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Structure/function correlations over binuclear non-heme iron active sites

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Abstract

Binuclear non-heme iron enzymes activate O₂ to perform diverse chemistries. Three different structural mechanisms of O₂ binding to a coupled binuclear iron site have been identified utilizing variable-temperature, variable-field magnetic circular dichroism spectroscopy (VTVH MCD). For the μ -OH-bridged Fe(II)₂ site in hemerythrin, O₂ binds terminally to a five-coordinate Fe(II) center as hydroperoxide with the proton deriving from the μ -OH bridge and the second electron transferring through the resulting μ -oxo superexchange pathway from the second coordinatively saturated Fe(II) center in a proton-coupled electron transfer process. For carboxylate-only-bridged Fe(II)₂ sites, O₂ binding as a bridged peroxide requires both Fe(II) centers to be coordinatively unsaturated and has good frontier orbital overlap with the two orthogonal O₂ π^* orbitals to form peroxo-bridged Fe(III)₂ intermediates. Alternatively, carboxylate-only-bridged Fe(II)₂ sites with only a single open coordination position on an Fe(II) enable the one-electron formation of Fe(III)–O₂[–] or Fe(III)–NO[–] species. Finally, for the peroxo-bridged Fe(III)₂ intermediates, further activation is necessary for their reactivities in one-electron reduction and electrophilic aromatic substitution, and a strategy consistent with existing spectral data is discussed.

Keywords

Binuclear non-heme iron enzymes; O₂ activation; Variable-temperature; variable-field magnetic circular dichroism; Frontier molecular orbitals; Peroxide activation

Introduction

The binuclear non-heme iron proteins and enzymes form expanding classes [1, 2] that play key roles in antibiotic biosynthesis [3, 4] are targets for drug inhibition [5, 6] and have important roles in biotechnology [7, 8] and bioremediation [9]. On a molecular level, they reversibly bind dioxygen [10], activate it for H-atom abstraction [11], desaturation [12], hydroxylation [13–15], and electrophilic aromatic substitution (EAS) [16, 17], and reduce

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O₂ to H₂O and NO to N₂O [18]. In contrast to the mononuclear non-heme iron enzymes which often require a cofactor [19, 20], the second Fe of the binuclear non-heme Fe proteins serves this role in providing the extra electron(s) required for reactivity [1]. We have recently reviewed our studies on mononuclear non-heme Fe enzymes [21]. Here, we focus on the binuclear non-heme Fe enzymes and the new modes of O₂ activation associated with utilizing pairs of interacting metal centers in O₂ binding and catalysis. The proteins we have studied, and results obtained are summarized in Table 1.

The binuclear non-heme Fe proteins mostly have a 2His/4carboxylate ligand set with bridging carboxylates and in some cases an oxygen (OH⁻ or oxo) bridge [22]; however, the reversible O₂ binding site in hemerythrin (Hr) has five His ligands [23]. It had been thought that this additional His ligation stabilizes the active site from O₂ activation; however, *p*-aminobenzoate *N*-oxygenase (AurF), myo-inositol oxygenase (MIOX), and the Flavin diiron proteins (FDPs) have more than two His ligands [24–29]. Most of the binuclear non-heme Fe proteins react from their 2Fe(II) state. However, MIOX is more reactive with O₂ from its mixed-valent Fe(II)Fe(III) state [30–32], and the 2Fe(II) state of the FDPs is the only binuclear non-heme Fe enzyme site capable of the reduction of 2NO to N₂O [33, 34]. In addition, in some enzymes, the resting 2Fe(II) state is relatively stable in O₂ and must be activated by substrate (stearoyl ⁹D-desaturase (⁹D) [35], arylamine oxygenase of the chloramphenicol biosynthetic pathway (CmlI) [36]) or coupling protein (component B in soluble methane monooxygenase (sMMO) [37, 38] and component D in toluene 4-monooxygenase (T4MO) [16]) binding for reaction with O₂. It should also be noted that in the class Ia ribonucleotide reductases (RNRs), the biferrous site reacts with O₂ (plus an electron) to generate a Tyr radical that transfers to an adjacent subunit for catalysis in DNA synthesis [11].

Over the years, we have developed variable-temperature, variable-field magnetic circular dichroism (VTVH MCD) spectroscopy in the near-IR (NIR) region as a probe of the geometric and electronic structures of ferrous active sites [1, 39]. In “VTVH MCD of biferrous active sites” of this Perspective, we focus on the new features associated with a binuclear Fe(II) center and in “Structure/function correlations over 2Fe(II) active sites”, we use these to define structure/ function correlations over the three structural types of 2Fe(II) active sites in Table 1. The rest of the Perspective focuses on peroxide-level intermediates in reversible O₂ binding and activation for H-atom abstraction and EAS. Spectroscopy coupled to electronic structure calculations provides insight into binding and activating peroxo intermediates by binuclear non-heme Fe enzymes for function.

VTVH MCD of biferrous active sites

As shown in Fig. 1a, a mononuclear high-spin Fe(II) site has two $d_{\pi} \rightarrow d_{\sigma}$ ligand field (LF) transitions at 10 Dq which for the N/O ligation of non-heme, Fe(II) is $\sim 10,000 \text{ cm}^{-1}$ [1, 39–41]. In a low symmetry protein site, these transitions split in energy dependent on the coordination geometry of the Fe(II). For a six coordinate (6C) LF, there are two transitions at $\sim 10,000 \text{ cm}^{-1}$ split by $\sim 2000 \text{ cm}^{-1}$; for a 5C square pyramidal LF, there are LF transitions at $>10,000$ and $\sim 5000 \text{ cm}^{-1}$. In a 5C trigonal bipyramidal LF, these transitions shift to below $10,000 \text{ cm}^{-1}$ and below 5000 cm^{-1} (the latter is often not observed due to instrumental

cutoff); for 4C distorted tetrahedral centers, there are only LF transitions in the 5000–7000 cm^{-1} region. This LF transition splitting is thus a direct probe of Fe(II) coordination. However, these transitions are parity forbidden and in the near-IR spectral region, and are generally too weak relative to the background to observe in absorption spectroscopy for metalloproteins. However, high-spin Fe(II) sites have $S = 2$ ground states, and, thus, are paramagnetic resulting in these LF transitions being intense in the MCD spectrum at low temperature, due to the C-term selection rule associated with degenerate ground states (see [42–44]). As shown in Fig. 1b, the intensity of these LF transitions in MCD at low temperature increases with increasing magnetic field eventually leveling off (i.e., a saturation effect due to the population of only the lowest magnetic-field-split sublevel of the ground state). A series of isotherms with increasing magnetic field taken over a range of temperatures gives the nested set of saturation magnetization curves for a non-heme Fe(II) site presented in Fig. 1c. The $S = 2$ ground state is five-fold degenerate in M_S which undergo a zero field splitting (ZFS) in a low symmetry protein site as given by the first two terms in the spin Hamiltonian in Eq. (1) [45, 46]. These are further split by an applied magnetic field, as shown in Fig. 1d. The saturation magnetization curves in Fig. 1c can be fit to the ZFS plus Zeeman splitting in Fig. 1d to obtain the spin Hamiltonian parameters (and δ and $g_{\parallel}$ of the lowest doublet) which in turn (i.e., through in-state spin–orbit coupling) reflect the low symmetry splitting of the 5T_2 d_{π} orbitals in Fig. 1a [47, 48].

Spin Hamiltonian for a non-heme Fe(II) center. D and E are the axial and rhombic ZFS parameters, respectively. β , g , and H are the Bohr magneton, g-factor, and the magnetic-field strength, respectively [45, 46]:

$$\mathbf{H} = D \left(S_z^2 - \frac{1}{3} S(S+1) \right) + E \left(S_x^2 - S_y^2 \right) + \beta \left(g_x H_x S_x + g_y H_y S_y + g_z H_z S_z \right). \quad (1)$$

Extension of the above to a binuclear non-heme Fe(II) enzyme active site leads to two $d_{\pi} \rightarrow d_{\sigma}$ LF transitions for each Fe(II) center (4 total), a different ZFS on each Fe(II), and the possibility of exchange coupling between the two Fe(II)s due to the presence of bridging ligation. The magnetic interaction between the two Fe(II)s due to their covalent interactions with the bridging ligands (called superexchange pathways) gives the $-2J\mathbf{S}_A \cdot \mathbf{S}_B$ term in Eq. (2) which in the absence of ZFS couples the two $S = 2$ monomer ground states to form total spin $S = 4, 3, 2, 1, 0$ states for the binuclear site [$S = 0$ is the antiferromagnetic (AF) ground state and $S = 4$ is the ferromagnetic (F) ground state reflecting orthogonal superexchange pathways through the bridge] [49]. For biferrous systems, the exchange and ZFS can be comparable leading to a series of J/D diagrams to describe the energies and wavefunctions of the spin sublevels of the coupled binuclear Fe(II) ground state depending on whether the coupling is AF [J negative in Eq. (2)] or F (J positive) and the sign of the ZFS on each Fe(II) and the relative magnitudes of J and D 's [1].

Spin Hamiltonian for an exchange-coupled (J) binuclear non-heme $\text{Fe}_A(\text{II})\text{Fe}_B(\text{II})$ site [49]:

$$\begin{aligned}
 H = & -2JS_A \cdot S_B + D_A \left[S_{z,A}^2 - \frac{1}{3}S_A(S_A+1) \right] \\
 & + E_A \left(S_{x,A}^2 - S_{y,A}^2 \right) + D_B \left[S_{z,B}^2 - \frac{1}{3}S_B(S_B+1) \right] \\
 & + E_B \left(S_{x,B}^2 - S_{y,B}^2 \right) + g_{x,A} \beta H_x S_{x,A} \\
 & + g_{y,A} \beta H_y S_{y,A} + g_{z,A} \beta H_z S_{z,A} \\
 & + g_{x,B} \beta H_x S_{x,B} + g_{y,B} \beta H_y S_{y,B} + g_{z,B} \beta H_z S_{z,B}. \quad (2)
 \end{aligned}$$

MCD data for two very different sets of bridging ligands found in binuclear non-heme Fe(II) active sites are shown in Fig. 2 top with associated J/D diagrams given at the bottom. For deoxyHr in contrast to the behavior of a mononuclear Fe(II) site where the C-term MCD intensity associated with the paramagnetism increases as $1/T$ (Fig. 2a top, dashed line) until saturation, deoxyHr has no MCD intensity at low temperature and high field [50]. The absence of MCD intensity at low temperature and high field requires that deoxyHr has a diamagnetic $M_S = 0$ ground state. Increasing the temperature leads to the appearance of and increase in MCD intensity which peaks at ~ 70 K and then decreases in going to higher temperatures (data points and solid curve fit in Fig. 2a top). This temperature-dependent MCD intensity variation requires the presence of a paramagnetic doublet at ~ 55 cm^{-1} above the ground state (insert in Fig. 2a top).

The right-hand side of the J/D diagram in Fig. 2a bottom gives the limit of no exchange coupling with positive ZFS on both Fe(II)s. Fitting the MCD data in Fig. 2a using only ZFS would require that $|D| > 35$ cm^{-1} in Eq. (2) (with $J = 0$), which is not physically reasonable. The left-hand side of Fig. 2a bottom gives the AF coupling of the 2Fe(II) with $D = 0$. Fitting the data in Fig. 2a to this limit would give a $-J \sim 25$ cm^{-1} , which is comparable to D 's of high-spin Fe(II)s. The middle of the J/D diagram gives the combination of AF J and positive D 's and fits the deoxyHr data with $-J = 12 \pm 2$ cm^{-1} . This antiferromagnetic coupling constant is large for a biferrous site and required that deoxyHr have an OH^- bridge capable of significant AF coupling of the two Fe(II) centers.

For the biferrous active site of T4MO, a very different ground-state behavior was observed (Fig. 2b) [51]. As the temperature is decreased, the MCD signal intensity increases consistent with T4MO having the C-term behavior of a paramagnetic ground state. The VTVH MCD data in Fig. 2b, however, show a very rapid saturation behavior for the low-temperature isotherms indicating a very high-effective g value for the ground doublet. For mononuclear Fe(II) sites, $g_{\parallel, \text{effective}}$ is close to 8 (dashed curve in Fig. 2b top), while for T4MO (data points and solid curves in Fig. 2b top), the $g_{\parallel}$ is close to 16, reflecting a ferromagnetically exchange-coupled biferrous ground state. The appropriate J/D diagram is given in Fig. 2b bottom. The center gives the energy-level splitting for a ferromagnetically coupled ground state with a positive D to the right and a negative D to the left. The arrow describes the region that fits the VTVH MCD data and indicates that the biferrous active site of T4MO is weakly ferromagnetically coupled with $J \sim 1$ cm^{-1} . This ferromagnetic coupling is associated with a μ -1,1 carboxylate bridge at the biferrous active site. Other biferrous active sites with only carboxylate bridges, but bridging in a μ -1,3 mode, show VTVH MCD

data indicating weak antiferromagnetic coupling (ground states with $M_S = 0, 1$, or 2 depending on the signs of D and the magnitude of D/J) on the order of $-J \sim 1 \text{ cm}^{-1}$ [1, 52].

It should finally be noted that in general, each Fe(II) ligand field is different with different ZFSs. Therefore, each contributes differently to the exchange-coupled sublevels of a 2Fe(II) active site (i.e., to the dimer spin states in the J/D diagrams in Fig. 2 bottom). VTVH MCD data taken at each LF transition energy of a biferrous active site (up to 4 in total) will then, in general, show different behaviors enabling the LF transitions to be correlated to each Fe(II) center, thus defining the coordination environment of each Fe(II) in the coupled binuclear Fe(II) active site [53, 54]. VTVH MCD studies have been performed on the biferrous active sites of all the proteins and enzymes included in Table 1 to define the coordination environment of each Fe(II) and the bridging ligation and their changes with perturbation (substrate binding, etc.). These results are summarized in Table 1 and generalized below to develop structure/function correlations.

Structure/function correlations over 2Fe(II) active sites

Based on VTVH MCD data and crystallography, we divide the proteins and enzymes in Table 1 into three categories based on geometric and electronic structures. All the enzyme active sites in category 2 have similar structural features: open coordination positions on both Fe(II) centers and weak exchange coupling ($|J| \sim 1 \text{ cm}^{-1}$) between the irons. RNR [55, 56], ferritin [57], bacterioferritin [58], and AurF [59] all rapidly react with dioxygen to generate peroxy intermediates. Alternatively, substrate binding in ^{99}D and component B(D) binding in sMMO (T4MO) are required for rapid reaction with O_2 to generate peroxy intermediates [16, 35, 37, 38]. For the latter enzymes, the VTVH MCD data show that a significant biferrous active-site structural change is associated with the increased O_2 reactivity [51, 60–62]. This structure/function correlation is illustrated with the VTVH MCD data for ^{99}D in Fig. 3 [60]. The resting 2Fe(II) ^{99}D site (Fig. 3a) shows only two LF transitions with temperature and field dependence indicating two Fe(II)s with equivalent negative ZFS and weak AF exchange coupling. This MCD analysis is consistent with the X-ray crystal structure showing two equivalent 5C Fe(II)s with μ -1,3-carboxylate bridges [63]. Figure 3b shows the large change in MCD spectral features associated with substrate [stearoyl bound acyl carrier protein (ACP)] binding to the biferrous enzyme. There are now three LF transitions and the two Fe(II)s which are weakly exchange coupled now have different ZFSs. These data show that an active-site structural change is required for O_2 activation to the peroxide level.

The above-mentioned analysis reflects the idea that, for the active sites of the enzymes in category 2, O_2 must bridge the 2Fe(II)s for $2e^-$ reduction to the peroxide level. As shown in Fig. 4 [64], if O_2 binds to a single Fe(II) in a biferrous site with only carboxylate bridges, it is reduced by $1e^-$ to superoxide that is AF coupled to the resultant high-spin Fe(III). Importantly, there is no significant e^- transfer from the second Fe(II) [labeled Fe(1) in Fig. 4]. The exchange coupling J relates to the superexchange pathways between the Fe centers which give the electronic coupling for electron transfer. The low J values associated with the carboxylate bridges reflect poor superexchange pathways for electron transfer (ET) from Fe(1) to the Fe(2)– O_2 complex. As shown in Fig. 4, O_2 end-on binding to a single Fe is

uphill relative to the biferrous site and O₂. Alternatively, if O₂ is allowed to bridge between the two Fe centers, it is now reduced by 2e⁻ to a peroxide bridging two Fe(III)s in an exothermic process. The significant energy difference reflects the energetically favored two 2e⁻ reduction of O₂ to peroxide (360 vs. -160 meV for 1e⁻ reduction of O₂) [65, 66], the ability of the peroxide to form strong donor bridges to each of the two Fe(III)s in a μ -1,2-bridged state (vide infra) and, as developed in [67], and the weak binding of superoxide to a high-spin Fe(III). Thus, there is the geometric requirement that the open coordination position on each Fe(II) is oriented, such that O₂ can bridge for its 2e⁻ reduction. There is also an electronic requirement. O₂ is a triplet with two half-occupied perpendicular π^* orbitals. Each Fe(II) is high-spin d^6 with an extra e⁻ in a d_{π}^* orbital, as shown in Fig. 5 for ⁹D, where there is spectral/ structural information available for the reactive biferrous state from Fig. 3b [60]. These β HOMOs on the two Fe(II) s should be oriented, such that one is in the Fe–O₂ plane and the other is perpendicular to this plane of a bridging O₂ such that there is efficient ET into its two perpendicular π^* orbitals.

MIOX and FDP are placed in a separate category 3, where there is again weak exchange coupling between the two Fe(II)s indicating only μ -1,3-carboxylate bridges with poor superexchange pathways for ET between the two Fe centers. However, now, one or both Fe(II)s is coordinatively saturated (i.e., 6C) [32]. For the FDPs, a 4C Fe(II) rapidly reacts with NO in a 1e⁻ process to form high-spin Fe(III)($S = 5/2$)–NO⁻($S = 1$) AF coupled, which is an exothermic reaction [68, 69]. Alternatively, MIOX must be oxidized by 1e⁻ to a mixed-valent Fe(II)Fe(III) state that upon substrate binding to the Fe(III) has an open coordination position on its Fe(II) that does react with O₂ to form a superoxide intermediate (G) [32]. The geometric and electronic structural changes that occur in going from the substrate-bound Fe(III)Fe(II) to the substrate-bound Fe(III) Fe(III)O₂⁻ state in MIOX that enable its 1e⁻ reduction of O₂ to form an Fe(III)-bound superoxide are currently unclear.

Finally, we include Hr by itself in category 1. It has an open coordination position on only 1 Fe(II), yet reversibly reacts with dioxygen to reduce it by 2e⁻ to a hydroperoxide [10]. However, as developed in “VT VH MCD of biferrous active sites”, deoxyHr is different from the other binuclear non-heme Fe enzymes in having strong AF coupling requiring an OH⁻ bridge. As shown in Fig. 6, O₂ binding at the open coordination position on one Fe induces a proton-coupled 2e⁻ transfer process, where the μ -OH bridge donates its proton to form the oxo bridge of oxyHr, thereby creating a very efficient superexchange pathway for ET [70]. The electronic structure enabling the reversible O₂ binding in Fig. 6 is summarized in the next section.

Reversible O₂ binding: Hr

As shown in Fig. 6, O₂ binding to deoxyHr to form oxyHr involves proton-coupled 2e⁻ transfer to O₂ binding at a single Fe center [10]. The geometric and electronic structures of deoxyHr are considered above. The electronic structure of oxyHr is defined by its charge transfer (CT) spectrum which reflects the covalent HOO–Fe(III) and Fe(III)– μ O–Fe(III) bonds. These CT transitions overlap each other in energy, but as shown in Fig. 7, could be resolved by polarized single crystal electronic absorption (Abs) spectroscopy on oxyHr [70, 71]. The HOO–Fe(III) CT transitions are polarized perpendicular to the Fe–Fe vector

(dashed line in Fig. 7b). Peroxide to Fe(III) CT generally involves intense peroxide π_{π}^* to d_{π} and peroxide π_{σ}^* to d_{σ} CT (see the next section). However, in oxyHr, there are two sets of two peroxo-to-Fe(III) CT transitions that are all comparably weak in intensity (Fig. 7b, dotted Gaussians). This difference in CT intensity reflects the effect of protonation of the peroxide, which mixes the peroxo π^* orbitals and reduces the donor interaction of the peroxide with the Fe(III) which decreases the CT intensity. Thus, electron transfer to Fe should be coupled to proton transfer from the hydroperoxide to the bridge. The μ -oxo-to-Fe(III) CT transitions are dominantly polarized along the Fe–Fe vector (solid line in Fig. 7b). We have shown that the CT transitions of bridging ligands reflect the superexchange pathways responsible for ground-state AF coupling [72, 73]. As observed by comparing Fig. 7b, c (solid curves), the CT transitions of the bent oxo bridge in Hr (125° due to the presence of two additional μ -1,3 carboxylate bridges) are quite different from those of linear oxo bridged biferric dimers. In the latter case, π to π superexchange dominates, while in the bent case, the π to π exchange pathway is limited and instead compensated by π to σ superexchange pathways. For this reason, the J values of oxo bridged biferric complexes are not very dependent on Fe–O–Fe angle [74]. As indicated above, superexchange pathways for AF coupling can be related to electronic coupling, and thus, the orbital interactions involved in ET. This point is developed below.

We can interpolate between the electronic structures of oxy and deoxy Hr for insight into this $2e^-$ proton-coupled electron transfer (PCET) process [75]. As shown in Fig. 8a, the elongation of the Fe(1)–(OOH) bond of oxyHr leads to the donation of a $\downarrow$ electron from the hydroperoxide π^* donor orbital to the d_{π} orbital on Fe(1) (on left). Associated with this bond elongation, the total energy increases, and the pK_a of the hydroperoxide decreases while that of the oxo bridge increases. At an Fe–(OOH) distance of $2.9 \text{ \AA}$, the complex has a ferrous(hydroxo-superoxo) electronic structure, and thus, it is isoenergetic to have the proton either on the superoxide or on the oxo bridge (red and blue curves in Fig. 8b). These energetics reflect a potential energy surface with a double minimum (Fig. 8c) that leads to rapid proton tunneling between these free bases and thus a low kinetic isotope effect (KIE) for O_2 binding [76]. Associated with the proton transfer from the now hydro-superoxide, as shown in Fig. 8a bottom, an $\uparrow$ electron is transferred adiabatically (i.e. continuously) from the HOO–Fe(1) π_{σ}^* orbital to the d_{π} orbital on the remote Fe(2). Importantly, while this transfer is a PCET process, it is not an H-atom transfer, as the e^- is transferred through the π/σ pathway of the bent oxo bridge. This description of the PCET process then correlates the AF coupling associated with the bent oxo bridge with its effectiveness as a superexchange pathway for ET between the Fe centers.

Peroxy intermediates

Enzymes in category 2 react with O_2 to form bridged peroxo-Fe(III)₂ intermediates, where the high-spin iron centers are antiferromagnetically coupled. Some of these intermediates (**P**) display a characteristic peroxo-to-Fe(III) charge transfer band at $\sim 700 \text{ nm}$ ($\epsilon \sim 1500 \text{ M}^{-1}\text{cm}^{-1}$) and Mössbauer parameters of $\delta \sim 0.66$ and $E_Q \sim 1.5 \text{ mm/s}$ [35, 55, 56, 77, 78], while others (**P'**) lack the absorption feature at $\sim 700 \text{ nm}$ and display a reduced Mössbauer isomer shift of $\delta \sim 0.52 \text{ mm/s}$ (Table 1) [59, 79, 80]. The **P'** intermediates exhibit

electrophilic reactivity in triggering the $1e^-$ oxidation of a nearby tryptophan (W48) to convert to a high-valent Fe(IV)Fe(III) intermediate **X** in RNR [79] and in performing EAS in ToMO [80]. In contrast, the **P** intermediates are less active; a chemical rescue experiment with the W48A variants of RNR and an indole electron source showed that **P** converts to **P'** for its reduction [79, 81], and **P** in 9D does not initiate substrate dehydrogenation [35, 77]. Based on the resonance Raman excitation profiles of the **P** intermediate in W48F/D84E RNR (Fig. 9), **P** was determined to have a *cis*- μ -1,2-peroxo bridge as in an equivalent structurally-defined model complex [82, 83]. DFT computational studies to evaluate the spectral and reactivity differences between **P** and **P'** suggested a hydroperoxo (i.e., protonated) structure for **P'** (Fig. 10; computational details in supporting information). Protonation of the peroxide in **P** would result in an increase in the peroxo-to-Fe CT band energy and a decrease in intensity and thus no prominent absorption at ~ 700 nm, and a diminished electron donation to Fe(III) and thus a decreased Mössbauer isomer shift [84–86].

Moreover, the presence of the protonated peroxide in **P'** relative to **P** can explain **P'**'s enhanced electrophilicity compared to **P** [84]. This protonation results in a stabilization of the peroxo- σ^* -based LUMO that accepts electrons for reductive O–O bond cleavage and EAS (Fig. 10, bottom). Accordingly, **P'** in the wt RNR active site is calculated to be capable of W48 oxidation with an exergonic G^0 of -4.4 kcal/mol, which results in the formation of an (Fe1(III)-hydroxo)(Fe2(IV) = oxo) initial species that would go on to form intermediate **X** (Fig. 11a, bottom coordinate). In contrast, the reduction of **P** by W48 and its subsequent O–O bond cleavage are calculated to have a large barrier and be endergonic with G^0 of $+5.0$ kcal/mol, leading to an Fe1(III)– μ O–(Fe2(IV) = oxo) initial structure.

The **P'** reactivity in ToMO can also be tested in the RNR active site; the O₂ reaction of the W48F/D84E variant of RNR leads to an increase in absorption first at ~ 700 nm, next at ~ 500 nm, and finally at ~ 550 nm; these appear to correlate with the formation of **P**, then **P'**, and finally, the Fe(III)₂ resting site bound by a nearby hydroxylated F208 residue [87]. In evaluating this reaction, the μ -1,2-hydroperoxo biferric structure in the D84E RNR active site is calculated to be almost isoenergetic to a μ -1,1-hydroperoxo biferric structure with a reasonable energy barrier for interconversion, $G^\ddagger$ of 7.6 kcal/mol (Fig. 11b). This μ -1,1-hydroperoxo structure can oxygenate F208 with a calculated energy barrier of $G^\ddagger \sim 17.5$ kcal/mol, consistent with the observed kinetics (2.75×10^{-5} /s; $G^\ddagger \sim 22$ kcal/mol) for the 500 nm-band decay to form the 550 nm bound hydroxylated product (Fig. 11c bottom reaction coordinate). Alternatively, the corresponding reaction with **P** is calculated to be highly unlikely with $G^\ddagger \sim 36.9$ kcal/mol (Fig. 11c top reaction coordinate). This significant reactivity difference with protonation again reflects its effect on the peroxo- σ^* orbital which is the frontier molecular orbital (FMO) in these reactions. The *cis*- μ -1,2 to μ -1,1 hydroperoxo conversion in Fig. 11b is required for this reactivity, as it orients the peroxo- σ^* orbital for effective overlap with the aromatic substrate.

Concluding comments

The studies summarized above are now being extended in a number of directions. Nuclear resonance vibrational spectroscopy (NRVS) is being used for the experimental structural

determination of the peroxo-level intermediates and the high-valent intermediates X in RNR and Q in sMMO to define geometric and electronic structure contributions to their reactivity [88, 89]. Spectroscopic studies on de novo binuclear Fe(II) proteins with systematic variation in His/CO₂⁻ ligation are being pursued to understand how this ligand variation affects structure and reactivity [90]. In addition, MnFe analogues of 2Fe sites are present in class Ic RNR [91] and the R2lox [92] enzymes. The Mn(III)/Mn(IV) center can be probed by MCD and the Fe(III) center by NRVS to correlate with the binuclear non-heme Fe enzymes [93]. Finally, a number of new biferrous enzyme classes are emerging (deoxyhypusine hydroxylase (DOHH) [94, 131], urease [95], CmlI [132], etc.) that are accessible to the VTVH MCD/DFT approach presented above to extend Table 1 and develop additional structure/function correlations.

Supplementary Material

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Fig. 1.

a LF splitting of a high-spin Fe(II) d^6 system with variations in coordination number and geometry. Adapted with permission from [41]. Copyright 2005 Royal Society of Chemistry. **b** Variable field at low temperature, below 5 K, and **c** variable-temperature, variable-field MCD spectra of $\text{Fe}(\text{HB}(3,5\text{-}i\text{Pr}_2\text{pz})_3)(\text{S-C}_6\text{H}_4\text{-4-}t\text{Bu})$. Reprinted with permission from [40]. Copyright 1998 American Chemical Society. **d** Ground-state energy-level diagram of a high-spin Fe(II) d^6 system with the effects of axial and rhombic zero field, and Zeeman splittings. *Left* positive ZFS with H perpendicular to z ; *right* negative ZFS with H parallel to z . Adapted with permission from [129]. Copyright 1992 American Chemical Society

Fig. 2.

a Temperature-dependent MCD intensity of deoxyHr (*top*) and its associated J/D diagram (*bottom*). Adapted with permission from [50]. Copyright 1987 American Chemical Society.

b VTVH MCD of T4MOH (*top*) [51] and its associated J/D diagram (*bottom*) [50]. Adapted with permission from [50] and [51]. Copyright 2008 and 1987 American Chemical Society

Fig. 3. CD spectra at 5 °C and MCD spectra at 7 T, 5 K (*left*) and VTVH MCD with fit energy-level diagram in insert (*right*) of ^9D in the absence (**a**) and the presence (**b**) of stearyl-ACP substrate Adapted with permission from [60]. Copyright 1999 American Chemical Society

Fig. 4. Energy diagram for the binding of O_2 to a biferrous site (a) terminal to $Fe(2)$ as superoxide and (b) *cis*- $\mu-1,2$ -bridged to the two Fe centers as peroxide Reprinted with permission from [64]. Copyright 2005 American Chemical Society

Fig. 5. Orbital interactions for the O_2 reaction of 9D in the absence (*left*) and the presence (*right*) of stearoyl-ACP substrate. The $\beta \pi^*$ orbitals of O_2 are unoccupied, while the d_{π} orbitals shown contain the high-spin $d^6 \beta e^-$. Adapted with permission from [60]. Copyright 1999 American Chemical Society

Fig. 6. Reversible O_2 binding to deoxyHr Reprinted with permission from [70]. Copyright 1999 American Chemical Society

Fig. 7.

a Electronic absorption spectrum of oxyHr in solution. **b** Single crystal absorption spectra of oxyHr with parallel (*solid*) and perpendicular (*dashed*) polarization. Gaussian deconvolution of the perpendicular absorption (corrected for an oxo CT contribution to the perpendicular polarization due to the Fe–O–Fe angle of 125°) is given as *dotted curves*. **c** Single crystal absorption spectra of enH₂[(FeHEDTA)₂]·6H₂O, which has a linear Fe–O–Fe angle, with parallel (*solid*) and perpendicular (*dashed*) polarization. Adapted with permission from [71, 130] and [70]. Copyright 1978, 1989, and 1999 American Chemical Society

Fig. 8.

a oxyHr FMO variations involved in the PCET process induced by the elongation of Fe(1)–(OOH) bond. **b** Potential energy surface (PES) for the elongation of the Fe–(O₂) bond with the proton on the O₂ (red circles) and with the proton on the oxo bridge (blue squares). **c** Proton PES as a function of the peroxo-proton distance to the oxo bridge Adapted with permission from [75]. Copyright 1999 American Chemical Society

Fig. 9. rR excitation profiles of the **P** intermediate in W48F/D84E RNR (*top*) and the $[\text{Fe}(\text{III})_2(\mu\text{-}1,2\text{-O}_2)(\text{OBz})_2\{\text{HB}(\text{pz}')_3\}_2]$ complex, where OBz is benzoate and $\text{HB}(\text{pz}')_3$ is hydrotris(3,5-diisopropyl-1-pyrazolyl)borate Adapted with permission from [82] and [83]. Copyright 1998 and 2004 American Chemical Society

Fig. 10.
μ-1,2-peroxo and μ-1,2-hydroperoxo structures for **P** (*left*) and **P'** (*right*) and their peroxo-σ-antibonding LUMOs (*bottom* with relative energies)

Fig. 11. Calculated peroxy reaction coordinates for RNR. **a** 1-electron reduction of P' (green) and P (red), **b** conversion of a μ -1,2- to a μ -1,1-hydroperoxy structure, and **c** F208 hydroxylation by peroxo (red) and hydroperoxo (green) intermediates

Table 1

Binuclear non-heme iron enzymes studied using VTVH MCD spectroscopy

Protein	Function	Coord. number ^a	Bridges	Ligation	Intermediates
Category 1					
Hemerythrin (Hr) [10, 23, 70, 75]	Reversible O ₂ binding	6C/5C	(μ-1,3-COO) ₂ (μOH)	5H/1E/1D	Fe ^{III} Fe ^{III} -OOH
Category 2					
Class Ia ribonucleotide reductase (RNR) [11, 55, 56, 79, 81, 82, 96–103]	Biferrous + Y → biferric + Y·	4C/5C	(μ-1,3-COO) ₂	2H/3E/1D	P (wt mouse and D84E), P' (W48A), X
Stearoyl ⁹ -desaturase (⁹ D) [12, 35, 54, 63, 77, 85, 104]	Stearoyl-acyl carrier → oleoyl-acyl carrier	5C/5C (without Stearoyl-ACP); 4C/5C (with Stearoyl-ACP)	(μ-1,3-COO) ₂	2H/4E	P
Soluble methane monooxygenase (sMMO) [13, 61, 105–115]	CH ₄ + O ₂ → CH ₃ OH + H ₂ O	5C/5C	(μ-1,1-COO) (μ-1,3-COO)	2H/4E	H _{peroxo} Q
Toluene 4-monooxygenase (T4MO) [17, 51, 116, 117]	Toluene → <i>p</i> -cresol	5C/5C	(μ-1,1-COO) (μ-1,3-COO)	2H/4E	[P'(wt), P(T201S)] ^b [78, 80]
Ferritin [118–120]	Fe ²⁺ → Fe ³⁺ and O ₂ → H ₂ O ₂ (Fe storage)	5C/5C	μ-1,3-COO	1H/3E/1D/1Q	P
Bacterioferritin [58, 121–123]	Fe ²⁺ → Fe ³⁺ and O ₂ → H ₂ O ₂ (Fe Storage)	5C/5C	(μ-1,3-COO) ₂	2H/4E	
DNA protection during starvation (DPS) proteins ^c [124, 125]	Fe ²⁺ → Fe ³⁺ and O ₂ /H ₂ O ₂ → H ₂ O	6C		1H/1D/1E	
<i>p</i> -aminobenzoate <i>N</i> -oxygenase (AurF) [24, 25, 59, 126]	<i>p</i> -Amino → <i>p</i> -Nitrobenzoate	5C/5C	(μ-1,3-COO) ₂	3H/4E	P'
Category 3					
Myo-inositol oxygenase (MIOX) [26, 27, 30–32, 127, 128]	<i>Myo</i> -inositol → d-glucuronate	6C Fe(II)/6C Fe(II) (without MI); 6C Fe(II)/6C Fe(III) (without MI); 5C Fe(II)/6C Fe(III) (with MI)	(μ-1,3-COO)	4H/2D	G, H
Flavin diiron proteins (FDP) [18, 28, 29, 68, 69]	O ₂ /2NO → H ₂ O/N ₂ O	4C/6C	(μ-1,3-COO)	4H/2D/1E	{FeNO} ⁷ Fe ^{II}

^aUnless otherwise indicated, ligation is for the biferrous state^bIn related enzyme, toluene/o-xylene monooxygenase ToMO^cOnly 1 Fe(II) binds with high affinity