetition rate of ≈ 1 kHz was used for all measurements. Q-band Davies ⁵⁵Mn-ENDOR and ¹H-ENDOR spectra were collected using the pulse sequence: $t_{inv} - t_{RF} - T - t_p - \tau - t_p - \tau$ -echo using an inversion microwave pulse length of t_{inv}

= 24 ns (⁵⁵Mn), and 128 ns (¹H), a radio frequency pulse length of $t_{RF} = 3.5$ μs (⁵⁵Mn), 20 μs (¹H). The length of the $\pi/2$ microwave pulse in the detection sequence was generally set to $t_p = 12$ ns (⁵⁵Mn), 64 ns (¹H) and the interpulse delays to $T = 0.6$ μs (⁵⁵Mn), 1.5 μs (¹H) and $\tau = 396$ –468 ns. The RF frequency was swept between 80–200 MHz in 50 kHz steps to collect ⁵⁵Mn-ENDOR spectra and 20 MHz around the ¹H-Larmor frequency of about 53 MHz (1.2 T) in 50 kHz steps to collect ¹H-ENDOR spectra.

2.3 EPR Spin Hamiltonian simulations. Spectra were simultaneously fitted assuming the two metals antiferromagnetically couple to yield an effective spin $S = 1/2$ ground state (for details see Supporting Information (SI)). The basis set that describes the interaction of the unpaired electron with the metal nuclei (⁵⁵Mn, ⁵⁷Fe) and its interaction with magnetic nuclei of the first coordination sphere ligands can be built from the product of the eigenstates of the interacting spins:

$$\left| \frac{1}{2} \ M \ I_1 \ m_1 \ I_2 \ m_2 \ I_3 \ m_3 \right\rangle$$

I_1 takes the value 5/2 for ⁵⁵Mn, I_2 takes the value 1/2 for ⁵⁷Fe and I_3 takes the value 1 for ²H/¹⁴N and 1/2 for ¹H/⁵⁷Fe. M_i refers to the electronic magnetic sub-level and takes the values $\pm 1/2$; m_i refers to the nuclear magnetic sublevel and takes the values $-I_i, 1-I_i, \dots, I_i-1, I_i$. As the magnetic interactions of the ligand nuclei with the electron spin are small, such that these interactions do not contribute to the inhomogeneous linewidth of the metallocofactor's EPR spectrum, and the nuclear-nuclear interactions are negligible in magnitude relative to the electron-nuclear interaction and as such, each ligand-electron interaction can be treated independently.

The Spin Hamiltonian that describes the nucleus-nucleus-electron spin manifold is:

$$\hat{H}_{Tot} = \hat{H}_{electron} + \hat{H}_{metal} + \hat{H}_{ligand}$$

Where the three components are:

$$\hat{H}_{electron} = \beta_e \vec{B}_0 \cdot \hat{g} \cdot \vec{S}$$

$$\hat{H}_{metal} = -g_{Mn} \beta_n \vec{B} \cdot \vec{I}_1 + \vec{S} \cdot \hat{A}_1 \cdot \vec{I}_1 + \vec{I}_1 \cdot \hat{Q}_1 \cdot \vec{I}_1 - g_{Fe} \beta_n \vec{B} \cdot \vec{I}_2 + \vec{S} \cdot \hat{A}_2 \cdot \vec{I}_2$$

$$\hat{H}_{ligand} = -g_L \beta_n \vec{B} \cdot \vec{I}_3 + \vec{S} \cdot \hat{A}_3 \cdot \vec{I}_3 + \vec{I}_3 \cdot \hat{Q}_3 \cdot \vec{I}_3$$

It contains: i) the Zeeman term for the total electronic spin; ii) the Zeeman term for the nucleus; iii) the hyperfine term for the nucleus; and iv) the quadrupole term for the nucleus. Spectral simulations were performed numerically using the EasySpin package^{41,42} in MATLAB.

2.4 Density functional theory (DFT) calculations. Model geometries were taken from Griese et al.²³ and used as published. Exchange coupling constants, hyperfine tensors and nuclear quadrupole tensors were computed using the broken-symmetry density functional theory (BS-DFT) approach.^{43–46} All calculations were performed with ORCA.⁴⁷ The hybrid meta-GGA TPSSh functional⁴⁸ was used because it has been extensively calibrated in recent studies of magnetic and spectroscopic parameters of manganese systems.^{39,49–53} Scalar relativistic effects were included with the zero-order regular approximation (ZORA).^{54–56} The resolution of the identity (RI) and the chain of spheres approximation (COSX)⁴⁵ were used for the Coulomb and exact exchange, respectively. The ZORA-contracted def2-TZVP(-f) SARC basis sets⁵⁷ were employed for all atoms, combined with fully decontracted def2-TZVP/J auxiliary basis sets.⁵⁸ Tight SCF convergence

and increased integration grids (Grid6 and GridX6 in ORCA notation) were applied. For the calculation of the hyperfine and quadrupole tensors, basis sets with added flexibility in the core region were used for the metal and ligand atoms. These basis sets are based on SARC ZORA-def2-TZVP but have fully decontracted s-shells with three additional functions obtained by multiplying the exponent of the steepest original primitive with 2.5, 6.25 and 15.625. As previously described,³⁹ this follows the procedure introduced in Ref.⁵⁹, with the added benefit that the higher angular momentum subspaces are consistently contracted⁵⁷ for the ZORA Hamiltonian. Picture change effects (the effect of using appropriate quasi-relativistic property operators for the quasi-relativistic wave function) were applied for the calculation of EPR parameters and the complete mean-field approach was used for the spin-orbit coupling operator. Radial integration grids were increased to an integration accuracy of 12 and 9 (ORCA convention) for the metal and ligand atoms, respectively.

3. RESULTS AND DISCUSSION

3.1 R2lox contains a Mn^{III}/Fe^{III} cofactor. A preliminary description of the electronic structure of the aerobically reconstituted cofactor of R2lox was recently provided in Griese et al.²³ Its frozen solution CW-X-band EPR spectrum is characteristic of a heterobimetallic Mn^{III}/Fe^{III} cofactor as shown in **Figure 2A and B** and is essentially the same for both R2lox proteins isolated to date, *MtR2lox*²² and *GkR2loxI*.²³ The metallocofactor X-band spectrum displays a characteristic seven line structure (**Figure 2A, B**). This discrete line splitting obscures additional spectral information including resolution of the g-tensor, which describes the asymmetry and anisotropy of the unpaired electron(s)’ chemical environment (**Figure 2D**). The EPR signal is consistent with the Fe^{III} and Mn^{III} ions ($S_T = 1/2$) antiferromagnetically coupled to yield an $S_T = 1/2$ spin state.²³ The spectrum can be considered the superposition of two six-line patterns, derived from the hyperfine interaction of the unpaired electron spin and the ⁵⁵Mn nucleus (natural abundance 100%) of the metallocofactor ($I(^{55}\text{Mn}) = 5/2$). An additional hyperfine structure due to the Fe nucleus can also be observed when reconstituting the cofactor using an ⁵⁷Fe salt (**Figure 2C**). To best visualize the effects of g-value anisotropy and hyperfine interactions, a decomposition of the simulation is shown in **Figure 2D**. The powder EPR spectrum of the cofactor in the absence of the hyperfine interaction is shown by the black trace, resolving the three turning points (g-values) describing the three unique orientations of the molecular frame, which define the limits of the ensemble (powder) spectrum. Each of these g-positions is split due to the hyperfine interaction into six evenly spaced lines. This decomposition demonstrates that the metallocofactor has approximately axial symmetry; there is one unique molecular axis with the other two appearing more similar, both in terms of the g-value (pattern position) and metal (⁵⁵Mn, ⁵⁷Fe) hyperfine splittings (peak spacing). The chemical significance of tensor axiality and colinearity forms the basis of our current study.

3.2 Multifrequency EPR measurements of the Mn/Fe

cofactor of R2lox. Pulse-EPR measurements were performed at multiple microwave frequencies (X-, Q- and W-band) to complement the CW-EPR measurements (**Figure 3**). Magnetic field swept spectra were collected using the standard two pulse (echo) detection sequence and by free-induction decay (FID).

Figure 2. CW-X-band EPR spectra (black lines) and spin Hamiltonian simulations (red dashed lines) of the R2lox cofactor. (A) The Mn/Fe metallocofactor observed in *MtR2lox* showing its characteristic seven line structure; (B) the homologous metallocofactor seen in *GkR2loxI*; (C) the *GkR2loxI* metallocofactor labeled with ⁵⁷Fe;²³ (D) a decomposition of the spin Hamiltonian simulation. The top black trace shows the inferred g-tensor of the metallocofactor. The lower colored traces show the hyperfine structure that superimposes the g-tensor. The sum of the hyperfine patterns results in the observed seven-line structure. Simulation parameters are given in **Table 1**.

Upon moving to higher microwave frequencies, the metallocofactor spectrum becomes more resolved, allowing the Zeeman and hyperfine interactions to be accurately determined. Indeed, the predictable frequency/field dependence of the metallocofactor spectrum suggests that it represents only one EPR species. Simulations using the spin Hamiltonian formalism (**Figure 3 red dashed lines**) reproduce all spectral features observed in the EPR datasets (see SI S1). ⁵⁵Mn-ENDOR was used to further characterize the Mn^{III} ion of the active site. ENDOR directly reports on the local properties of metal ions in spin exchange coupled systems.^{31,32} In these experiments, the hyperfine splitting seen in the EPR spectrum is measured directly via radio-frequency irradiation.

Figure 3. EPR spectra (black lines) and spin Hamiltonian simulations (red dashed lines: inclusive of background, blue dashed lines: exclusive of background. Green dashed line represent the background) of the R2lox cofactor. (A, D) Pulse X-band EPR spectra of the Mn^{III}/Fe^{III} metallocofactor observed in *Gkr2loxI*. (B, E) Corresponding pulse Q-band EPR spectra.²³ (C, F) Corresponding pulse W-band EPR spectra. Data on the left hand side (LHS) represent the measured absorption spectra. Data on the right hand side represent pseudomodulated (CW-like) spectra shown on the LHS. The parameters for the simulation are given in **Table 1**. Experimental parameters are given in the **Experimental Section** and **SI S1**.

The ⁵⁵Mn-ENDOR spectrum essentially consists of a doublet centered about half the ⁵⁵Mn hyperfine coupling interaction, split by twice the nuclear Larmor frequency [$\nu(^{55}\text{Mn}) = 12.9$ MHz at 1.22 T]. ⁵⁵Mn-ENDOR signals are observed between 110 and 160 MHz, centered at ~130 MHz, yielding a hyperfine coupling of 260 MHz (~10 mT), consistent with the EPR data (**Figure 4A**). Importantly, the magnetic field dependence of the ⁵⁵Mn-ENDOR dataset constrains the orientations of both the ⁵⁵Mn hyperfine and the quadrupole tensor relative to the molecular (g-axis) frame, resolving the local electronic structure of the Mn ion (see **section 3.4**).

3.3 A photoinduced state. The absorption spectrum of *Gkr2loxI* shows a shoulder in the wavelength region 300–400 nm ($\epsilon_{350} \approx 3000 \text{ M}^{-1} \text{ cm}^{-1}$) trailing into the visible region that likely represents a ligand-to-metal charge transfer state

(**SI S2**).^{60,61} Interestingly, photoexcitation into this transition using broadband visible light at cryogenic temperatures leads to the loss of the Mn^{III}/Fe^{III} cofactor signal described and emergence of a new EPR signal (**Figure 5**). This new species, denoted R2lox_{photo}, is stable as long as the sample remains frozen (**SI S2**). Its EPR spectrum is centered about $g \approx 2$ with no additional signals observed at lower magnetic fields. It displays a simpler six line structure at both X- and Q-band but with larger average line spacings of 140 mT. This larger apparent hyperfine interaction was confirmed by ⁵⁵Mn-ENDOR (**SI S3**). ⁵⁷Fe-labelling of the cofactor led to further isotropic line broadening, confirming the signal arises from a spin-coupled Mn/Fe system (**Figure 5**). In addition to the metal centered signal described above, a radical-like signal centered at $g \approx 2$ was also observed.

Figure 4. Q-band ^{55}Mn -ENDOR (A), ^1H -ENDOR (B) and ^2H -HYSCORE (C) spectra of the R2lox cofactor at three field positions within the EPR absorption envelope (see **Figure 3**): Low Field (LF) - 1169 mT, Center Field (CF) - 1210 mT, and High field (HF) - 1251 mT. The black solid line represents the experimental data; dashed red lines represent simultaneous simulation of the entire dataset shown in **Figures 2** and **3**. The ^1H -ENDOR spectra were symmetrized about the respective $\nu(^1\text{H})$ Larmor frequency. Experimental parameters are given in the **Experimental Section** and **SI S4**.

Spectral simulations demonstrate that the R2lox_{Photo} signal differs from the precursor R2lox signal in terms of i) its g-tensor, with a larger isotropic g-value (g_{iso}) and a smaller g-anisotropy; ii) its isotropic ^{55}Mn hyperfine coupling (A_{iso}), which is significantly larger (253 vs. 353 MHz); iii) the anisotropic component of its ^{55}Mn hyperfine tensor, which now takes the opposite sign; and iv) its ^{57}Fe hyperfine tensor which is approximately isotropic. Interestingly, it is noted that the photolyzed R2lox state displays similar EPR parameters as the Mn/Fe cofactor of R2c^{5,6} (see **Table 1**, **SI S2**).

Table 1. EPR parameters used to simulate the R2lox and R2lox_{Photo} signals.

		Hyperfine /MHz				
			Projected (A_i)		Intrinsic (a_i)	
	g		Mn	Fe	Mn	Fe
R2lox	x	1.953	257	62.5	270	32.0
	y	1.968	249	51.6	260	26.4
	z	2.034*	282*	93.9*	127*	29.2
	iso	1.985	263	69.3	219	29.2
	$aniso$	0.073	29.3	36.85	-138	0
R2lox- Photo	x	2.005	331		248	
	y	2.004	379*	≈ 60	284*	≈ 26
	z	1.990*	353		264	
	iso	2.000	354	≈ 60	266	≈ 26
	$aniso$	-0.015	36.8	0	27.6	0

*Indicates unique axis. The isotropic g_{iso} and A_{iso} values are the average of the individual values: $g_{\text{iso}} = (g_x + g_y + g_z)/3$ and $A_{\text{iso}} = (A_x + A_y + A_z)/3$. The anisotropy in the g and A_i values is expressed as the difference between the unique axis component and the average of other two components. For calculation of the intrinsic hyperfine parameters see **SI S3**.

3.4 Local EPR parameters for the Mn and Fe ions of the R2lox metallocofactor. The EPR parameters deduced from the above spectral simulations (**Table 1**) demonstrate that the local electronic structure of the Mn site in R2lox is fundamen-

tally different from R2lox_{photo}. The ^{55}Mn hyperfine parameters determined for the R2lox require the Mn^{III} ion of the cofactor to have a $^5\text{B}_1$ local electronic state consistent with a tetragonally elongated metal site (**Figure 6**, **SI S3**). This is the same Mn site geometry seen in the X-ray crystal structure, confirming that the crystal structure is a valid model for the solvated metallocofactor. In contrast, ^{55}Mn hyperfine parameters determined for the R2lox_{Photo} require the Mn^{III} ion of the cofactor to have a $^5\text{A}_1$ local electronic state as is seen for the Mn superoxide dismutase (Mn-SOD).^{62,63} This change in local electronic structure should manifest in terms of an altered ligand geometry of the Mn^{III} ion, with the Mn site now displaying structural compression along its unique axis (**Figure 6**, **SI S3**). In practice such an assignment may be complicated by the influence of metal-ligand interactions of varying degrees of π -bonding. A tetragonally elongated and compressed Mn^{III} site geometry have both been observed in synthetic μ -oxo, bis- μ -carboxylato bridged $\text{Mn}^{\text{III}}/\text{Mn}^{\text{III}}$ dimer complexes. In structures which contain a tetragonal elongation of the Mn^{III} ion, the unique (Jahn–Teller) axis lies perpendicular to the Mn- μ -oxo bridge vector,^{64,65} as seen for the R2lox cofactor. In contrast, in structures which contain a tetragonally compressed Mn^{III} ion, the unique axis lies in the direction of the Mn- μ -oxo bridge vector.^{66,67} The compression is inferred from the short nitrogen donor atom distance *trans* to the Mn- μ -oxo bond, which has the same distance as the nitrogen donors being *cis* to that bond. This is a strong indication of a compressed geometry as the *trans* donor distance should be about 0.1–0.2 Å longer due to the *trans* influence of the oxo-donor, as observed in related iron-dimers.^{68–70} Thus, photolysis likely rotates the unique axis of the cofactor.

3.5 Development of a structural model: a μ -OH bridged cofactor with a terminal water ligand. The protonation state of the R2lox cofactor was assessed using two techniques, ^1H -Davies ENDOR and ^2H -ESEEM/HYSCORE. **Figure 4B** shows the ^1H -ENDOR spectrum of R2lox measured at the low-, center-, and high-field edges of the absorption envelope. These spectra represent the superposition of multiple ^1H signals, which appear symmetrically centered about the ^1H nuclear Larmor frequency [$\nu(^1\text{H}) = 51.9$ MHz at 1.22 T] with their width/splitting defined by the hyperfine coupling.

Figure 5. ESE-detected Q-band (A and B), CW-X-band EPR (C) and Q-band ^{55}Mn -ENDOR (D) spectra of the photolyzed $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ R2lox sample reconstituted with natural abundance and ^{57}Fe -enriched iron. Simulations are overlaid as red dashed traces; spin Hamiltonian parameters given in **Table 1**. ^{55}Mn -ENDOR were collected at three field positions within the EPR absorption envelope: Low Field (LF) - 1169 mT, Center Field (CF) - 1210 mT, and High field (HF) - 1251 mT. Experimental parameters are given in the **Experimental Section** and **SI S2**.

The coupling between a ^1H nucleus and an electron spin is usually dominantly a through space, dipole-dipole interaction (A_{dip}). Thus, the magnitude of the hyperfine interaction informs on the distance between the proton and the metal ions of the cofactor. The two largest hyperfine couplings observed in the ^1H -ENDOR spectra are both ≈ 7 MHz, consistent with two protons 2.5 Å away from the Mn^{III} ion, i.e., the two protons of a water ligand. The magnetic field dependence of the ENDOR profile supports this assignment and demonstrates that the two water protons are inequivalent: one proton signal shows axial symmetry, while the second displays rhombic symmetry (**SI S4**). This inequivalence may be a consequence of the protein pocket. One of the water protons most likely hydrogen bonds to Glu202, locating it proximal to the Fe^{III} site, whereas the second proton would point away from the Fe^{III} site (**Figure 1C**). The remaining ^1H -ENDOR envelope represents protein residue protons in the immediate vicinity of the metallocofactor (< 5 MHz, < 5 Å).

A third, larger hyperfine coupling was observed by deuterating the cofactor and detection via ^2H -ESEEM/HYSCORE (**Figure 4C**). The ^2H -HYSCORE spectrum measured in the center of the EPR spectrum reveals a doublet signal in the (+,+) quadrant centered around the diagonal at the ^2H Larmor frequency ($\nu(^2\text{H}) = 7.97$ MHz at 1.22 T), split by a hyperfine coupling of ≈ 3 MHz. It is noted that to directly compare ^2H and ^1H hyperfine couplings the ^2H value should be multiplied by 6.51 (owing to the difference in their intrinsic magnetic moment), yielding an effective ^1H coupling of ≈ 21 MHz. This very large proton coupling can be assigned to a $\mu\text{-OH}$ bridge proton. Its magnitude comes about because the bridging oxygen to which it is bound carries significant spin density as it mediates the metal-metal exchange interaction (see **SI S4**).

3.6 Possible mismetallation under aerobic conditions: exchange of the metal sites? The X-ray structure of R2lox

identifies a histidine residue coordinated to each metal ion (**Figure 1C**). In the coupled spin system, these two histidines should give rise to two inequivalent ^{14}N signals and thus provide a novel means to test if mismetallated (Fe/Mn) centers exist under solution loading conditions. The ^{14}N His105 signal of the Mn^{III} ion was observed by X-band ESEEM. Its hyperfine and quadrupole couplings are typical of synthetic mixed-valent $\text{Mn}^{\text{III}}/\text{Mn}^{\text{IV}}$ complexes, such as the superoxidized state of the Mn-catalase³⁵ (**SI S5**). Similarly, the ^{14}N His205 signal of the Fe^{III} ion was observed by W-band ESEEM, with hyperfine couplings now similar to those measured for $\text{Fe}^{\text{III}}/\text{Fe}^{\text{IV}}$ complexes⁷¹ (**SI S5**). In addition to the two anticipated nitrogen signals, a third ^{14}N signal was observed using Q-band ESEEM, but only when measuring ESEEM between the low-field edge and center field of the EPR spectrum (**SI S5**). This signal is taken as evidence for a second underlying cofactor signal, representing $\approx 5\%$ of centers. DFT calculations shown in the next section support the assignment of this second species to a mismetallated $\text{Fe}^{\text{III}}/\text{Mn}^{\text{III}}$ cofactor.

3.7 DFT calculations. Using the spectroscopic data above, DFT models were developed for the R2lox and R2lox_{photo} cofactor. Three basic structures were examined, representing optimized geometries of the crystal structure assuming a III/III oxidation state for both ions.²³ Two features were varied: the metal identity of site 1 and 2 and the protonation of the bridging ligand (oxo/hydroxo). A set of three DFT models described below is sufficient to explain all EPR observations (**Figure 7**).

3.7.1 A $\text{Mn}^{\text{III}}-(\mu\text{-OH})\text{-Fe}^{\text{III}}$ cofactor: Mn is bound at site 1 with its ligand sphere completed by a water ligand and Fe is bound at site 2 (**Figure 7**).

Figure 6. Top: Idealized orbital splitting diagram for tetragonal elongation (left) and tetragonal compression (right) of a perfect octahedron, leading to electronic states $^5B_{1g}$ and $^5A_{1g}$, respectively, in the case of a high-spin Mn^{III} ion. **Bottom:** Proposed local site geometry of the Mn^{III} ion of the Mn/Fe cofactor of R2lox and R2lox_{Photo}. The R2lox cofactor shows a tetragonal elongation of bonds perpendicular to the μ -O(H) bridge, whereas the R2lox_{Photo}/R2c cofactor shows a tetragonal compression parallel to the μ -O(H) bridge.

The two metal sites are linked by a μ -OH bridge. This structure represents the aerobically prepared R2lox cofactor, reproducing all crystallographic and spectroscopic constraints, namely: i) the metal-metal distance: 3.55 Å (exp) vs. 3.48 Å (DFT); ii) the metal-OH distance: 2.12 Å (exp) vs. 2.08 Å (DFT); iii) the metal-bridge-metal angle: 107° (exp) vs. 126° (DFT); iv) the local site geometry of the Mn ion, with elongation along the Mn-OH₂ axis, perpendicular to the Mn^{III} -(μ -OH)-Fe^{III} plane (Figure 7); v) the local site geometry of the Fe ion, which is approximately spherically symmetric;

vi) the small exchange coupling, inferred from EPR simulations, (see SI S3) typical of μ -OH dimers;^{38,39} and vii) the complete set of EPR observables described above (Table 2 and SI S6). It is noted that the Mn^{III} ion displays a local high spin $d(xy)^1 d(xz)^1 d(yz)^1 d(z^2)^1$ electronic configuration (a “ 5B_1 ”-like ground state). The HOMO, of Mn $d(z^2)$ origin, is oriented along the Mn-OH₂ axis. Its strongly antibonding metal-ligand σ^* character leads to the observed elongation of the corresponding bonds. Accordingly, the LUMO of the cofactor is of Mn $d(x^2-y^2)$ origin (Figure 7). Since this orbital, which corresponds to the σ^* interaction of the Mn ion with the equatorial ligands, remains unoccupied, the equatorial bonds such as Mn-N are exceptionally short in this structure.

3.7.2 A Fe^{III}-(μ -OH)-Mn^{III} cofactor: Fe is bound at site 1 with its ligand sphere completed by a water ligand, while Mn is bound at site 2 (Figure 7). This mismetallated cofactor displays a ^{14}N hyperfine coupling of approximately the same magnitude for the minority species described in section 3.6, assigned to the ^{14}N His205 of what is now the Mn^{III} ion. Compared with the R2lox model discussed above, a change in the local electronic configuration of the Mn ion is observed, with the frontier orbitals swapping metal character, i.e., the HOMO is now of Mn $d(x^2-y^2)$ origin and the LUMO is the Mn $d(z^2)$, oriented along the Mn-OH axis (Figure 7). In line with this change in electronic configuration, the local geometry of Mn^{III} is tetragonally compressed, with the unique, short axis lying along the Mn-OH vector. It is also noted that this structure displays the same ground electronic state ($S = 1/2$), the same spin density distribution and a similar ^{55}Mn -hyperfine coupling, so it should yield a similar EPR spectrum of approximately the same spectral width. However, owing to the change in the electronic configuration of the Mn^{III} ion (a local “ 5A_1 ”-like ground state as opposed to a local “ 5B_1 ”-like state seen for the oxidized R2lox) its g-tensor is altered, as also inferred from experiment. This mismetallated structure is predicted to be of slightly higher energy compared to the R2lox model described above (+ 2.2 kcal mol⁻¹) and as such is not thermodynamically favored, consistent with its apparent small population in solution.^{23,72}

3.7.3 A Mn^{III}-(μ -O)-Fe^{III} cofactor: As in the first model, Mn is bound at site 1 with its ligand sphere completed by a water ligand and Fe is bound at site 2. However, the two metal sites are now linked by a μ -oxo bridge. This bridging motif leads to a larger calculated exchange interaction between the two metal ions, as inferred from EPR simulations, see SI S3.

Table 2. Calculated onsite ^{55}Mn and ^{57}Fe hyperfine tensors for the three model R2lox cofactors (μ -hydroxo bridged, mismetallated, μ -oxo bridged); comparison to experimental values

	J / cm^{-1}	Calculated onsite metal nuclei hyperfine tensors $ a_i / \text{MHz}$									
		Mn^{III}					Fe^{III}				
		a_1	a_2	a_3	a_{iso}	a_{aniso}	a_1	a_2	a_3	a_{iso}	a_{aniso}
Mn-(μ -OH)-Fe	-16.8	274	207	129	203	-112	25.2	26.0	26.6	25.9	1.1
Fe-(μ -OH)-Mn	-16.9	194	202	326	240	128	26.2	26.4	27.0	26.6	0.7
Mn-(μ -O)-Fe	-82.7	124	199	278	201	116	21.4	22.1	24.1	22.5	2.3
Exp.	-1.8 to -8.0*	270	260	127	219	-138	32.0	26.4	29.2	29.2	0
Exp. (Photo)	>20	248	264	284	266	27.6	26	26	26	26	0

*assuming an onsite $d(Mn^{III}) = -1.1$ to -5.0 cm^{-1} ; $\rho(Fe) = [\rho_1 \rho_2 \rho_3] = [1.95 \ 1.95 \ 3.21]$; $\rho(Mn) = [\rho_1 \rho_2 \rho_3] = [-0.95 \ -0.95 \ -2.21]$

Figure 7. Top: Calculated onsite ^{55}Mn hyperfine tensor orientations for the three model R2lox cofactors; **Middle:** the corresponding LUMO for each structure; and **Bottom:** the local site geometry of the Mn^{III} ion. The three structures differ in terms of their electronic structure, with the character of the Mn-centered LUMO changing from predominantly $d(x^2-y^2)$ for the oxidized Mn/Fe cofactor to predominantly $d(z^2)$ for the mismetallated Fe/Mn cofactor. The oxo-bridged species serves as a model for the photolyzed state; it forms an intermediate state that more closely resembles the mismetallated cofactor. The arrows showing the hyperfine tensor axis are colored: green, blue, red in order of increasing magnitude.

This structure represents a good candidate for the photolyzed state of the R2lox cofactor and appears to display many of the characteristics associated with the $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ cofactor of R2c. Unlike the Mn^{III} ion of the R2lox and mismetallated cofactor, DFT calculations suggest that the photolyzed state represents a mixed state from a structural as well as an electronic point of view. Specifically, both the HOMO and the LUMO have mixed $d(z^2)$ and $d(x^2-y^2)$ Mn character, and the analysis of the metal–ligand interactions is complicated by the presence of a π^* interaction in the HOMO between the oxo bridge and the Mn ion (Figure 7, SI S6). Nevertheless, it is clear from the optimized geometry that the Jahn–Teller axis of the tetragonally elongated R2lox model is lost in the photolyzed state. It is less clear from inspection of the structure alone whether there is a simple reorientation of the Jahn–Teller axis along the Mn–His vector (in which case this should be assigned as the local z axis with respect to Mn orbital character), or whether the elongation of the Mn–His and Mn–OCO bonds is a result of the significant $d(x^2-y^2)$ metal–ligand σ^* character in the HOMO. The presence of tetragonal compression (along the Mn–oxo bond) in the latter case would be masked to some extent by the simultaneous presence of the Mn–oxo π^* interaction in the HOMO (Figure S6). The picture is greatly clarified when the calculated EPR parameters are considered: the com-

puted ^{55}Mn hyperfine tensors more closely resemble those seen for the mismetallated cofactor (Table 2), allowing us to assign an effective “ $^5\text{A}_1$ ”-like local ground state to the Mn^{III} ion of the model for the photolyzed cofactor. This change in the local electronic structure implies (as described in section 3.6) that the unique axis of the system rotates into the plane of the Mn–($\mu\text{-O}$)–Fe bridge, lying along the Mn–($\mu\text{-O}$) bond (Figure 7, Table 2, SI S6). This structure is thus fully consistent with EPR data on the photolyzed state. Thus it is proposed that the photolyzed state represents the photoinduced migration of the $\mu\text{-OH}$ bridge proton, as has been previously observed for the heterobimetallic metal cofactors of the [NiFe] hydrogenases^{73,74} and may be related to light-induced changes seen in myoglobin.⁷⁵ In R2lox, it is hypothesized that the displaced proton may migrate to a nearby protein residue. The non-coordinating oxygen of Glu202, which is only 2.7 Å away from the bridging $\mu\text{-OH}$, could potentially fill this role. This residue bridges the two metals in the reduced state, rotating out of this bridging position upon cofactor activation (Figure 1C).

3.8. Comparison of R2c and R2lox. The photolyzed R2lox state appears to share many of the same EPR properties ob-

served for the $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ state of the cofactor of R2c,^{5,6} as described above. This correspondence suggests that the photo-lyzed state of R2lox represents an electronic model for R2c, especially in terms of the Mn^{III} site. The comparison implies that the $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ state of R2c most likely contains a Mn^{III} ion with a tetragonally compressed ligand field, such that the unique axis of the Mn^{III} ion is aligned along the Mn- μ -oxo bridge bond vector, not along the Mn-OH₂ bond vector as seen for the R2lox cofactor. Since the reorientation of the unique axis of the Mn^{III} ion is driven by bridge deprotonation, it is postulated that the R2c cofactor contains at least one μ -oxo bridge in the $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ state. We note that a μ -oxo/ μ -hydroxo bridge cofactor with a terminal water ligand was favoured as the most likely R2c cofactor in a recent NRVs/MCD study,⁷⁶ in line with our results.

The NRVs/MCD study thus assigns the total charge of the R2c cofactor to -1 in both its $\text{Mn}^{\text{IV}}/\text{Fe}^{\text{III}}$ and $\text{Mn}^{\text{III}}/\text{Fe}^{\text{III}}$ oxidation states.⁷⁶ The same negatively charged $\text{Mn}^{\text{IV}}\text{Fe}^{\text{III}}$ active cofactor structure was in an earlier DFT study shown to have the correct redox properties to replace the tyrosyl radical of class Ia R2. A total charge of -1 is also found here for the R2lox_{Photo} cofactor, in line with earlier DFT studies on R2lox.⁷⁷ Thus, from an electronic perspective, the R2lox and R2lox_{Photo} cofactors, and by extension the R2lox and R2c cofactors, primarily differ in terms of the local electronic state of the Mn^{III} ion. This property alone must dictate the different redox properties of the two cofactors and their divergent catalytic function.

5. CONCLUSION

The analysis presented above suggests the Mn/Fe cofactor of R2lox and that of its homologue R2c represent similar, but not identical, electronic motifs. This electronic flexibility must in part explain the chemical versatility of the Mn/Fe cofactor, directing its chemistry towards either $1e^-$ /hole transfer and storage (at the Mn site) in the case of R2c or $2e^-$ oxidation of a potential substrate occupying the open (water) coordination site in the case of R2lox. Chemical versatility is achieved by tuning the local electronic structure and thus redox properties of the Mn ion itself. In R2lox, the μ -hydroxo/ μ -carboxylato bridging network stabilizes a tetragonally elongated Mn^{III} ion with the Jahn-Teller axis located perpendicular to the Mn- μ -hydroxo bond, whereas in R2c, a μ -oxo/ μ -hydroxo bridging network stabilizes a tetragonally compressed Mn^{III} ion with the unique axis located parallel to the Mn-oxo bond. Both cofactors have a terminal water bound at the Mn^{III} site. As seen in **Figure 7**, the electronic structure of the cofactor interchanges between a local “ 5A_1 ”-like ground state with a LUMO of predominantly $d(z^2)$ character to a “ 5B_1 ”-like ground state with a LUMO of predominantly $d(x^2-y^2)$ character. This interchange leads to a re-orientation of the unoccupied orbital such that it does not coincide with the open (water) coordination site in the R2lox structure, favoring $2e^-$ vs. $1e^-$ redox chemistry.

ASSOCIATED CONTENT

This contains additional material and is arranged as follows: S1) Multifrequency CW- and pulse-EPR data of the Mn/Fe cofactor of R2lox; microwave nutation curves; S2) Optical absorption data of the activated R2lox and the time-course of the induction of the photo-lyzed state (R2lox_{Photo}); S3) Estimates of the metal-metal exchange and fine structure parameters of the R2lox Mn/Fe cofactor: comparison to literature R2c data; S4) Q-band $^1\text{H}/^2\text{H}$ ESEEM/ENDOR data of

the R2lox cofactor; S5) ^{14}N ESEEM of the R2lox cofactor, evidence for a mismetallated cofactor; S6) Tabulated DFT parameters; S7) References. The material is available free of charge via the Web at <http://pubs.acs.org>

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Notes

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