Spectroscopic Studies of Single and Double Variants of M Ferritin: Lack of Conversion of a Biferrous Substrate Site into a Cofactor Site for O₂ Activation

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ABSTRACT: Ferritin has a binuclear non-heme iron active site that functions to oxidize substrate as a substrate for formation of an iron mineral core. Other enzymes of this class have tightly bound diiron cofactor sites that activate O₂ to react with substrate. Ferritin has an active site ligand set with 1-His/4-carboxylate/1-Gln rather than the 2-His/4-carboxylate set of the cofactor site. This ligand variation has been thought to make a major contribution to this biferrous substrate rather than cofactor site reactivity. However, the Q137E/D140H double variant of M ferritin, has a ligand set that is equivalent to most of the diiron cofactor sites, yet did not rapidly react with O₂ or generate the peroxy intermediate observed in the cofactor sites. Therefore, in this study, a combined spectroscopic methodology of circular dichroism (CD)/magnetic CD (MCD)/variable temperature, variable field (VTVH) MCD has been applied to evaluate the factors required for the rapid O₂ activation observed in cofactor sites. This methodology defines the coordination environment of each iron and the bridging ligation of the biferrous active sites in the double and corresponding single variants of frog M ferritin. Based on spectral changes, the D140H single variant has the new His ligand binding, and the Q137E variant has the new carboxylate forming a μ-1,3 bridge. The spectra for the Q137E/D140H double variant, which has the cofactor ligand set, however, reflects a site that is more coordinately saturated than the cofactor sites in other enzymes including ribonucleotide reductase, indicating the presence of additional water ligation. Correlation of this double variant and the cofactor sites to their O₂ reactivities indicates that electrostatic and steric changes in the active site and, in particular, the hydrophobic nature of a cofactor site associated with its second sphere protein environment, make important contributions to the activation of O₂ by the binuclear non-heme iron enzymes.

In contrast, the two ferrous ions are the “substrate” in the ferritins, as these enzymes perform a ferroxidase reaction to generate an oxo/hydroxo diferric product, which is then released from the site and stored in a ferric mineral core.¹⁶ Each catalytic subunit contains a ferroxidase site that catalyzes this reaction between the biferrous center and O₂. A similar DFP intermediate from fast dioxygen reaction has been observed during the ferroxidation reaction.¹⁷,¹⁸

To understand the unique ferroxidase reaction in the substrate site of the ferritins, crystallographic and spectroscopic studies on bullfrog M ferritin have been performed.¹⁷⁻³⁰ Crystal structures of the active sites are shown in Figure 1. These crystal structures were obtained with divalent proxies (i.e., Mg(II), Co(II), and Cu(II)) for Fe(II), since the Fe(II)
substrate rapidly reacts with O\textsubscript{2}, and thus only ferric structures have been observed in the vicinity of the ferritin active sites after soaking ferritin protein crystals in solutions of Fe(II).\textsuperscript{30,31} For this reason, protein crystallography of ferritin is a less informative probe of metal binding at the active site than for the related proteins where diiron sites remain occupied. Although the three crystal structures with different divalent metals display variations in the ligand set of one metal site (M2 in Figure 1) and the binding mode of the terminal carboxylate to the other metal site (M1, Figure 1),\textsuperscript{20,27,30} it is clear that the substrate site has a coordinating environment that is distinct from that of the cofactor sites, as it has only one $\mu$-1,3 carboxylate bridge and is more weakly bound to the protein (Figure 1). This structural difference allows the diferric product to leave the active site for formation of the ferric mineral core.

A combined spectroscopic methodology of circular dichroism (CD), magnetic CD (MCD), and variable-temperature, variable-field (VTVH) MCD has been applied to define the wild-type (WT) substrate site in M ferritin under biologically relevant solution conditions,\textsuperscript{24} enabling its comparison to the binuclear non-heme iron cofactor sites.\textsuperscript{8–10,24} CD and MCD spectroscopies probe the ligand-field (LF) transitions characteristic of the coordination of each iron in the active site. These transitions can be correlated to a specific iron center based on their VTVH MCD behavior.\textsuperscript{32} This methodology also determines the zero-field splitting (ZFS, $D$ (axial), $E$ (rhombic)) of each Fe(II) and exchange coupling ($J$) between two iron centers (reflecting bridging).

The spectra obtained for the biferrous active site of the ferritin showed 4 LF transitions that were paired by their VTVH MCD behaviors. Based on their LF splitting patterns, the two sets of transitions identified two inequivalent 5-coordinate iron sites (Figure 2b). This result indicates that the substrate site is ligated by one water on each iron in addition to the protein-based ligands, as shown in Figure 2a, with an open coordinate position on each iron that could react with O\textsubscript{2} in a bridged structure. The water in the active site was thought to be important in the formation of the diferric oxo/hydroxo species, since the protons provided by the coordinated water would facilitate the decay of the DFP intermediate.

In a study by Tosha et al., the substrate site in M ferritin was modified by substituting two of the natural amino acids of the Fe2 site with those found in the cofactor sites, creating the Q137E/D140H double variant.\textsuperscript{25} However, the kinetic results indicate that despite having the same ligand set, the double variant was not converted into a cofactor site in that the double variant exhibited very slow O\textsubscript{2} reaction without the observed formation of a DFP intermediate.

In the present study, we have applied CD/MCD/VTVH MCD spectroscopic methodology to define the geometric and electronic structure of the biferrous active site in the Q137E/D140H double variant of M ferritin and to correlate this structure with its slow O\textsubscript{2} reactivity. The CD/MCD/VTVH MCD spectra of two single variants (D140H and Q137E) were obtained in parallel and compared to the WT M ferritin spectra (Figure 2b) to evaluate the structural contribution associated with each ligand substitution, in order to elucidate the double-variant active site ligation. Comparison of the ferritin double variant active site obtained in this study to that of D84E RNR, which has the same ligand set but rapidly reacts with O\textsubscript{2}, revealed important factors required for an effective O\textsubscript{2} reaction.

### EXPERIMENTAL SECTION

#### 1. Sample Preparation.
MOPS buffer (Sigma-Aldrich), sodium chloride (EMD Chemicals, Inc.), deuterium oxide (99.9 atom % D, Cambridge Isotope Laboratories), $d_3$-glycerol (98 atom % D, Cambridge Isotope Laboratories), and ferrous sulfate heptahydrate (Baker) were purchased commercially and...
used as obtained. Before being cycled into the glovebox (<1 ppm O₂), D₂O was made anaerobic by pumping it with argon gas (99.9% pure) for ~5 h, and d₁-glycerol was degassed by heating it for ~8 h under vacuum (10⁻³ Torr). Fe(II) stock solutions, used for reconstitution of the apoprotein, were freshly prepared before each experiment by adding anaerobic D₂O to a premeasured amount of solid ferrous sulfate in the glovebox.

A QuickChange Mutagenesis kit (Stratagene) was used for site-directed mutagenesis on frog M-ferritin in pET-3a, and recombinant ferritin variants were expressed in *Escherichia coli*, and then purified as previously described.²²,²⁵ To remove iron from the core and potential binding sites, anaerobic reductive dialysis was performed in the presence of dithiothreitol.²⁴ For near-infrared (NIR) MCD measurements, the protein buffer solution was exchanged with a deuterium oxide buffer solution of 100 mM MOPS buffer (with 100 mM NaCl) at pD = 7.0 until D₂O comprised more than 99.5% of the solvent. This protein solution was concentrated to 1–7 mM diiron active centers (0.04–0.3 mM protein nanocages) and degassed by rapidly purging with vacuum and argon cycles for ~30 min.

Samples were studied in the absence of dioxygen (glovebox with an N₂ atmosphere). CD samples were prepared by adding incremental amounts of Fe(II) stock solution to the degassed apoprotein solution and incubating for ~15 min. An anaerobic quartz cuvette was used, and the sample temperature was kept constant by a circulating water bath. The biferrous active site was also monitored by CD for any effect on the active site due to the glycerol. MCD samples were prepared by adding 2 Fe(II)/active site (determined based on anaerobic CD titration results; see Supporting Information) and rapidly mixing the protein solution with d₁-glycerol (50% v/v for the final concentration) until the sample became homogeneous. The protein samples were then injected into the MCD cell (with a 0.2–0.3 cm⁻¹ thick spacer between two quartz windows), and immediately frozen in liquid N₂.

2. Spectroscopic Methods. NIR (600–2000 nm; 5000–16 666 cm⁻¹) CD and MCD data were collected on a Jasco J730 spectropolarimeter operating with a liquid N₂-cooled InSb detector. The MCD spectra were obtained at various fields (0 T, 0.7 T, 1.4 T, 2.1 T, 2.8 T, 3.5 T, 4.2 T, 4.9 T, 5.6 T, 6.3 T, 7.0 T), and normalized to the observed intensity at lowest temperature and highest field (1.8 K, 7 T) for all isotherms taken at a given wavelength.

The CD and MCD spectra were simultaneously fit with Gaussian bandshapes to find the minimum number of peaks required. The VTVH MCD data were fit using the approaches described in the Supporting Information. A goodness-of-fit parameter and visual comparison of the simulations to the data with error bars were used to determine final fit results.

### RESULTS AND ANALYSIS

1. D140H Variant (Reported as EQH mutant in ref 25).

The D140H single amino acid substitution changes the Asp140 in Figure 2a to a His residue. If this His binds to the Fe₂ site, only one of the two Fe(II) centers should be affected by this ligand change. The CD and MCD spectra of this variant are shown in Figure 3a and are different from those of the biferrous active site in WT ferritin in Figure 2b. The CD data show changes in more than two features in the spectrum, reflecting the sensitivity of CD to conformational changes in the vicinity of the site. MCD, by contrast, focuses directly on the electronic structure of the biferrous center. All the transitions in the D140H ferritin MCD spectrum are at energies similar to those of the WT site, indicating that this site has two inequivalent 5-coordinate (5C) iron centers. However, relative to the WT MCD spectrum in Figure 2b, this alteration perturbs the feature at 7600 cm⁻¹, which loses intensity, and the feature at 11 100 cm⁻¹, which changes sign from positive to negative (Figure 3a, bottom). This perturbation of two LF transitions indicates that the His ligand binds to one of the two ferrous centers of the
active site and that the two perturbed LF transitions are associated with that Fe. From Figure 2, the His would coordinate to Fe2.

The VTVH MCD data on the D140H ferritin were collected at 5530 cm\(^{-1}\) (arrow in Figure 3a) and fit using the two methods (equations S1–S3) in Supporting Information. Each ferrous center has a ground state with \( S = 2 \). These two iron centers can undergo both single center ZFS and exchange coupling between the centers, thus leading to a complicated set of ground state non-Kramers doublets for the biferrous active site (see SI pages S2 – S3). In the first method, sums of non-Kramers doublets are used to fit the ground state parameters (\( g_\| \)-tensor with \( M_s \) value) and the excited state energies of the combined metal site (equation S1, doublet model). Because exchange coupling (\( J \)) between two ferrous centers is comparable to the ZFS of each iron (\( D, E \)), both \( J \) and ZFS (\( D, E \)) were considered together in a spin-Hamiltonian analysis of the doublet fit results to describe the ground state electronic structures (equation S2). In the second method, each electronic transition was correlated to a specific iron center using the fact that the MCD intensity is proportional to the spin-expectation values of the single iron center in the biferrous wavefunctions (equation S3, spin-projection model).

From the doublet fit, the lowest doublet gave the parameters of \( g_\| = 8 \) and \( \delta_0 = 4.1 \) cm\(^{-1}\), which indicate an \( M_s = \pm 2 \) ground state. At around 15 K, a low-lying excited state starts to populate, requiring an additional excited-state doublet to fit the VTVH MCD data in Figure 3b. The best fit was obtained with \( g_\| = 4 \), which is associated with the \( M_s = \pm 1 \) sublevel, at an excited state energy of \( \sim 6 \) cm\(^{-1}\).

A spin-Hamiltonian analysis was then used to define the range of \( J, D \), and \( E/D \) values for the biferrous site associated with the doublet fit parameters obtained above. A fit of the VTVH MCD data in Figure 3b to the spin-Hamiltonian gives \( -15 \) cm\(^{-1} < D_1 < -12 \) cm\(^{-1} \) (\( E_1/D_1 = 0.3 - 0.33 \)), \( 10 \) cm\(^{-1} < D_2 < 12 \) cm\(^{-1} \) (\( E_2/D_2 = 0.2 - 0.25 \)) with \( -1 \) cm\(^{-1} < J < 0 \) cm\(^{-1} \), showing that the two Fe(II) centers are weakly antiferromagnetically coupled and that the single mutation did not significantly perturb the weak bridging interaction present in the WT enzyme (\( J \approx -0.3 \) cm\(^{-1}\)).

The saturation magnetization behavior of the data in Figure 3b was then correlated to a specific Fe(II) center, using the spin-projection model described in Supporting Information. From this analysis, the Fe(II) center associated with the transition at 4775 cm\(^{-1}\) in Figure 3a was shown to have a ZFS of \( -14.6 \pm 1 \) cm\(^{-1}\) and \( E/D \approx 0.33 \) (Supporting Figure S4). This spin projection analysis also gives the exchange coupling \( J \approx -0.16 \pm 0.15 \) cm\(^{-1}\), which is consistent with the spin-Hamiltonian analysis of the doublet model fit to the VTVH MCD data in Figure 3b.

2. Q137E Single Variant (reported as EED mutant in ref 25). The Q137E single amino acid substitution changes Gln137 (amide) in Figure 2a to a Glu residue (carboxylate), which is capable of binding to both iron sites (i.e., to form a second bridge). The CD and MCD spectra for this variant are shown in Figure 4a. Compared to the spectrum of WT ferritin (Figure 2b), the CD and MCD spectral features from both iron centers are perturbed. Their simultaneous Gaussian peak fit requires 3 LF transitions, at 4500, 8400, and 10 300 cm\(^{-1}\). The band at 10 300 cm\(^{-1}\) could reflect an Fe site that is SC (paired with the 4500 cm\(^{-1}\) transition) or 6C (paired with the 8400 cm\(^{-1}\) transition). However, the two bands at 10 300 cm\(^{-1}\) and 8400 cm\(^{-1}\) showed different binding behaviors in a CD titration of the apoprotein with Fe(II) (Supporting Figure S5), indicating that these two transitions are from different iron centers. Based on the different binding behaviors, the band at 10 300 cm\(^{-1}\) (paired with 4500 cm\(^{-1}\)) reflects 5C iron site, and the band at 8400 cm\(^{-1}\) reflects another 5C iron site. Therefore, the biferrous active site coordination is determined to be SC + SC iron centers. Although the coordination numbers of both iron are the same as the WT ferritin, the large LF changes for both Fe(II) centers indicate that both iron are perturbed by the Q to E mutation, indicating formation of a second carboxylate bridge between the two Fe centers.

VTVH MCD data taken at 7570 cm\(^{-1}\) (arrow in Figure 4a) were analyzed using the methods (equations S1–S3) in Supporting Information. The doublet analysis gave ground-state parameters of \( g_\| = 8 \) and \( \delta_0 = 3.0 \) cm\(^{-1} \) (\( M_s = \pm 2 \)) and an excited-state energy of \( \sim 6 \) cm\(^{-1}\), with \( g_\| = 4 \) (\( M_s = \pm 1 \)). The

Figure 4. Spectra of the Q137E ferritin single variant: (a) CD (290 K) spectra (top) and MCD (1.8 K, 7 T) spectra (bottom) of the biferrous ferritin single variant. The experimental data are shown in the black line and the Gaussian bands from simultaneous peak fit are shown in color (overall fit, red; each peak, blue, pink, and cyan). Spectra require 3 LF transitions from two inequivalent iron centers. Arrow indicates the energy where VTVH data were collected. (b) VTVH data collected at 7570 cm\(^{-1}\) (pink points with error bars) and the obtained doublet fit (solid line) (* is the baseline due to the protein precipitation and is not affected by the magnetic field, indicating it is not a metal centered transition).
spin-Hamiltonian analysis associated with these ground- and excited-state parameters gave the ZFS parameters, $-13 \text{ cm}^{-1} < D_1 < -10 \text{ cm}^{-1}$ ($E_1/D_1 = 0.30 – 0.33$), $9 \text{ cm}^{-1} < D_2 < 11 \text{ cm}^{-1}$ ($E_2/D_2 = 0.20 – 0.25$), and an exchange coupling $-1 \text{ cm}^{-1} < J < 0 \text{ cm}^{-1}$. It is noted that the $J$ value is determined to be small and negative, which is similar to that of the WT enzyme and the D140H single variant. Based on proteins and model studies, $\mu$-$1,3$ carboxylate bridges generally show small negative $J$’s that are not necessarily additive (i.e., the number of $\mu$-$1,3$ carboxylate bridges does not determine the magnitude of $J$). However, the data do show at least one $\mu$-$1,3$ carboxylate bridge is present for all three active sites (WT and both single variants), because $\mu$-$1$ carboxylate and $\mu$-$\text{H}_2\text{O}$ bridges both give small positive $J$ values and an $\mu$-$\text{OH}$ bridge would have strong antiferro-coupling (i.e., $-J > 10 \text{ cm}^{-1}$).$^{34–36}$

These VTVH MCD data were further correlated to a specific Fe(II) center, using the spin-projection model in equation S3, with the ZFS and $J$ range obtained from the spin-Hamiltonian analysis. From this fitting, it was determined that the Fe(II) center associated with the $8400 \text{ cm}^{-1}$ transition has a ZFS of $-11.7 \pm 0.8 \text{ cm}^{-1}$ and $E/D \approx 0.33$ (Supporting Figure S9). The exchange coupling was determined to be $J \approx -0.18 \pm 0.15 \text{ cm}^{-1}$, consistent with the results of fitting the doublet model.

3. Q137E/D140H Double Variant (reported as EEH mutant in ref 25). From the above results, the D to H mutation perturbed one Fe, indicating His binding to Fe2, and the Q to E mutation perturbed both iron, indicating bridge formation between two iron centers. Based on the above results, the double variant Q137E/D140H of ferritin should, in principal, correlate to the cofactor site of D84E RNR, which has the same ligand set. The CD, low-temperature (LT) CD, and MCD data for this double variant are shown in Figure 5. Compared to WT ferritin, the MCD spectrum of the double variant clearly shows the effect of His binding (MCD sign change for one iron site, Figure 2b vs Figure 5) and the LF transition energy slightly changed, which could be associated with the bridge formation. The simultaneous Gaussian fit for these spectra requires 4 LF transitions, at $4540, 8240, 9460$, and $11 100 \text{ cm}^{-1}$. The two Fe sites are inequivalent. The band at $9460 \text{ cm}^{-1}$ is either $5C$ or $6C$, as the LF splitting for that iron site $J = 0.15 \text{ cm}^{-1}$, and the transition at $11 100 \text{ cm}^{-1}$ is associated with an iron site with $D \approx +13.6 \pm 0.8 \text{ cm}^{-1}$ and $E/D \approx 0.2$ (Supporting Figure S14). The exchange coupling is $J \approx -0.15 \pm 0.15 \text{ cm}^{-1}$. The Fe(II) with LF transitions at $4540 \text{ cm}^{-1}$ and $9460 \text{ cm}^{-1}$ is $5C$, and the second Fe(II) with transitions at $8240 \text{ cm}^{-1}$ and $11 000 \text{ cm}^{-1}$ is either $5C$ or $6C$, as the LF splitting for that iron site ($\sim 3000 \text{ cm}^{-1}$) is in a range that can be consistent with both. The ground and excited state parameters obtained for the single and double variants are summarized and compared to those of WT ferritin in Table 1.

**DISCUSSION**

The combination of CD, MCD, and VTVH MCD spectroscopies has been applied to the biferrous active site in the Q137E/D140H double variant of M ferritin that converts the ligand set of the ferroxidase site into one that is equivalent to the diron cofactor site in D84E RNR. However, this double variant site has a different geometric and electronic structure compared to that of the cofactor site in D84E RNR that correlates with its much decreased $O_2$ reaction rate. The ferritin double variant has 4 LF transitions, which are paired as shown in Figure 7a, indicating that the two iron sites are $SC + SC$ (or $6C$), and the transition at $11 100 \text{ cm}^{-1}$ is associated with an iron site with $D \approx +13.6 \pm 0.8 \text{ cm}^{-1}$ and $E/D \approx 0.2$ (Supporting Figure S14). The exchange coupling is $J \approx -0.15 \pm 0.15 \text{ cm}^{-1}$. The Fe(II) with LF transitions at $4540 \text{ cm}^{-1}$ and $9460 \text{ cm}^{-1}$ is $5C$, and the second Fe(II) with transitions at $8240 \text{ cm}^{-1}$ and $11 000 \text{ cm}^{-1}$ is either $5C$ or $6C$, as the LF splitting for that iron site ($\sim 3000 \text{ cm}^{-1}$) is in a range that can be consistent with both. The ground and excited state parameters obtained for the single and double variants are summarized and compared to those of WT ferritin in Table 1.

**Figure 5.** Spectra of the Q137E/D140H ferritin double variant: CD (280 K) spectra (top), low temperature CD (3K) spectra (middle), and MCD (1.8 K, 7T) spectra (bottom) of the biferrous active site in the Q137E/D140H ferritin double variant. The experimental data are shown in the black line and the Gaussian bands from simultaneous peak fit are shown in color (overall fit, red; each peak, green, pink, orange, and purple). Spectra require 4 LF transitions from two inequivalent iron.
Coordination would require that the two terminal carboxylates of ferritin double variant did not have a water ligand, the 5C + 5C terminal carboxylate or from water. If the active site of the extra ligand in the double variant in ferritin either from the presence of at least one active site structure resulting from the spectroscopic studies in WT ferritin has the optimal orientation of the open coordination positions on both Fe(II) to allow O2 to react in the bridging mode required for 2e− reduction. Interestingly, WT ferritin, which has a SC + SC active site, thus an additional ligand relative to the D84E RNR cofactor site, has comparable DFP formation and decay rates to D84E RNR (Table 2). These rates suggest that WT ferritin has the optimal orientation of the open coordination positions on both Fe(II) to allow O2 to react in the bridging mode required for 2e− reduction. Based on the active site structure resulting from the spectroscopic studies in ref 24 (Figure 2a), the orientations of the open coordination positions in WT ferritin would derive from H-bonding of the coordinated water to the Q137 and D140 ligands. These water ligands would also contribute to the rapid DFP decay through proton donation to efficiently complete the ferroxidase reaction.

While the hydrophilic environment of the active site, which provides the additional water ligand in the double variant, is important in limiting the O2 reaction rate, electrostatic and while the biferrous site in D84E RNR has 3 LF transitions, which are paired as shown in Figure 7b, indicating 5C + 4C iron coordination. Therefore, the coordination environment at the active sites of ferritin double variant is more saturated than that of D84E RNR. This requires the presence of at least one extra ligand in the double variant in ferritin either from the terminal carboxylate or from water. If the active site of the ferritin double variant did not have a water ligand, the 5C + 5C coordination would require that the two terminal carboxylates have bidentate binding at both iron centers. In this case, only two LF transitions from two similar ferrous centers would be observed, as found for the resting Δ⁵D active site (Figure 7c). However, the four LF transitions observed in the ferritin double variant indicate that the two ferrous ions in the active site possess inequivalent coordination environments. Therefore, with two bridging carboxylates, if one iron has a terminal bidentate carboxylate then the second would possess at least one water ligand.

Additional water coordination to one of the iron’s in the active site of the ferritin double variant would explain the difference in O2 reactivities between this double variant site and a cofactor site as in D84E RNR. While it is possible to form the oxidized ferric species by iron auto-oxidation, the fact that the iron oxidation rate of this double variant (0.2 s−1) is still an order of magnitude faster than auto-oxidation in the L subunit recombinant ferritin (~0.01 s−1), which has no functional catalytic site, indicates that the iron oxidation observed in the double variant reflects the reaction of O2 with the biferrous active site, likely through a DFP intermediate. The rate constants for the O2 reaction with the biferrous site (through a DFP, kD) and the decay of the DFP (kD) were estimated for the double variant (eq 1) and compared to those of D84E RNR (Table 2).

\[
2\text{Fe}(II) + \text{O}_2 \xrightarrow{k_1} \text{Diferric-peroxo (DFP)} \\
\xrightarrow{k_D} \text{Fe(III)} - \text{O(H)} - \text{Fe(III)}
\]

Since a peroxo intermediate was not observed in the double variant, the DFP formation rate should be limiting, and the DFP rapidly decay (relative to kD) to form the dfferic–oxo(hydroxo) species. Therefore, kD is calculated from the kinetic data in ref 25 based on the initial diferric–oxo(hydroxo) formation rate (monitored ΔA_{565 nm}) and the lower limit of kD is estimated by assuming that the DFP intermediate would not be observed when A_{565 nm} < 0.01. As shown in Table 2, the kD for the rate of the O2 reaction of the double variant is 2 orders of magnitude slower than that of D84E RNR, while the lower limit of the kD value is similar between the double variant and D84E RNR. This decreased rate of DFP-formation could result from the presence of an active site water ligand in the double variant relative to D84E RNR, as it could block O2 binding or alter the orientation of the redox active orbitals required for O2 bridging. Therefore, although the protein-derived ligands are the same as those of a cofactor site, the presence of additional water ligation in the double variant active site of ferritin limits its ability to activate O2. As shown in Table 2, the kinetic data in ref 25 based on the initial diferric–oxo(hydroxo) formation rate (monitored ΔA_{565 nm}) and the lower limit of kD is estimated by assuming that the DFP intermediate would not be observed when A_{565 nm} < 0.01. As shown in Table 2, the kD for the rate of the O2 reaction of the double variant is 2 orders of magnitude slower than that of D84E RNR, while the lower limit of the kD value is similar between the double variant and D84E RNR. This decreased rate of DFP-formation could result from the presence of an active site water ligand in the double variant relative to D84E RNR, as it could block O2 binding or alter the orientation of the redox active orbitals required for O2 bridging. Therefore, although the protein-derived ligands are the same as those of a cofactor site, the presence of additional water ligation in the double variant active site of ferritin limits its ability to activate O2. Interestingly, WT ferritin, which has a SC + SC active site, thus an additional ligand relative to the D84E RNR cofactor site, has comparable DFP formation and decay rates to D84E RNR (Table 2). These rates suggest that WT ferritin has the optimal orientation of the open coordination positions on both Fe(II) to allow O2 to react in the bridging mode required for 2e− reduction. Based on the active site structure resulting from the spectroscopic studies in ref 24 (Figure 2a), the orientations of the open coordination positions in WT ferritin would derive from H-bonding of the coordinated water to the Q137 and D140 ligands. These water ligands would also contribute to the rapid DFP decay through proton donation to efficiently complete the ferroxidase reaction.

While the hydrophilic environment of the active site, which provides the additional water ligand in the double variant, is important in limiting the O2 reaction rate, electrostatic and
Table 1. Comparison of Ground State Parameters, Spectral Features, Coordination Numbers of Each Iron, and the Exchange Coupling between Irons in the Active Site of WT M Ferritin, the D140H, the Q137E, and the Q137E/D140H Ferritin Variants

|                  | WT ferritin<sup>a</sup> | D140H single variant | Q137E single variant | Q137E/D140H double variant |
|------------------|-------------------------|----------------------|----------------------|----------------------------|
| δ<sub>i</sub>(G<sub>i</sub>) | 8                       | 8                    | 8                    | 8                          |
| δ<sub>0</sub>    | 4.3                     | 4.1                  | 3.0                  | 4.6                        |
| δ<sub>i</sub>(G<sub>i</sub>) | 4                       | 4                    | 4                    | 4                          |
| ES energy (cm<sup>−1</sup>) | ~3.4                    | ~6                   | ~6                   | ~7                         |
| Fe<sub>1</sub> Coord. Number/D (cm<sup>−1</sup>) | 5C/−9.9                 | 5C/−14.6             | 5C/−11.7             | 5C/−14.9                   |
| MCD Transitions (cm<sup>−1</sup>) | (5025, 9900)            | (4775, 9500)         | (4500, 10300)        | (4540, 9460)               |
| Fe<sub>2</sub> Coord. Number/D (cm<sup>−1</sup>) | 5C/+4.6                 | 5C/+11.4             | C/+10.5              | 5 or 6C/+13.6              |
| MCD Transitions (cm<sup>−1</sup>) | (7600, 11500)           | (7570, 11070)        | (8400)               | (8240, 11100)              |
| Exchange coupling J (cm<sup>−1</sup>) | −0.3 ± 0.3              | −0.16 ± 0.15         | −0.18 ± 0.15         | −0.15 ± 0.15               |

<sup>a</sup>Fit parameters are obtained from ref 33. Transitions for Fe1 and Fe2 are corrected based on the result from the single variant spectra in this work.

Table 2. Comparison of Initial O<sub>2</sub> Reaction Constants for Peroxo-Formation and Peroxo-Decay of the Q137E/D140H Ferritin Double Variant, D84E RNR, and WT M Ferritin

|                  | Q137E/D140H variant<sup>ac</sup> | D84E RNR<sup>b</sup> | WT M ferritin<sup>ac</sup> |
|------------------|----------------------------------|----------------------|-----------------------------|
| k<sub>1</sub> (peroxo-formation, s<sup>−1</sup>) | 0.20 ± 0.04                      | 58 ± 13              | 89 ± 2                      |
| k<sub>2</sub> (peroxo-decay, s<sup>−1</sup>) | >2.2                             | 3.7 ± 0.7            | 7.4 ± 0.2                   |

<sup>a</sup>Rate constant obtained (WT M ferritin) and calculated (Q137E/D140H variant) from ref 25 (20 °C); <sup>b</sup>Rate constant obtained from ref 11, adjusted for subunit base for comparison with WT ferritin and the Q137E/D140H variant, 25 °C.

Figure 7. Comparison of MCD spectra and inferred active site structures of the Q137E/D140H ferritin double variant, D84E RNR, and resting Δ<sup>ΔD</sup>.

(a) MCD spectrum (top) and the active site structure (5C + 5C, bottom) of the Q137E/D140H ferritin double variant. Since Glu137 forms a second bridge, the water molecule on Fe1 (Figure 2) was replaced with the carboxylate oxygen ligand. Due to the His140 ligand replacing Asp140, the water molecule on Fe2 site is likely redirected relative to WT (Figure 2), which could affect the O<sub>2</sub> reactivity. There could be additional water binding or a change in the carboxylate binding mode (mono- vs bidentate E23 and E103); (b) D84E RNR, MCD spectrum (top) and matching crystal structure of the active site in D84E RNR (bottom), adapted and modified from refs 8 and 39; (c) Δ<sup>ΔD</sup>, MCD spectrum (top) and matching crystal structure of the active site (bottom), adapted and modified from refs 6 and 10.

Table 2. Comparison of Initial O<sub>2</sub> Reaction Constants for Peroxo-Formation and Peroxo-Decay of the Q137E/D140H Ferritin Double Variant, D84E RNR, and WT M Ferritin

- Steric effect must also be considered. Mutation of the ligands could change the charge distribution (with no net charge change) around the active site, thus affect the dioxygen accessibility. In addition, steric effects, mostly caused by the mutation of the aliphatic chain into a ring (D to H), could reduce the O<sub>2</sub> reaction rate by blocking access to the active site, as suggested in the previous kinetic studies.<sup>25</sup> Because the dioxygen channel in ferritin is not well-defined, we focus on the active site pocket in WT ferritin and the double variant (WT ferritin obtained from crystal structure, RCSB 1MFR, ref 20; the double variant obtained by substituting the ligands from the WT crystal structure (Q137 to E and D140 to H), optimizing this structure with α-carbon position constraints using DFT,<sup>38</sup> and placing the optimized structure into the protein active site). The pocket volume of WT ferritin is calculated as ~70–100 Å<sup>3</sup> within 6 Å from the center of two irons. This volume is reduced to ~40–70 Å<sup>3</sup> in the double variant, which, however, is still large enough to bind both water and dioxygen (total ~30 Å<sup>3</sup>). Therefore, the volume change in the active site pocket does not make a major contribution to the slower O<sub>2</sub> reaction rate of the double variant. Alternatively, this mutation does change in the shape of the active site pocket (Figure 8a vs b), which could
reduce O$_2$ accessibility to the proper binding mode. However, D84E RNR (obtained from crystal structure, RCSB 1PIZ, ref 39) has an even smaller active site pocket (<30 Å$^3$) as shown in Figure 8c, yet shows a fast O$_2$ reaction rate (Table 2) compared to that of the double variant. This indicates that the dynamic properties of the protein can provide flexibility at the active site for O$_2$ binding. Therefore, although electrostatic and steric effects could contribute to the low $k_1$ in eq 1, the additional water ligand present in the double variant is likely the major contributor in limiting this O$_2$ reaction.

To obtain insight into the origin of the water ligation difference between the double variant of ferritin and D84E RNR, the overall protein scaffolds and their water accessibilities were compared (Figure 9). Even though both active sites share 4-helix bundle structures, D84E RNR has additional protein segments surrounding its 4-helix bundle, while the 4-helix bundles in the ferritin subunits are more exposed (Figure 9a). This difference in protein scaffolds would isolate the active site in D84E RNR from the solvent environment, relative to the active site in the double variant (Figure 9b). In addition, hydrophilic residues near the active site in ferritin could contribute to the water access through a channel observed in the crystal structure (Figure 10). (It was recently shown that these hydrophilic residues are important for Fe(II) transfer from the ion channel to the active sites; R.K. Behera and E.C. Theil, in review).

Therefore, ferritin active sites are optimized for iron substrate access, which is consistent with the hydrophilic protein environment near the biferrous site. Alternatively, the cofactor sites activate O$_2$ for reactions with substrate with a slower decay of the peroxo intermediate that allows its control for enzymatic function. The hydrophobic environment required in biferrous cofactor sites and the hydrophilic environment required in diiron substrate sites are achieved through first and second sphere contributions from the protein scaffold. Thus, the contributions of iron ligands to protein catalytic activity can be sufficiently modulated by the protein scaffold, to limit the ferritin double variant in its cofactor reactivity with the O$_2$.

**ASSOCIATED CONTENT**

* Supporting Information

VTVH MCD data fit methods, binding affinity calculation methods, spectra from CD Fe(II) titration, spectra from MCD field dependence and temperature dependence, and VTVH MCD data fit with spin projection model. This material is available free of charge via the Internet at http://pubs.acs.org.
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Notes
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ABBREVIATIONS
CD, circular dichroism; MCD, magnetic circular dichroism; VTTH MCD, variable temperature, variable field magnetic circular dichroism; ZFS, zero field splitting (D: axial ZFS, E: rhombic ZFS); J, exchange coupling; RNR, ribonucleotide reductase; MMO, methane monoxygenase; Δ^D, Δ^D Desaturase; DFP, diferric-peroxo; WT, wild-type; LF, ligand field; NIR, near-infrared; LT, low temperature; ES, excited state; GS, ground state

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