Published in final edited form as:

J Am Chem Soc. 2017 August 30; 139(34): 12009–12019. doi:10.1021/jacs.7b06546.

Spectroscopy and DFT Calculations of a Flavo-diiron Enzyme Implicate New Diiron Site Structures

Andrew C. Weitz[¶], Nitai Giri[‡], Jonathan D. Caranto[‡], Donald M. Kurtz Jr.^{*,‡}, Emile L. Bominaar^{*,¶}, and Michael P. Hendrich^{*,¶}

[¶]Department of Chemistry, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213, United States

[‡]Department of Chemistry, University of Texas at San Antonio, San Antonio, Texas 78249, United States

Abstract

Flavo-diiron proteins (FDPs) are non-heme iron containing enzymes that are widespread in anaerobic bacteria, archaea, and protozoa, serving as the terminal components to dioxygen and nitric oxide reductive scavenging pathways in these organisms. FDPs contain a dinuclear iron active site similar to that in hemerythrin, ribonucleotide reductase, and methane monooxygenase, all of which can bind NO and O₂. However, only FDP competently turns over NO to N₂O. Here, EPR and Mössbauer spectroscopies allow electronic characterization of the diferric and diferrous species of FDP. The exchange-coupling constant $J(H_{\text{ex}} = \mathcal{S}_1 \cdot \mathcal{S}_2)$ was found to increase from +20 cm⁻¹ to +32 cm⁻¹ upon reduction of the diferric to the diferrous species, indicative of (1) at least one hydroxo bridge between the iron ions for both states and (2) a change to the diiron core structure upon reduction. In comparison to characterized diiron proteins and synthetic complexes, the experimental values were consistent with a dihydroxo bridged diferric core, which loses one hydroxo bridge upon reduction. DFT calculations of these structures gave values of J and Mössbauer parameters in agreement with experiment. Although the crystal structure shows a hydrogen bond between the iron bound aspartate and the bridging solvent molecule, the DFT calculations of structures consistent with the crystal structure gave calculated values of J incompatible with the spectroscopic results. We conclude that the crystal structure of the diferric state does not represent the frozen solution structure and that a mono- μ -hydroxo diferrous species is the catalytically functional state that reacts with NO and O₂. The new EPR spectroscopic probe

*Corresponding Authors: hendrich@andrew.cmu.edu, eb7g@andrew.cmu.edu, donald.kurtz@utsa.edu.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b06546. Mössbauer spectra of deflavo-FDP_{red}, Mössbauer parameters of FDP_{red} in zero-field as a function of temperature, DFT-calculated values of J , δ , E_Q , η and diiron structural metrics for the models of Figure 6, experimental and DFT-calculated values of J and diiron structural metrics for synthetic diferric and diferrous complexes, experimental and resultant DFT-calculated δ and E_Q values used for calibration of DFT, figures showing deviation from DFT-calculated and experimental values of δ , E_Q , and J , figure showing fixed atoms of DFT optimization for models of Figure 6, and reference to Gaussian 09 (PDF)

ORCID

Donald M. Kurtz Jr.: 0000-0003-1179-1875

Emile L. Bominaar: 0000-0002-5125-265X

Michael P. Hendrich: 0000-0003-4775-0389

Notes

The authors declare no competing financial interest.

of the diferric state indicated that the diferric structure of FDP prior to and immediately after turnover with NO are flavin mononucleotide (FMN) dependent, implicating an additional proton transfer role for FMN in turnover of NO.

Graphical abstract

INTRODUCTION

Flavo-diiron proteins (FDPs) are non-heme iron containing enzymes that are widespread in anaerobic bacteria, archaea, and protozoa, serving as the terminal components to dioxygen and nitric oxide reductive scavenging pathways in these organisms. Most FDPs show both dioxygen reductase (O₂R) and nitric oxide reductase (NOR) activities, but the relative turnover rates vary significantly among FDPs.^{1,2}

The role of FDPs in scavenging NORs has become particularly relevant as a mechanism for pathogenic bacteria to metabolize and detoxify nitric oxide to prevent it from reaching toxic levels. Nanomolar amounts of nitric oxide are capable of inhibiting aerobic respiration and energy metabolism.³ Additionally, nitric oxide in aerobic environments can react with O₂ to produce more reactive species, such as NO₂, peroxynitrite (ONOO⁻), and NO⁻.⁴ Consequently, nitric oxide levels in organisms are tightly regulated. In humans, NO synthases produce nitric oxide in response to the presence of pathogens. Bacteria in turn can utilize FDPs to scavenge and eventually detoxify free nitric oxide to the less potent N₂O. The active site of FDPs contains a non-heme diiron active site, which catalyzes NO reduction, in contrast to the dinuclear heme/non-heme iron active sites found in the respiratory NORs found in some bacteria or the mononuclear P450-type heme NORs of fungi.⁵

FDPs show a characteristic head-to-tail homodimer structure, in which a flavin mononucleotide cofactor (FMN) is located approximately 4 Å from the diiron site across the subunit interface (Figure 1).⁶ The homodimer thus contains two symmetrically disposed active sites ~40 Å apart. The Fe_P–Fe_D distance within the diiron site is ~3.4 Å, where Fe_P and Fe_D refer to the irons located proximal and distal to the FMN, respectively. Nearly all structurally characterized FDPs show each iron with two histidine ligands and a terminal monodentate carboxylate ligand from either aspartate or glutamate. A bridging bidentate carboxylate from an aspartate residue and a bridging solvent ligand complete the diiron coordination sphere.^{1,7} The bridging solvent in the oxidized state has been presumed to be OH but has not yet been proven. The hydrogen bond between the terminally coordinated

carboxylate of D89 coordinated to Fe_D and the bridging solvent species is conserved in all FDP_{ox} crystal structures, which are presumed to be diferric. The amino acid sequence numbering is for *Thermotoga maritima* (Tm) FDP, which is the enzyme used for the present study. This hydrogen bonding to a diiron bridging molecule is a rare opportunity to explore its effect on the electronic properties of the diiron core. Although the protonation state of the solvent bridge is not always obvious from protein crystal structures, monohydroxo-bridged diferric sites seem to be relatively rare in their resting states. The crystal structures without exogenous anions, such as that of Tm FDP, show a solvent ligand, presumed to be H₂O (O_w in Figure 1), coordinated to Fe_D to give 5- and 6-coordinate iron centers in the diferric state. Second coordination sphere species, which could conceivably have functional relevance, are also shown in Figure 1. A noncoordinating water (O_{pw} in Figure 1) is observed to occupy a pocket above the diiron site in some FDP crystal structures. O_{pw} and O_w are sometimes replaced by an exogenous anionic bridging ligand (nitrate, acetate, or phosphate) coordinated trans to H85 and H90. These exogenous coordinating ligands occupy a pocket above the diiron site and are the likely positions for NO or O₂ coordination in the diferrous state. Some FDPs can be prepared without the FMN (deflavo-FDP).⁸ The crystal structure of Tm FDP contains no FMN, but the structure of the diiron core (Figure 1) is essentially the same as FDP crystal structures with high FMN occupancy; including the hydrogen bond between D89 and the bridging solvent.⁹ FMN can be readily inserted into Tm deflavo-FDP to produce a catalytically functional protein. The diferrous site in the deflavo-FDP can also turn over NO to N₂O, albeit noncatalytically.^{8,10}

The carboxylate/solvent-bridged diiron site of FDP shows similarities to those in several other O₂ binding and activating enzymes.^{11,12} Prototypical examples are ribonucleotide reductase (RNR), methane monooxygenase (MMO), and hemerythrin (Hr). These active sites contain a diiron center, bridged by either one or two carboxylates, and a solvent-derived ligand (oxo, hydroxo, or aqua). The diferric Fe–Fe distance in FDPs (3.3–3.5 Å) is comparable to those in RNR (3.4 Å) and Hr (3.2 Å) but longer than that in MMO (3.1 Å).¹³ More recent examples of 3- and 4His-ligated, carboxylate-bridged diiron sites in O₂-activating enzymes have been reported.^{11,14} The diferrous sites in RNR, MMO, Hr, and FDP all bind NO;^{15–17} however, only FDPs have been shown to be competent NORs. Detailed kinetic and spectroscopic investigations of Tm FDP have identified key active site oxidation states and intermediates in the NOR pathway.^{8,18} The fully oxidized FDP (FDP_{ox}) contains a FMN/diferric active site and is unreactive with NO or O₂. The fully reduced FDP (FDP_{red}) contains a diferrous site and two-electron reduced FMN (FMNH₂). The four-electrons stored in the Tm FDP_{red} active site (FMNH₂/Fe²⁺Fe²⁺) were shown to be capable of turning over four equivalents of NO.¹⁸ Rapid kinetics monitoring of reactions of Tm FDP_{red} with NO showed sequential accumulation of antiferromagnetically coupled *S* = 1/2 diferrous mononitrosyl [Fe²⁺{FeNO}⁷] and *S* = 0 diferrous dinitrosyl [{FeNO}⁷]₂ species prior to two-electron oxidation of FMNH₂ to FMN (where {FeNO}⁷ is the Enemark–Feltham notation for ferrous nitrosyl).¹⁸ A mechanism consistent with kinetic measurements involves rate-limiting NN bond formation from the diferrous dinitrosyl intermediate, generating N₂O and reformation of the diferric site.

The FDP-catalyzed reduction of NO to N₂O requires two electrons and two protons in accordance with the reaction $2\text{NO} + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$. The source of these two

protons is unclear. Possibilities include FMNH₂, protein residues in the first and second coordination sphere, the diiron solvent bridge, or directly from solvent. The carboxylate ligand (D89) H-bonded to the solvent bridge could conceivably facilitate proton transfer during turnover.

Here, spectroscopic and computational methods are used to characterize the electronic properties of the diferric and diferrous species of FDP and identify the molecules that bridge the iron ions. EPR and Mössbauer spectroscopies have allowed determination of the electronic exchange interaction between the iron ions of the diiron site for these species. The value of the exchange-coupling constant J is strongly dependent on the nature of the bridging ligands and can provide information on specific structural changes at the active site during turnover. The value of J is likely influenced by hydrogen bonding to the bridging solvent. DFT calculations of conformations with and without hydrogen bonding to the bridging ligands were correlated to spectroscopic results to test this influence. In combination with DFT calculations, the spectroscopic characterization of FDP provides an opportunity to demonstrate a more quantitative correlation of J with the diiron site structure.

EXPERIMENTAL SECTION

FDP Preparation and Manipulation

All experiments were conducted on Tm FDP, either flavinated or deflavo-FDP, prepared as previously described.^{8,9,18} Samples of FDP_{ox}, FDP_{red}, and rapid freeze quenched samples for spectroscopies were prepared as reported previously.^{8,18} All reactions were carried out under anaerobic conditions in an N₂ atmosphere. All spectra were obtained in frozen solutions at the indicated temperatures.

EPR and Mössbauer Spectroscopies

X-band EPR spectra were recorded on a Bruker ELEXSYS spectrometer equipped with an Oxford ESR-910 liquid helium cryostat and a Bruker bimodal cavity for generation of microwave fields parallel and transverse to the applied magnetic field. The quantification of all signals was measured relative to a CuEDTA spin standard prepared from a copper atomic absorption standard (Sigma-Aldrich). The microwave frequency was calibrated with a frequency counter and the magnetic field with a NMR gaussmeter. The sample temperature was calibrated against a calibrated cernox sensor (Lakeshore CX-1050) mounted inside an EPR tube. A modulation frequency of 100 kHz was used for all EPR spectra. Mössbauer spectra were recorded on either a strong-field or a weak-field spectrometer operating in a constant acceleration mode in a transmission geometry using Janis Research Inc. cryostats that allow for a variation in temperature from 1.5 to 300 K. One of the dewars housed a superconducting magnet that allowed for the application of magnetic fields up to 8 T parallel to the γ -radiation. Isomer shifts are reported relative to Fe metal at 298 K.

The simulation software *SpinCount* was written by one of the authors.¹⁹ The software diagonalizes the electronic terms of the spin Hamiltonian:

$$\begin{aligned}
 H &= JS_1 \cdot S_2 + H_1 + H_2 + H_{\text{dip}} \quad (1) \\
 H_i &= \beta S_i \cdot \mathbf{g}_i \cdot \mathbf{B} + S_i \cdot \mathbf{D}_i \cdot S_i + S_i \cdot \mathbf{A}_i \cdot \mathbf{I}_i + g_n \beta_n \mathbf{B} \cdot \mathbf{I}_i + \mathbf{I}_i \cdot \mathbf{P}_i \cdot \mathbf{I}_i \\
 (i &= 1, 2) \\
 S_i \cdot \mathbf{D}_i \cdot S_i &= D_i [S_{zi}^2 - S_i(S_i + 1)/3 + (E/D)_i (S_{xi}^2 - S_{yi}^2)] \\
 \mathbf{I}_i \cdot \mathbf{P}_i \cdot \mathbf{I}_i &= (eQV_{zi}/12) [3I_{zi}^2 - I_i(I_i + 1) + \eta_i (I_{xi}^2 - I_{yi}^2)] \\
 H_{\text{dip}} &= (\beta^2/4\pi r^3) [(\mathbf{g}_1 \cdot \mathbf{S}_1) \cdot (\mathbf{g}_2 \cdot \mathbf{S}_2) - 3(\mathbf{g}_1 \cdot \mathbf{S}_1 \cdot \mathbf{r}) \cdot (\mathbf{g}_2 \cdot \mathbf{S}_2 \cdot \mathbf{r}) / (r^2)]
 \end{aligned}$$

where the parameters have the usual definitions,²⁰ and performs least-squares fitting of simulations to the spectra. The spin $\mathbf{S}_i$ refers to individual Fe^{3+} ($S = 5/2$) or Fe^{2+} ($S = 2$) sites depending on the FDP oxidation state. Euler angles (α , β , γ) rotate $\mathbf{D}_2$ (coaxial with $\mathbf{g}_2$) relative to $\mathbf{D}_1$ (coaxial with $\mathbf{g}_1$).²¹ The tensors $\mathbf{P}_i$ (coaxial with $\mathbf{A}_i$) can each be independently rotated relative to $\mathbf{D}_i$. The dipolar term, H_{dip} , was only in use for calculation of EPR spectra of FDP_{ox} . The quantitative simulations are generated with consideration of all intensity factors, which allows computation of simulated spectra for a specified sample concentration.

DFT

The DFT calculations were performed on various states of the FDP diiron site with *Gaussian* '09 (see SI for reference), using the hybrid functional B3LYP and basis set 6-311G.^{22,23} First and second coordination sphere atoms were included, as described in Results. The FDP crystal structures show that the FMN has no direct contact with any of the iron ligands, and the FMN was, therefore, excluded from the DFT models. The exchange-coupling constants were calculated with the expression $J = 2(E_{\text{F}} - E_{\text{BS}})/(n_{\text{F}}n_{\text{D}})$ where n_{F} and n_{D} are the numbers of unpaired electrons on Fe_{F} and Fe_{D} , respectively, and E_{F} and E_{BS} are the energies for the optimized geometries of the ferromagnetic state (F) and broken-symmetry configuration (BS).^{24,25} The BS configurations were constructed using the fragment approach implemented in *Gaussian* '09. The J value calculations for the diferrous state required a careful inspection of the orbital configuration to ascertain that the broken symmetry configuration and ferromagnetic state have the same 3d orbital ground state. The calculations for the ^{57}Fe isomer shifts (δ) and quadrupole splitting (E_{Q}) were calibrated using a set of previously characterized ferrous and ferric model complexes (Table S6).

RESULTS

Oxidized FDP (FDP_{ox})

Mössbauer spectra of as-isolated FDP_{ox} at 4.2 K in low and high magnetic fields are shown in Figure 2. The low field spectrum is similar to that of our previous work.¹⁸ The high field spectrum is new, and the combination of the two spectra allowed resolution of parameters for the individual irons. The simulations of both the low and high field spectra have been resolved to require inequivalent irons with the parameters listed in Table 1. The parameters confirm that both irons are high-spin Fe^{3+} . The visible absorption spectrum corresponding to this sample was consistent with the oxidized state of FMN.¹⁸ The lack of a magnetic pattern

in the Mössbauer spectrum at low field and the nuclear Zeeman pattern at high field indicated that the two $S = 5/2$ Fe^{3+} ions are exchange-coupled antiferromagnetically to give a diamagnetic ground state. The close match of the simulation to the data for a spin $S = 0$ species indicated that the lowest spin-coupled $S = 0$ state has the majority of population at 4.2 K. Mössbauer spectra at higher temperatures displayed intermediate relaxation, where the $S > 0$ states acquire thermal population, preventing determination of the exchange-coupling constant from Mössbauer spectroscopy.

EPR signals were detected from excited spin states of the diferric site in FDP_{ox} . Parallel- and perpendicular-mode EPR spectra of FDP_{ox} at various temperatures are shown in Figure 3. In parallel-mode (Figure 3, red traces, lower panel), a signal was observed near $g = 8$ for $T > 20$ K, and as the temperature was increased, a new signal grew in near $g = 12$. These signals originate from transitions within the $S = 2$ and $S = 3$ spin manifolds, respectively, of the exchange-coupled diferric system as proven below. Measurements of the growth and decay of these signals as a function of temperature allowed determination of the exchange-coupling constant J . Figure 4 shows a plot of the population of the system $S = 2$ and 3 multiplets versus temperature. The data points are signal intensity $\times$ temperature (proportional to spin multiplet population) at various temperatures of the $g = 8$ and 12 signals. The curves overlaid on the data points were computed from eq 1 for two $S_{\text{Fe}^{3+}} = 5/2$ ions coupled antiferromagnetically with $J = 20(3) \text{ cm}^{-1}$.

The zero-field parameter $D_{\text{Fe}^{3+}}$ for non-heme ferric sites is typically $\sim 1 \text{ cm}^{-1}$, thus $J \gg D_{\text{Fe}^{3+}}$ and each spin manifold can be described by system spin states $S = 0, 1, \dots, 5$, with the energies of the relevant spin states indicated in the inset of Figure 4. Though the $S = 2$ and 3 manifolds have integer-spin, the small value of $D_{\text{Fe}^{3+}}$ permits many transitions within these manifolds for X-band (microwave quantum, 0.3 cm^{-1}) microwave fields oscillating either parallel ($\mathbf{B}_1 \parallel \mathbf{B}$) or perpendicular ($\mathbf{B}_1 \perp \mathbf{B}$) to the static magnetic field. Indeed, perpendicular-mode EPR transitions are observed, with temperature dependence matching the parallel mode $g = 12$ resonance. A representative spectrum is shown in the top panel of Figure 3. Both parallel- and perpendicular-mode signals were simulated with a simultaneous set of parameters for a spin-coupled diferric system as shown in Figure 3 (black traces). The simulations of the resonances are sensitive to the magnetic dipolar interaction between the two Fe^{3+} ions, and accordingly, a precise match to the simulation was not pursued owing to the large number of parameters. Nevertheless, a set of parameters consistent with a diferric species, using the Fe–Fe distance from the DFT structure (see below, $3.2 \text{ \AA}$), gave simulations in approximate agreement with experiment for $J = 20 \text{ cm}^{-1}$ and the concentration of the FDP_{ox} sample. This provides an unambiguous assignment of the signals to the $S = 2$ and 3 spin manifolds of the diferric active site of FDP_{ox} , which corresponds to the majority species of the sample.

The spectroscopic values δ , E_Q and J of flavinated FDP_{ox} are all close to the values reported for the diferric site of MMO_{ox} (Table 1), which shows a bis(μ -hydroxo) structure. This value of J is also, within error, the same as those of the four available characterized synthetic diferric complexes that have either bis(μ -hydroxo) or μ -hydroxo/ μ -alkoxo structures (Table S5).

Similar measurements were performed on the form of FDP, which lacks FMN (deflavo-FDP). The diferric state of deflavo-FDP (deflavo-FDP_{ox}) also showed a parallel-mode EPR signal at $g = 8$ (Figure 3, blue traces) that is broader than the FDP_{ox} $g = 8$ signal. The $g = 12$ signal may be present at higher temperatures but was too broad to accurately measure. EPR spectra of deflavo-FDP_{ox} were recorded as a function of temperature, and the relative intensity of the $g = 8$ signal is plotted in Figure 4. The calculated blue curve in Figure 4 is a fit of the deflavo-FDP_{ox} experimental signal intensity, which gave an exchange-coupling constant of $J = 30(3) \text{ cm}^{-1}$. The significantly higher exchange-coupling constant for deflavo-FDP_{ox} shifts the energy of the $S = 3$ multiplet to 180 cm^{-1} . The higher energy of the $S = 3$ manifold and broader resonance explains the weakness of the $g = 12$ signal of deflavo-FDP_{ox}. The increase in the value of J indicates a change in the structure of the diiron core relative to that for FDP_{ox}.

The Diferric Site after Turnover with NO

Our previous Mössbauer studies of FDP demonstrated that a diferric species returned to nearly full yield $\sim 120 \text{ s}$ after the $[\{\text{FeNO}\}^7]_2$ concentration had reached its maximum (at $\sim 130 \text{ ms}$ after RFQ mixing with excess NO).¹⁸ These RFQ experiments were repeated here with EPR samples. FDP_{red} was mixed with 2.5 equiv of NO per diiron site and frozen at 130 ms and 120 s. The EPR spectrum of the FDP_{ox} species observed prior to sample reduction is shown in Figure 5A. At a quench time of 130 ms, the FDP_{ox} signal is absent (Figure 5B). This is consistent with our previous work, which showed the sample to be a mixture of $[\text{Fe}^{2+}\{\text{FeNO}\}^7]$ and $[\{\text{FeNO}\}^7]_2$ transient species at 130 ms. At 120 s, the signal from FDP_{ox} has returned in nearly full yield (Figure 5C). The signals of Figure 5, spectra C and A, are the same, and the intensities of both have the same temperature dependence, indicating that the magnitude of the exchange interaction for the diferric site after turnover is the same as that of the starting FDP_{ox}. This observation indicates that the bridging ligands of the diferric core structure return to that of the resting state structure after turnover with NO.

Similar RFQ experiments were performed for deflavo-FDP. The EPR spectrum of the deflavo-FDP sample prior to reduction is shown in Figure 5D, which reproduces that of Figure 3. At the 120 s time point, the EPR signal of deflavo-FDP_{ox} did not return (Figure 5E), in contrast to the RFQ experiments of flavinated FDP. As previously shown, Mössbauer samples of deflavo-FDP prepared at 120 s indicated that the irons are oxidized and spin-coupled and NO is not bound.⁸ Despite the Mössbauer spectra showing spin-coupled oxidized irons, EPR spectra of parallel samples did not show evidence for regeneration of the deflavo-FDP_{ox} species. Upon anaerobically thawing the sample for 20 min, a minor amount of the deflavo-FDP_{ox} signal reappeared. The perpendicular mode EPR spectra showed a small amount of mononuclear Fe^{3+} , indicating that the majority of the irons remained exchange coupled. Thus, a short time after turnover with NO, the diiron site of deflavo-FDP is diferric but has not reverted to the starting deflavo-FDP_{ox} state, whereas for flavinated FDP, the FDP_{ox} state has been recovered. The evidence for exchange-coupled oxidized irons in Mössbauer and lack of the distinctive EPR signals indicate that the coupling strength has increased, preventing a significant population of the $S = 2$ and $S = 3$ manifolds to be detectable by EPR.

DFT Calculations of FDP_{ox}

DFT calculations have been performed to assess the bridging mode of the diiron centers of FDP on the basis of their exchange-coupling constants (J) and Mössbauer parameters (δ and E_Q). From the more than six X-ray crystal structures of the diferric state available to date,² we used PDB 1VME (of Tm deflavo-FDP) as a representative structure to create our DFT model. The structural model adopted in the DFT calculations of FDP was modified from that in Figure 1 as shown in Figure S8 with fixed atom positions indicated. Although Fe_p is 5-coordinate, there is a crystallographic solvent (O_{pw} in Figure 1) in the putative substrate binding pocket at 2.7 Å from Fe_p. The O_{pw} is within hydrogen bonding distance (2.8 Å) of the terminal solvent ligand (O_w) of Fe_D. O_{pw} and O_w were both modeled as waters. To make the structure more amenable to DFT analysis, the iron coordinating residues were truncated at the α -carbons, which were replaced by H atoms placed at a typical C–H distance from the β -carbons. These substituted H atoms were fixed in space, and the remainder of the side chains were allowed to rotate freely (with the exception of the tyrosine side chains) during the geometry optimizations. The hydroxyl H atoms of the tyrosines were not fixed in order to simulate hydrogen bonding with the active site core. The orientations of the ligand histidine ring planes in the protein structure are influenced by hydrogen bond interactions of the N δ H moieties. In the computational structure (Figure S8), these hydrogen bond acceptors were modeled as water molecules; the O atoms of which were placed 1.7 Å from the proton of the histidine N δ Hs. The O atom positions of these substituted waters were frozen, while the H atoms were left free in the geometry optimizations. These hydrogen bonds provide flexible constraints on the orientations of the histidine planes. This core diiron model contains the dominant interactions required for calculation of the parameters J , δ , and E_Q .

There are a number of characterized synthetic diferric model complexes that provide a basis for comparison of the DFT and experimental results for J , δ , and E_Q . We evaluated the reliability of our DFT method by calculating J values of a set of synthetic hydroxo- and alkoxo-bridged, N,O-ligated diferric compounds (Table S4) for which experimental J values have been reported. The empirical Gorun–Lippard (GL) relation, which correlates J with the shortest superexchange pathway, reproduced the DFT-calculated order of magnitude change in J between oxo- and alkoxo/hydroxo-bridged species.³⁷ However, as Table S4 and Figures S2, S3 show, the agreement within the alkoxo/hydroxo-bridged complexes is poor for the GL relation. Satisfactory agreement with experimental J values was obtained with broken-symmetry calculations, $J = (E_F - E_{BS})/12.5$, where E_F and E_{BS} are the B3LYP/6-311G system energies obtained for optimized ferromagnetic (F) and optimized broken symmetry (BS) geometries. The calibration constants for the values of δ and E_Q were based on a best fit to a number of mono- and dinuclear Fe²⁺ and Fe³⁺ complexes. The DFT-calculated values of J , δ , and E_Q (Tables S5, S6, and Figures S4–S7) were in good agreement with experimental values for the 9 complexes listed, with average errors of 3 cm^{−1}, 0.045 mm/s, and 0.2 mm/s, respectively.

Differing protonation states and hydrogen bonding interactions of the D89 carboxylate and the various bridging solvent-derived ligands for the diiron site of FDP_{ox} are shown in Figure 6 along with values of J calculated by DFT for the optimized structures (Mössbauer parameters of the optimized structures are listed in Table S1). Although Figure 6 shows only

the diiron core fragments, the full set of atoms in Figure S8 were used to generate the DFT optimized structures. The optimized structures obtained using the BS or F configurations were very similar to each other, and the structural relaxation between the two had only a minor effect on J ($\sim 1 \text{ cm}^{-1}$). For the diferric and diferrous structures **1–5**, the calculations were repeated to include a model of the hydrogen bond between the peptide backbone NH of D89 to the hydrogen-bonded D89 carboxylate oxygen shown in Figure 1. The backbone NH does not directly interact with the hydroxo bridge, and its inclusion had only a minor effect on the optimized structures. The calculated values of J for these structures were all within 3 cm^{-1} of the values given in Figure 6 for the structures without the additional peptide fragment. The inclusion of the peptide fragment generally resulted in extended calculation times owing to the additional structural degrees of freedom. Since the inclusion had an insignificant effect on the results, the set of values stated in Figure 6 are all based on optimized structures without this peptide fragment.

The calculated value of J for a bridging oxo (**1₃**, where the subscript refers to the valence of both irons) was 208 cm^{-1} , which is comparable to values for synthetic oxo-bridged diferric complexes and oxo-bridged diferric sites in proteins but is an order of magnitude larger than the experimental value for FDP_{ox} (20 cm^{-1}). The calculation for the aqua-bridged species (**4₃**) yielded a J value of 1 cm^{-1} , which is also incompatible with experiment. Structures **1** and **4** are therefore dismissed as possibilities for FDP_{ox}.

The calculated value of J for the hydrogen-bonded hydroxo bridge model (**2₃**) was 45 cm^{-1} , which exceeds the experimental value by a factor ~ 2 and is higher than the J values of the diferric hydroxo-bridged synthetic complexes listed in Table S4. We have found no monohydroxo-bridged diferric or diferrous complexes with hydrogen bonding to the bridge for which the value of J has been reported. The optimized diiron structure **2₃** was close to that in the 1VME crystal structure of FDP_{ox} but had a longer Fe–Fe distance ($3.6 \text{ \AA}$) than that in the crystal structure ($3.4 \text{ \AA}$). Reoptimization of **2₃** with the Fe–Fe distance constrained to $3.4 \text{ \AA}$ gave a higher value of $J = 60 \text{ cm}^{-1}$. The unusually high calculated value of J for **2₃** can be explained by hydrogen bond donation from the bridging hydroxo to the noncoordinating oxygen atom of the carboxylate ligand. This hydrogen bonding weakens the hydroxo O–H bond and confers some oxo character to the bridging hydroxo, leading to an increase in the value for J . This explanation was tested by rotating the noncoordinating carboxylate oxygen away from the bridging hydroxo group (**6₃**), thereby removing the hydrogen bond. Indeed, the calculated J value dropped to 30 cm^{-1} , which is in the middle of the range ($15 < J < 43 \text{ cm}^{-1}$) observed for synthetic N,O-ligated alkoxo/hydroxo-bridged diferric complexes and close to the J values of 34 and 43 cm^{-1} for the monohydroxo-bridged synthetic complexes in Table S4.

We also considered crystallographically compatible models **3** and **5** in which the noncoordinating oxygen of D89 is protonated but retains a hydrogen bond with the hydroxo bridge. The calculated J value for **3₃** decreased to 11 cm^{-1} . This lower J value compared to that of **2₃** is consistent with a protonated carboxylate donating a hydrogen bond to the hydroxo bridge, thus making the bridge more aqua-like. Alternatively, **5₃** contains a protonated carboxylate ligand functioning as a weak hydrogen bond acceptor from the bridging hydroxo. The value of J (33 cm^{-1}) for **5₃** is essentially the same as that for the

structure without the hydrogen bond (**63**, 30 cm^{-1}). Both **33** and **53** give J values bracketing the experimental value (20 cm^{-1}) while retaining the hydrogen bond between the bridging hydroxo and carboxylate ligand. The DFT calculations of the δ and E_Q parameters for models **33** and **53** are in agreement with the experimental data (Table S1). However, coordination to Fe^{3+} is expected to significantly lower the carboxylic acid $\text{p}K_a$. While a protonated carboxylate ligand has been proposed as a potential intermediate in peroxo complexes of diiron hydroxylases,^{38,39} a stable protonated carboxylate ligand to Fe^{3+} near neutral pH has no precedent; thus **3** and **5** were not considered further.

With the exception of **63**, all the models discussed above are compatible with the crystal structure of Tm FDP. Given the close experimental values of J , δ , and E_Q for FDP_{ox} and MMO_{ox} (Table 1), we also considered a bis(μ -hydroxo) diferric model, **73**. This model was derived from **23** but with a hydroxo bridge replacing the aqua ligand to Fe_D . The optimized structure for **73** gave an Fe–Fe distance of $3.14\text{ \AA}$ and average Fe–O–Fe angle of 102° , which are comparable to those for MMO_{ox} ($3.1\text{ \AA}$, 95°).⁴⁰ Structure **73** gave a calculated J value (19 cm^{-1}) in good agreement with experiment. This calculated value of J was essentially independent of the presence or absence of the hydrogen bond to D89. The DFT-calculated δ and E_Q values for **73** were also in agreement with the experimental values (Table S1). For deflavo- FDP_{ox} , the higher experimental J value (30 cm^{-1}) is predicted by **63**.

Reduced FDP (FDP_{red})

Mössbauer spectra of FDP_{red} recorded at 4.2 K in zero applied field show a single quadrupole doublet with parameters indicative of high-spin ($S = 2$) non-heme Fe^{2+} (Table 1). The spectrum at 45 mT was nearly identical with no indication of Fe^{3+} impurities. The narrow line width of the doublet (0.29 mm/s) indicates low heterogeneity in the structure and equivalent electronic environments for the individual Fe^{2+} centers of the active site. A simulated fit to the spectrum can tolerate no more than 0.10 mm/s difference in the quadrupole parameters of the two iron centers ($|E_Q^{\text{FeP}} - E_Q^{\text{FeD}}| < 0.10\text{ mm/s}$), which as discussed below was an important constraint for DFT generated structures.

Figure 7 shows variable temperature Mössbauer spectra of FDP_{red} for an applied field of 7 T . The doublet splits into a magnetic pattern indicative of two antiferromagnetically coupled $S_{\text{Fe}^{2+}} = 2$ iron centers. The sign of E_Q for both sites was found to be positive, indicative of a doubly occupied $3d_{xy}$, $3d_{yz}$, or $3d_{xz}$ orbital. In contrast to FDP_{ox} , the electronic relaxation for FDP_{red} becomes fast for $T > 25\text{ K}$ such that the magnetic pattern depends on the thermal average over the internal magnetic fields for the individual electronic spin states. The population of the excited spin states $S > 0$ generates an internal magnetic field, which opposes the external applied field, allowing variable temperature Mössbauer spectra to be used to determine the exchange coupling between the Fe^{2+} ions. As Figure 7 shows, the overall splitting of the 7 T Mössbauer spectra decreases as the temperature was increased, demonstrating that $S > 0$ spin states were populated at higher temperatures.

The temperature dependence of the spectra in Figure 7 was dominated by the product of the thermal spin expectation and the isotropic value of the ^{57}Fe A-tensor (A_{iso}), with minor dependence on $D_{\text{Fe}^{2+}}$. The thermal spin expectation value is dependent on the value of J .

The value of A_{iso} cannot be independently determined, but the range of values of characterized non-heme Fe^{2+} centers in proteins with similar N/O coordination is -18 to -27 MHz (-13 to -20 T).^{29,41,42} Fits of the variable temperature data using eq 1 and A_{iso} constrained to this range gave a corresponding range for the exchange-coupling constant of $+28 < J < +37 \text{ cm}^{-1}$ or $J = +32(5) \text{ cm}^{-1}$. The value of E_Q used for each simulation was determined from zero-field spectra recorded at the corresponding temperatures (Table S8). These values ranged from $+2.42 \text{ mm/s}$ at 4.2 K to $+2.28 \text{ mm/s}$ at 150 K .

Similar Mössbauer spectra were collected for deflavo-FDP_{red} (Figure S1). The examined samples of deflavo-FDP_{red} typically had 40% Fe^{2+} impurity species, which may be a combination of mononuclear ferrous and weakly interacting diferrous ions, which increased the uncertainty in the measurement of the J value. Nevertheless, 60% of the iron belongs to a species showing approximately the same spectra and temperature dependence as FDP_{red}, indicating a similar value for J for the majority species.

DFT Calculations of FDP_{red}

As for the diferric case, we compared the DFT and experimental values of J from characterized synthetic complexes. There are relatively few structurally characterized non-heme diferrous OH_x -bridged synthetic complexes with known experimental values for J ; we found four in the literature, which are listed in Table S7.^{43–46} The calculated values of J were in good agreement with experiment for three of these complexes. The agreement with the fourth complex was not as good but of the correct sign, owing to a significantly different orbital configuration and a ferromagnetic J value.

For isostructural diiron cores, it is expected that the exchange-coupling constant for the diferrous state is approximately equal to or slightly lower than that for the diferric state.⁴⁷ The doubly occupied, weakly antibonding 3d orbital of high spin Fe^{2+} can introduce a ferromagnetic contribution to J associated with the metal-to-metal transfer of the minority-spin electron in the doubly occupied orbital. Fe^{2+} is expected to have longer metal-bridging ligand bond lengths than Fe^{3+} . These two effects would tend to lower the value of J for diferrous relative to diferric. Remarkably, however, the experimental value of J for FDP_{red} (32 cm^{-1}) is significantly *larger* than that of FDP_{ox} (20 cm^{-1}), suggesting a structural change upon reduction that affects the exchange interaction.

For the sake of comparison, DFT calculations were performed for the FDP diferrous state in the same conformations **1–7** considered for the diferric state (Figure 6 and Table S2). The optimized structures of **1₂–7₂** display the expected longer Fe–O bridge bonds relative to those of the diferric complexes (Table S3). The DFT-calculated J values for **1₂–7₂** (Figure 6) were found to be comparable to or weaker than those of the corresponding diferric models, **1₃–7₃**. For diferrous, the smaller energy difference between the F and BS states, owing to the longer bond lengths and ferromagnetic minority spin contributions, is compensated by the smaller spin projection factor (diferric 12.5, diferrous 8). We also considered structures without the water ligand to Fe_D. The calculated J values for **2₂^{NW}**, **3₂^{NW}**, **5₂^{NW}**, and **6₂^{NW}** (Figure 6) (superscript NW = no water) are approximately the same as those for the corresponding diferric structures. All states of **3** gave relatively small J values, which can be attributed to hydrogen bond donation from the protonated carboxylate. For the diferric state,

the presence or absence of the Fe_D terminal water ligand had an insignificant effect on the value of J because all d orbitals are singly occupied. In contrast, for the diferrous state, this water ligand can affect which d orbital is doubly occupied and thereby J . For example, Figure 8 shows that for **6₂**, the doubly occupied orbital on Fe_D is oriented roughly parallel to the Fe–Fe vector, whereas in the absence of the terminal water, **6₂^{NW}**, the doubly occupied orbital is roughly perpendicular to the Fe–Fe vector. The alignment of the doubly occupied d-orbital of Fe_D in **6₂** contributes a ferromagnetic exchange interaction, which lowers the value of J when water is bound. For isostructural diiron cores, these calculations show that the significant increase in J from FDP_{ox} to FDP_{red} cannot be attributed to a valence change without attendant structural rearrangements. The necessary structural arrangements cannot be the presence or absence of a terminal water ligand, since the presence of the water only lowers the value of J of FDP_{red} relative to FDP_{ox}.

We rule out **1**, **3**, **4**, **5**, and **7** for diferrous because either the calculated values of J do not agree with experiment (**1**, **3**, **4**, and **7**) or a protonated carboxylate ligand is unlikely (**3** and **5**). This leaves diferrous **2** and **6** as possibilities. We add a constraint from the Mössbauer quadrupole splitting. The individual irons of the diferrous state of FDP_{red} have identical Mössbauer parameters. In contrast, the calculated values for δ and E_Q differ significantly when water is bound to Fe_D in **1₂–6₂** (Table S2). While the calculated Mössbauer parameters for the 5-coordinate Fe²⁺ of the optimized structures agree with the experimental values ($\delta = 1.15$ mm/s, $E_Q \approx 2.4$ mm/s), the 6-coordinate water-bound Fe²⁺ had calculated values ($\delta = 1.24$ mm/s, $E_Q \approx 3$ mm/s) that are significantly outside the experimental tolerances. The agreement with experimental Mössbauer parameters improved significantly when the ligand water of Fe_D was removed. For some of these solely 5-coordinate diferrous models, including **2₂^{NW}** and **6₂^{NW}**, the calculated values of E_Q for the two Fe²⁺ are nearly equal to each other and within the allowed experimental difference. This additional constraint and the predicted value of J show agreement of **6₂^{NW}** (35 cm⁻¹) with experiment, whereas **2₂^{NW}** (48 cm⁻¹) does not.

DISCUSSION

It is a misconception that EPR signals cannot be obtained for exchange-coupled dinuclear high-spin Fe³⁺ sites. While this is true for oxo- or sulfido-bridged diferric sites in which the strong exchange interaction shifts the paramagnetic states to energies too high for detection, this is not true for bridging groups that induce weaker exchange interactions. The lower value of J positions the system $S = 2$ and 3 multiplets at thermally accessible energies. Non-heme high-spin Fe³⁺ sites have $D \approx 1$ cm⁻¹, and owing to small spin projection factors, the system $S = 2$ and 3 manifolds have small zero-field splittings. Consequently, these spin manifolds have at least one pair of sublevels split by less than 0.3 cm⁻¹ and thus can show X-band EPR signals. Such a resonance was first observed from the diferric cluster of methane monooxygenase.²⁹ Our observation of an analogous signal for FDP_{ox} allowed determination of the exchange-coupling constant, $J = 20(3)$ cm⁻¹, which indicates the presence of at least one hydroxo bridge between the Fe³⁺ ions. Extensive studies of N,O-ligated non-heme high spin diferric sites in enzymes and synthetic complexes have found exchange-coupling constants $J > 200$ cm⁻¹ for an oxo-bridge, $15 < J < 43$ cm⁻¹ for

hydroxo-/alkoxo-bridges, and J near 0 cm^{-1} for water or solely carboxylate bridged irons.^{48–50} Previous reports have suggested a hydroxo bridge between the irons of FDPox.⁷

Table S5 lists experimental values of J for synthetic hydroxo-/alkoxo-bridged diferric complexes. Two of the complexes (CSD COCJIN, CEPBEF) have monohydroxo-bridged irons and values of J (+34, +43 cm^{-1}) significantly higher than that of FDPox. Three of the diferric complexes (CSD CAHJEA, HXAPFE, DEWPIE10) are dihydroxo- or hydroxo-/alkoxo-bridged having J values of +21, +23, and +24 cm^{-1} which agree with that of FDPox. The MMOox diferric site is dihydroxo-bridged and has $J = 15\text{ cm}^{-1}$, which is also within error equal to that of FDPox. DFT models of the FDP diferric site showed that a second hydroxo bridge lowers the Fe–O–Fe angle (average of 102°) and significantly lowers the value of J to +19 cm^{-1} for **73** (Figure 6), which is in agreement with the spectroscopic value of +20 cm^{-1} . The calculated values of δ and E_Q for **73** are also in agreement with experiment. The relatively low value of J for FDPox is consistent with the dihydroxo-bridged diferric site of Figure 9 and is not compatible with a monohydroxo-bridged diferric site. The DFT-calculated value of J was found to be independent of the hydrogen bond to D89 and thus the uncertainty indicated by the parentheses around the bond of Figure 9. The resting diferric active sites of most other known non-heme carboxylate-bridged diiron sites in proteins contain either an oxo or dihydroxo solvent-bridging structure. The higher number of N donors to the irons of FDP in place of O donors for the dihydroxo-bridged diferric site of MMO is expected to favor a dianionic bridging species (oxo or dihydroxo) over a monoanionic single hydroxo. The crystal structure of as-isolated, presumably diferric *Moorella thermoacetica* (Mt) (PDB 1YCF)⁶ does have additional density in a position for a second bridging molecule, which could not be described accurately and was modeled as a diatomic.

In both the presence and absence of FMN, six crystal structures of FDP from various sources show a conserved hydrogen bond between D89 (Figure 1, numbering for Tm) and the bridging solvent molecule. The hydrogen bonding interaction with a bridge is rare in His/carboxylate-ligated protein diiron sites. These crystal structures do not show a dihydroxo-bridged diiron complex, and we initially considered structures (**1–5**) that were compatible with the crystal structures of FDP. It is expected that the D89 hydrogen bond with the bridging solvent affects the value of J . The hydrogen bond modulation of the exchange-coupling constant in carboxylate-bridged diiron proteins has precedence in the met and oxy states of Hr, both of which are formulated as μ -oxo diferric. The value of J decreases from 270 cm^{-1} in metHr to 155 cm^{-1} in oxyHr, which has been attributed to hydrogen bond donation from a hydroperoxide to the oxo bridge in oxyHr.²⁶ For the crystallographic FDP structure shown in Figure 1, the hydroxo bridge would donate a hydrogen bond to D89, and the value of J should accordingly increase. Indeed, DFT calculations of structures **23** and **63** show that this hydrogen bond increases the value of J by 15 cm^{-1} . However, the experimental value of $J = 20\text{ cm}^{-1}$ for FDPox is significantly *lower*, not higher, than that of synthetic diferric complexes with a monohydroxo bridge unperturbed by hydrogen bonding (Tables S4 and S5). The experimental value of J for FDPox is not compatible with a monohydroxo-bridged diferric structure; even more so, the J value is not compatible with a monohydroxo bridge functioning as a hydrogen bond donor.

Crystal structures of diiron FDPs other than Tm FDP all have the FMN site occupied, and they all have the diiron core bridging structure shown in Figure 1, including the bridging solvent ligand within hydrogen bonding distance to D89. Our measurements of defflavo-FDP_{ox} indicate an increase of 10 cm⁻¹ in the J value relative to that of FDP_{ox} (in which the FMN site is occupied). This increase indicates a change in the diiron core bridging structure, which has not been previously noted. The higher experimental J value (30 cm⁻¹) is accurately predicted by **63** in which one of the hydroxo bridges is lost relative to **73**, as illustrated in Figure 9, and this value agrees with that of synthetic complexes. The hydrogen bond to the amide NH of D89 may stabilize the terminal O of D89 out of hydrogen bonding range to the now monohydroxo-bridged site. Although FMN has no direct interactions with the diiron core ligands, these results indicate that the diiron core structure of FDP_{ox} changes in the absence of FMN.

Turning to FDP_{red}, two crystal structures are currently available for a reduced FDP. The diiron active site from *Moorella thermoacetica* (Mt) (PDB 1YCG)⁶ is very similar to that of the oxidized Mt enzyme (PDB 1YCF), with the solvent bridge hydrogen bonded to D89. However, this cannot be true for the active site of Tm studied here. DFT calculations of **1–7** show that diferrous species have values of J that are similar to or lower than that of their corresponding diferric state. The significant observed *increase* in the experimental value of J upon reduction of FDP therefore requires a significant structural change of the bridging species. The diiron active site from *Escherichia coli* FDP (PDB 4D02) appears to lose the solvent bridge upon reduction, but the lower resolution of the structure complicated the analysis.⁵¹ The loss of the solvent bridge altogether would significantly lower the value of J for the reduced state. For Tm FDP_{red}, independent of FMN occupancy, the value of J indicates retention of a monohydroxo bridge between the ferrous ions as indicated in Figure 9.

The value of J for FDP_{red} is compared to several diferrous proteins listed in Table 1. The bridges in the reduced state of MMO and RNR have been identified as H₂O or carboxylate ligands and have values of J near zero. In contrast, FDP_{red} and deoxyHr have much larger J values consistent with a monohydroxo bridge. The values for the two proteins are the same within experimental error, and these values agree with a synthetic complex having a hydroxo bridge (Table S7, DIBWUG10).⁴⁵ These results are consistent with the absence of a hydrogen bond between D89 and the hydroxo bridge in Tm FDP_{red} as shown in Figure 9.

The equivalent Mössbauer quadrupole splittings (E_Q) for the two Fe²⁺ ions in Tm FDP_{red} requires the same coordination number for both Fe_P and Fe_D. The condition is satisfied when water is removed from both irons, which agrees with the absence of water coordination to the irons in the Mt FDP_{red} structure. The calculated value of J for the structure that most closely agrees with the crystal structure (**22^{NW}**) is much higher than experiment. Rotation of D89 so that it no longer hydrogen bonds to the bridging hydroxyl (**62^{NW}**) produces a lower calculated value of J , which agrees with experiment. In addition, the calculated values of δ and E_Q and the small difference in E_Q between the iron sites are all in agreement with experiment for **62^{NW}**. The DFT calculations also support the Tm FDP_{red} structure of Figure 9.

The structures presented in Figure 9 are not compatible with the crystal structures of FDPs from various organisms. Similarly, our calculated J values for the crystallographically determined diiron structure do not agree with experiment. We conclude that the frozen solution structures of Tm FDP, and in particular the diiron core properties, are not accurately represented by the crystal structure of Tm FDP. Photo-reduction of the irons by the X-ray beam is a possibility, and we note that the DFT-calculated Fe–Fe distance and Fe–O–Fe angle for a reduced diiron center are in better agreement with the crystal structure than those of the oxidized diiron center. Further work is needed to measure values of J for other FDPs. We have introduced EPR and Mössbauer spectroscopic methods that demonstrate that such measurements are possible. Through the measurement of exchange coupling, comparison of the FDP_{ox}, deflavo-FDP_{ox}, and FDP_{red} spectroscopic data indicate that the diiron core changes among these species; changes that are not apparent in the crystallographic data.

A recent report stated that FDP_{red} is exclusively bridged by carboxylate ligand(s) on the basis of a weak exchange interaction.¹¹ Our finding is not in agreement with this report. The Mössbauer spectra of Figure 7 clearly indicate a large value of J for FDP_{red} and deflavo-FDP_{red}. These large values of J are incompatible with only carboxylate bridges and require a hydroxo bridge.

The state of FDP after turnover with NO was also probed here using the new integer-spin EPR signal of the diferric state. Reaction of FDP_{red} with >4 equiv of NO resulted in full conversion to the diferric state and oxidized FMN 120 s after mixing. This fully reoxidized FDP had the same Mössbauer parameters and same EPR signal and, therefore, the same value of J , showing that the bridging structure of the diferric site in FDP after reaction with NO is the same as that of the starting FDP_{ox}. Thus, if the bridging hydroxo proton is consumed in the reduction of NO, this proton must have been replaced within 120 s. Unlike the flavinated FDP enzyme, the deflavo-FDP spectroscopic data for the diiron core differs after turnover. Specifically, the EPR signal of deflavo-FDP_{ox} signal did not reappear even though the irons are oxidized and spin-coupled as determined from Mössbauer spectroscopy. A plausible explanation for the disappearance of the EPR spectrum is a change in the bridging species that results in a larger J value. The loss of the proton from the hydroxo bridge would raise J to >100 cm⁻¹ and raise the energy of the EPR active $S = 2$ multiplet too high for detection. This change in protonation state in the absence of FMN implicates a role for the FMN in proton transfer during reduction of NO to N₂O.

CONCLUSIONS

The exchange-coupling constants between the iron sites of FDP have been determined for the diferric and diferrous species from EPR and Mössbauer spectroscopies, respectively. In comparisons with diferric and diferrous proteins and synthetic complexes, the exchange-coupling constants are consistent with dihydroxo- and monohydroxo-bridged diiron active sites for FDP_{ox} and FDP_{red}, respectively. DFT calculated values of J , δ , and E_Q for these structures were found to be in agreement with the spectroscopic values. Our results indicate that a monohydroxo-bridged diferrous species is the catalytically functional state that reacts with NO and O₂. The crystal structure of oxidized Tm FDP, which is similar to several structures from other sources, does not show a dihydroxo-bridged diiron core suggesting a

discrepancy between the crystalline and frozen solution structures. Furthermore, our experimental results demonstrate that the structure of the diiron center changes between FDP_{ox} and FDP_{red} and is FMN dependent, changes that have not been noted in crystal structures. Spectroscopic studies of FDPs from other sources are needed to resolve the apparent discrepancy. The new EPR spectroscopic probe of the diferric state indicated that the diferric structure of FDP prior to and immediately after turnover with NO are FMN dependent, the latter implicating a proton transfer role for FMN, in addition to electron transfer, during enzymatic turnover of NO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work was funded by National Institutes of Health Grants R01 GM077387 (M.P.H.) and GM040388 (D.M.K.). Funding for the EPR spectrometer was from National Science Foundation Grant CHE1126268.

References

1. Kurtz DM Jr. Dalton Trans. 2007; 4115
2. Romao CV, Vicente JB, Borges PT, Frazao C, Teixeira M. J Biol Inorg Chem. 2016; 21:39. [PubMed: 26767750]
3. Gardner AM, Helmick RA, Gardner PR. J Biol Chem. 2002; 277:8172. [PubMed: 11751865]
4. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Proc Natl Acad Sci U S A. 1990; 87:1620. [PubMed: 2154753]
5. Berto TC, Speelman AL, Zheng S, Lehnert N. Coord Chem Rev. 2013; 257:244.
6. Silaghi-Dumitrescu R, Kurtz DM Jr, Ljungdahl LG, Lanzilotta WN. Biochemistry. 2005; 44:6492. [PubMed: 15850383]
7. Silaghi-Dumitrescu R, Coulter ED, Das A, Ljungdahl LG, Jameson GNL, Huynh BH, Kurtz DM Jr. Biochemistry. 2003; 42:2806. [PubMed: 12627946]
8. Caranto JD, Weitz A, Giri N, Hendrich MP, Kurtz DM Jr. Biochemistry. 2014; 53:5631. [PubMed: 25144650]
9. Fang H, Caranto JD, Mendoza R, Taylor AB, Hart PJ, Kurtz DM Jr. J Biol Inorg Chem. 2012; 17:1231. [PubMed: 22990880]
10. Hayashi T, Caranto JD, Wampler DA, Kurtz DM, Moenne-Loccoz P. Biochemistry. 2010; 49:7040. [PubMed: 20669924]
11. Solomon EI, Park K. J Biol Inorg Chem. 2016; 21:575. [PubMed: 27369780]
12. Krebs C, Bollinger JM Jr, Booker SJ. Curr Opin Chem Biol. 2011; 15:291. [PubMed: 21440485]
13. Que L, True AE. Prog Inorg Chem. 1990; 38:97.
14. Han Z, Sakai N, Bottger LH, Klinke S, Hauber J, Trautwein AX, Hilgenfeld R. Structure. 2015; 23:882. [PubMed: 25865244]
15. Coufal DE, Tavares P, Pereira AS, Huynh BH, Lippard SJ. Biochemistry. 1999; 38:4504. [PubMed: 10194372]
16. Haskin CJ, Ravi N, Lynch JB, Münck E, Que L Jr. Biochemistry. 1995; 34:11090. [PubMed: 7669766]
17. Hayashi T, Caranto JD, Matsumura H, Kurtz DM Jr, Moenne-Loccoz P. J Am Chem Soc. 2012; 134:6878. [PubMed: 22449095]
18. Caranto JD, Weitz A, Hendrich MP, Kurtz DM Jr. J Am Chem Soc. 2014; 136:7981. [PubMed: 24828196]
19. Petasis DT, Hendrich MP. Methods Enzymol. 2015; 563:171. [PubMed: 26478486]

20. Abragam, A., Bleaney, B. Electron Paramagnetic Resonance of Transition Ions. Clarendon Press; Oxford: 1970.
21. Brink, DM., Satchler, GR. Angular Momentum. 2nd. Clarendon Press; Oxford: 1968.
22. Becke AD. J Chem Phys. 1993; 98:1372.
23. Lee CT, Yang WT, Parr RG. Phys Rev B: Condens Matter Mater Phys. 1988; 37:785.
24. Noodleman L, Case DA. Adv Inorg Chem. 1992; 38:423.
25. Han WG, Liu TQ, Lovell T, Noodleman L. J Am Chem Soc. 2005; 127:15778. [PubMed: 16277521]
26. Dawson JW, Gray HB, Hoenig HE, Rossman GR, Schredder JM, Wang RH. Biochemistry. 1972; 11:461. [PubMed: 5059123]
27. Kurtz DM, Shriver DF, Klotz IM. Coord Chem Rev. 1977; 24:145.
28. Petersson L, Graslund A, Ehrenberg A, Sjoberg BM, Reichard P. J Biol Chem. 1980; 255:6706. [PubMed: 6248531]
29. Fox BG, Hendrich MP, Surerus KK, Andersson KK, Froland WA, Lipscomb JD, Münck E. J Am Chem Soc. 1993; 115:3688.
30. Brunold TC, Solomon EI. J Am Chem Soc. 1999; 121:8277.
31. Clark PE, Webb J. Biochemistry. 1981; 20:4628. [PubMed: 7295637]
32. Hendrich MP, Pearce LL, Que L Jr, Chasteen ND, Day EP. J Am Chem Soc. 1991; 113:3039.
33. Hendrich MP, Münck E, Fox BG, Lipscomb JD. J Am Chem Soc. 1990; 112:5861.
34. Strand KR, Yang YS, Andersson KK, Solomon EI. Biochemistry. 2003; 42:12223. [PubMed: 14567684]
35. Lynch JB, Juarez-Garcia C, Münck E, Que L Jr. J Biol Chem. 1989; 264:8091. [PubMed: 2542262]
36. Pulver SC, Tong WH, Bollinger JM, Stubbe J, Solomon EI. J Am Chem Soc. 1995; 117:12664.
37. Gorun SM, Lippard SJ. Inorg Chem. 1991; 30:1625.
38. Do LH, Hayashi T, Moenne-Loccoz P, Lippard SJ. J Am Chem Soc. 2010; 132:1273. [PubMed: 20055391]
39. Banerjee R, Meier KK, Münck E, Lipscomb JD. Biochemistry. 2013; 52:4331. [PubMed: 23718184]
40. Rosenzweig AC, Brandstetter H, Whittington DA, Nordlund P, Lippard SJ, Frederick CA. Proteins: Struct, Funct Genet. 1997; 29:141. [PubMed: 9329079]
41. Zimmermann R, Huynh BH, Münck E, Lipscomb JD. J Chem Phys. 1978; 69:5463.
42. Rodriguez JH, Ok HN, Xia YM, Debrunner PG, Hinrichs BE, Meyer T, Packard NH. J Phys Chem. 1996; 100:6849.
43. Korendovych IV, Kryatov SV, Reiff WM, Rybak-Akimova EV. Inorg Chem. 2005; 44:8656. [PubMed: 16296818]
44. Hendrich MP, Day EP, Wang C-P, Synder BS, Holm RH, Münck E. Inorg Chem. 1994; 33:2848.
45. Hartman JAR, Rardin RL, Chaudhuri P, Pohl K, Wieghardt K, Nuber B, Weiss J, Papaefthymiou GC, Frankel RB, Lippard SJ. J Am Chem Soc. 1987; 109:7387.
46. Menage S, Brennan BA, Juarez-Garcia C, Münck E, Que L Jr. J Am Chem Soc. 1990; 112:6423.
47. Rodriguez JH, Xia YM, Debrunner PG, Chaudhuri P, Wieghardt K. J Am Chem Soc. 1996; 118:7542.
48. Kurtz DM. Chem Rev. 1990; 90:585.
49. Que L Jr, True AE. Prog Inorg Chem. 1990; 38:97.
50. Solomon EI, Brunold TC, Davis MI, Kemsley JN, Lee SK, Lehnert N, Neese F, Skulan AJ, Yang YS, Zhou J. Chem Rev. 2000; 100:235. [PubMed: 11749238]
51. Romao CV, Vicente JB, Borges PT, Victor BL, Lamosa P, Silva E, Pereira L, Bandejas TM, Soares CM, Carrondo MA, Turner D, Teixeira M, Frazao C. J Mol Biol. 2016; 428:4686. [PubMed: 27725182]

Figure 1.
Schematic structure of the diferric Tm FDP active site based on PDB entry 1VME. O_{pw} indicates a pocket water, which is hydrogen bonded to the Fe_D ligand water, O_w .

Figure 2.

Mössbauer spectra (red) and simulations (black) of 1 mM Tm FDP_{ox} recorded at 4.2 K and magnetic fields of (A) 50 mT and (B) 7.5 T. Simulations for system spin $S = 0$ of two Fe³⁺ sites: $\delta = 0.48, 0.43$ mm/s, $E_Q = +1.04, 0.94$ mm/s, $\eta = 0.6, 1$, $\Gamma = 0.35$ mm/s.

Figure 3.

EPR spectra and simulations of ~ 1 mM Tm FDP_{ox} (red lines, A–D) and deflavo-FDP_{ox} (blue lines, E, F) at the temperatures listed. Conditions: microwaves (A) $\mathbf{B}_1 \perp \mathbf{B}$, 9.645 GHz at 20 mW, (B–F) $\mathbf{B}_1 \parallel \mathbf{B}$, 9.326 GHz at 20 mW. Simulations for two identical $S = 5/2$ Fe³⁺ sites (black lines, A–D): $J = 20$ cm⁻¹, $D_{\text{Fe}^{3+}} = 1.1$ cm⁻¹, $(E/D)_{\text{Fe}^{3+}} = 0.15$, $g = 2$, Fe–Fe vector $\mathbf{r} = 3.2$ Å, $\mathbf{r}_\theta = 56^\circ$ relative to $D_{\text{Fe}^{3+}}$. The intensity scales of the experimental spectra are all for equal protein concentration. The vertical bars indicate the relative scale between modes $\mathbf{B}_1 \perp \mathbf{B}$ and $\mathbf{B}_1 \parallel \mathbf{B}$.

Figure 4.

Signal intensity $\times$ temperature versus temperature of Tm FDP_{ox} parallel mode EPR signals: (●) $g = 8$, (■) $g = 12$, and (+) deflavo-FDP_{ox}, $g = 8$. No data points could be obtained for the deflavo-FDP_{ox} $g = 12$ signal. The solid lines are the percent populations using eq 1 for the $S = 2$ and 3 manifolds for $J = 20 \text{ cm}^{-1}$ (red, FDP_{ox}) and 30 cm^{-1} (blue, deflavo-FDP_{ox}). The dashed lines, representing the range of uncertainty, are calculations for $J = 17$ and 23 cm^{-1} of the $S = 3$ manifold.

Figure 5.

EPR spectra of ~ 1 mM Tm FDP (A,B,C) or deflavo-FDP (D,E) active sites before and after NO reactions. (A) FDP_{ox} ; (B,C) FDP_{red} quenched 130 ms (B) or 2 min (C) after NO addition; (D) deflavo- FDP_{ox} ; (E) deflavo- FDP_{red} quenched 2 min after NO addition. Conditions: temperature, 70 K; microwaves, 20 mW at 9.33 GHz.

Figure 6.

Diiron core conformations of DFT-optimized structural models **1** to **7** using the atoms and constraints in the model structure shown in Figure S8. For clarity, only the irons, the terminal carboxylate ligand to Fe_D, protons, and Fe_D-coordinated water are shown. The subscripts refer to iron valences, and the numbers following the subscripts are the DFT-derived values of J in cm⁻¹ for each optimized structure. The superscript NW refers to the structure optimized without the terminal water ligand to Fe_D. See Table S3 for additional structural metrics.

Figure 7.

Mössbauer spectra of Tm FDP_{red} (red bars) and simulations (black lines) for a magnetic field of 7 T recorded at the temperatures listed. Simulations parameters for identical $S_{\text{Fe}^{2+}} = 2$ Fe^{2+} sites: $J = +32 \text{ cm}^{-1}$; $D_{\text{Fe}^{2+}} = -7 \text{ cm}^{-1}$, $(E/D)_{\text{Fe}^{2+}} = 0.2$, $\mathbf{A}_{\text{Fe}^{2+}}/g_n\beta_n = (-15, -26, -7)$ T, $A_{\text{iso}} = -16$ T, $\delta = +1.16 \text{ mm/s}$, $E_Q = +2.41 \text{ mm/s}$, $\eta = 0.2$. The gray vertical lines are a visual aid.

Figure 8.

DFT contour plots of the doubly occupied 3d orbitals for the diferrous structures 6_2 ($J = 24$ cm^{-1}) and 6_2^{NW} ($J = 35$ cm^{-1}), showing the effect of the water ligand to Fe_D . In each panel, Fe_P and Fe_D are on the left and right, respectively. Hydrogens, including that of the bridging hydroxide, have been omitted for clarity.

Figure 9.

Proposed diiron core structures of Tm FDP. The bridging carboxylate is not shown for any of the structures. The experimental values of J for each complex are given in cm^{-1} .

Table 1

Selected Properties of Non-heme Diiron Sites in Proteins That React with NO

protein	$J, \text{ cm}^{-1}$	bridging ligands ^b	$\delta, \text{ mm/s}$	$E_Q, \text{ mm/s}$	ref
$\text{Fe}^{3+}\text{Fe}^{3+}$					
FDP _{ox}	20(3)	bis($\mu\text{-OH}^-$)	0.48, 0.43	+1.04, 0.94	<i>d</i>
deflavo-FDP _{ox}	30(3)	OH^-	0.44	0.92	<i>d</i> ₈
methr	268	O^{2-}	0.46	1.57	26,27
oxyHr	154	O^{2-}	0.46, 0.47	1.87, 0.94	26,27
RNR _{ox}	216	O^{2-}	0.55, 0.45	-1.62, -2.44	28,17
MMO _{ox}	15	bis($\mu\text{-OH}^-$)	0.50	1.07	29
$\text{Fe}^{2+}\text{Fe}^{2+}$					
FDP _{red}	32(5)	OH^-	1.15	+2.39	<i>d</i>
deflavo-FDP _{red}	32(10)	OH^-	1.15	+2.5	<i>d</i>
deoxyHr	28	OH^-	1.14	2.76	30,31
deoxyHrN ₃	-3	OH_2			32
MMO _{red}	-1	COO^-	1.30	3.14, 2.4	29,33
RNR _{red}	1		1.26	3.13	34-36

^a $H_{\text{ex}} = 15 \text{ J} \cdot \text{S}^2$.^b All diiron sites have either one or two bidentately bridging carboxylates, which are not listed.^c Signs of E_Q are omitted if not determined.^d This work.^e Monodentate carboxylate.