

# Second coordination sphere effects on the mechanistic pathways for dioxygen activation by a ferritin: involvement of a Tyr radical and the identification of a cation binding site

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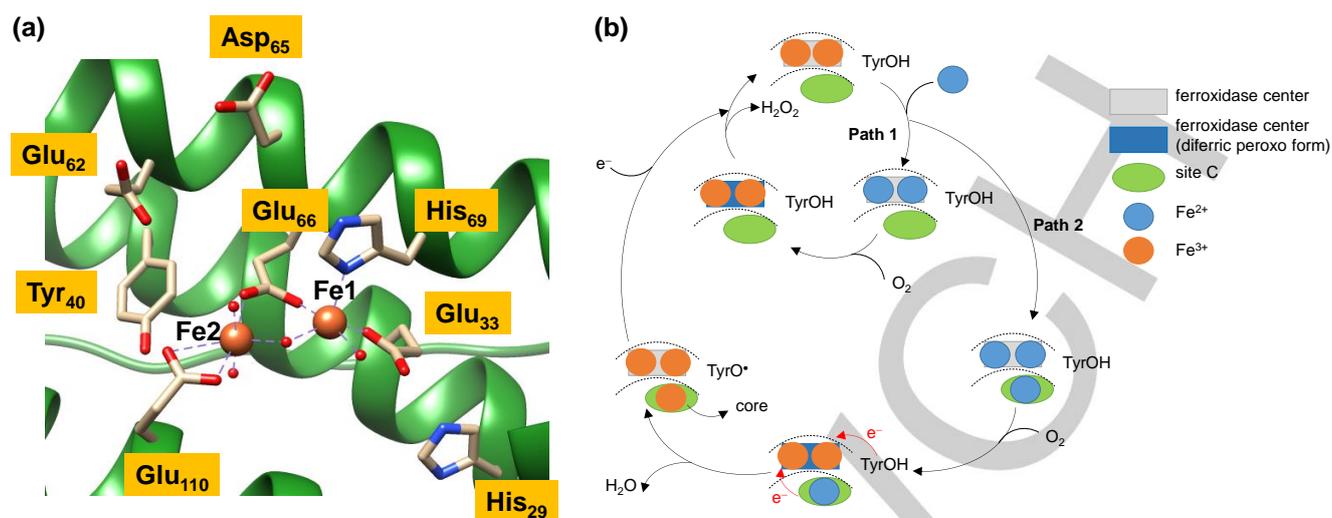
**Abstract:** Ferritins are ubiquitous diiron enzymes involved in iron(II) detoxification and oxidative stress responses and can act as metabolic iron stores. The overall reaction mechanisms of ferritin enzymes are still unclear, particularly concerning the role of the conserved, near catalytic center Tyr residue. Thus, we carried out a computational study of a ferritin using a large cluster model of well over 300 atoms including its first- and second-coordination sphere. The calculations reveal important insight into the structure and reactivity of ferritins. Specifically, the active site Tyr residue delivers a proton and electron in the catalytic cycle prior to iron(II) oxidation. In addition, the calculations highlight a likely cation binding site at Asp<sub>65</sub>, which through long-range electrostatic interactions, influences the electronic configuration and charge distributions of the metal center. The results are consistent with experimental observations but reveal novel detail of early mechanistic steps that lead to an unusual mixed-valent iron(III)-iron(II) center.

## Introduction

O<sub>2</sub> activating metalloenzymes are common catalysts in biology and found in all forms of life. Many of these enzymes utilize mononuclear iron in their active site and react as either monooxygenases or dioxygenases.<sup>[1,2]</sup> In addition to these mononuclear iron enzymes, there are a number of nonheme diiron enzymes that activate O<sub>2</sub> in their catalytic cycles.<sup>[3]</sup> Among these oxygen activating nonheme diiron enzymes are the soluble methane monooxygenases involved in the hydroxylation of methane in soil,<sup>[4]</sup> as well as the aldehyde deforming dioxygenases,<sup>[5]</sup> and the toluene monooxygenases.<sup>[6]</sup> Another O<sub>2</sub> activating nonheme diiron enzyme is ferritin, which has various biological roles and has been implicated in iron(II) detoxification and protection against oxidative stress,<sup>[7]</sup> in addition to providing an accessible store of iron under conditions of limitation. Ferritins are widely distributed across all kingdoms of life due to their importance in iron homeostasis.<sup>[8]</sup> Animal ferritins are composed of a mixture of H- and L- chain subunits that co-assemble to produce a hetero-polymeric protein cage in which the H-chains harbor a diiron site, the ferroxidase center, and the L-chains do not.<sup>[7a,9]</sup> Ferritins isolated from other organisms are polymers containing only H-chain like subunits.<sup>[10]</sup>

Structural models derived from high resolution X-ray diffraction data are now available for many ferritins. These reveal an identical 4  $\alpha$ -helical fold in all examples and similar coordination of the metal ions at the ferroxidase center in the H-chain proteins, illustrated in Figure 1a. The two ions are bridged by the carboxylate group of a glutamate residue (Glu<sub>66</sub>). Studies in which ferrous iron exposure times were varied identified one high-affinity (Fe1) and one low-affinity iron site (Fe2).<sup>[11]</sup> The coordination of the high affinity Fe1 site is completed by the sidechain of a second carboxylate (Glu<sub>33</sub>), a histidine (His<sub>68</sub>) and a water molecule. The lower affinity Fe2 site is coordinated only by a second carboxylate (Glu<sub>110</sub>) and a water molecule. A strictly conserved tyrosine residue (Tyr<sub>40</sub>) is located close to, but not within bonding distance of, Fe2 and has been identified as important in the mechanism of several ferritins.<sup>[12]</sup>

The similarity of protein structure together with the identification of a common reaction intermediate and product<sup>[13]</sup> has been invoked to argue in favor of a 'universal mechanism' of ferritin activity involving 2 reaction pathways.<sup>[14]</sup> In pathway 1, dioxygen binds to a diiron(II) center and is converted into H<sub>2</sub>O<sub>2</sub>, concomitant with oxidation of the diiron center to Fe<sup>3+</sup> (Figure 1b). An alternative dioxygen activation channel (through pathway 2, Figure 1b) requires binding and oxidation of 3 equivalents of Fe<sup>2+</sup> together with oxidation of the active site Tyr residue (Tyr<sub>40</sub>) coupled to the reduction of dioxygen to water. However, this proposal remains controversial for several reasons. Prokaryotic ferritins contain a third iron binding site, site C, close to the catalytic center that is clearly defined in crystal structures of proteins exposed to iron.<sup>[15]</sup> Disruption of this binding site by mutagenesis has been shown to affect catalysis in prokaryotic ferritins<sup>[16]</sup> and the binding of a third equivalent of iron is invoked in the universal mechanism under high iron loadings. In the H-chain like proteins of prokaryotes three conserved glutamate residues act as ligands to site C. One of these acts as a bridging ligand between site C and the low affinity site of the ferroxidase center. In contrast there is considerable variation in the equivalent residue of the true H-chains, with a non-coordinating side chain at this position in many instances (Figure S1, Supporting Information). It has been shown that the site C of prokaryotic ferritins can be disrupted by using site directed mutagenesis to introduce a non-coordinating residue in place of Glu<sub>52</sub> calling into question the existence of such a site in the animal H-chains.<sup>[16,17]</sup>



**Figure 1.** (a) Extract of the diiron active site of ferritin as taken from the 6GKA pdb file. (b) The universal reaction mechanism of dioxygen reduction to hydrogen peroxide or water at the diiron site of ferritins through pathway 1 (independent of site C occupation) and pathway 2 (with  $\text{Fe}^{2+}$  occupation at site C).

In the universal mechanism both site C and the conserved tyrosine residue are proposed to play identical roles in all ferritins.<sup>[12c]</sup> However, the consequences of substitution of Tyr for the non-oxidizable Phe or of a site C ligand for a non-coordinating residue vary between proteins. It is also known that the stoichiometry of ferritin catalyzed iron- $\text{O}_2$  reaction varies between the extremes of 2:1 and 4:1 for different proteins.<sup>[10]</sup> Consequently the view persists that a degree of mechanistic diversity exists within the ferritin family, perhaps reflecting variation in primary role between iron storage and oxidative stress response but also the environmental niche occupied by the organism in which the protein was identified.<sup>[7b]</sup>

*SynFtn* is a ferritin isolated from the prokaryotic marine cyanobacterium *Synechococcus sp.* CC9311 but bears some resemblance to the H-chain subunits of the animal proteins. Specifically the three glutamate residues that comprise site C are absent from the peptide chain with a serine residue, Ser<sub>146</sub>, in place of the glutamate that bridges site C and the low affinity site of the ferroxidase center in other prokaryotic ferritins. Structural models derived from X-ray diffraction data from iron enriched crystals revealed only two areas of electron density associated with metal ion binding at *SynFtn* ferroxidase centers and that Ser<sub>146</sub> does not act as a ligand to the low affinity site.<sup>[12a]</sup> Therefore, the first coordination sphere of iron bound to the ferroxidase center of *SynFtn* is identical to that reported for animal H-chains. Indeed, one of the PDB entries for iron enriched *SynFtn* crystals, 6GKA,<sup>[12a,18]</sup> was used to produce the illustration in Figure 1. The first detectable intermediate in *SynFtn* reactivity is a previously unreported mixed valent iron(II)/iron(III) ferroxidase center together with a protein based radical assigned to the side chain of Tyr<sub>40</sub>. The diiron(III)-peroxo species thought to be common to all other ferritins and related diiron enzymes<sup>[19]</sup> was not reported as a reaction intermediate for this protein. *SynFtn* reactivity therefore appears to contradict one of the central arguments in favor of a universal mechanism of ferritin function – similarity in structure inevitably results in similarity of reaction mechanism.

Several computational studies on ferritin and analogous non-heme  $\text{O}_2$ -activating diiron enzymes have been reported.<sup>[20]</sup> However, most of these studies used small cluster models or QM/MM with a modest QM region. To be specific, none of the reported computational studies included the Tyr<sub>40</sub> residue in the models and hence no pathway was ever studied that includes the involvement of this residue. To gain insight into the mechanism of ferritin activity we conducted a computational study into the various possible reaction mechanisms, including the use of large active site models to probe second coordination sphere effects. The latter is relevant because in recent work we showed the importance of the second-coordination sphere in quantum chemical calculations, whereby the long-range electrostatic, dipole and electric field perturbations influence optimized geometries and energetics.<sup>[21]</sup> Our calculations indicate that Tyr<sub>40</sub> is rapidly oxidized and deprotonated upon  $\text{O}_2$  binding and enables facile reduction of  $\text{O}_2$  to  $\text{H}_2\text{O}_2$  on the diiron center. Our studies also imply an important role of the second-coordination sphere Asp<sub>65</sub> residue that can hold a cation or may be protonated during the catalytic cycle.

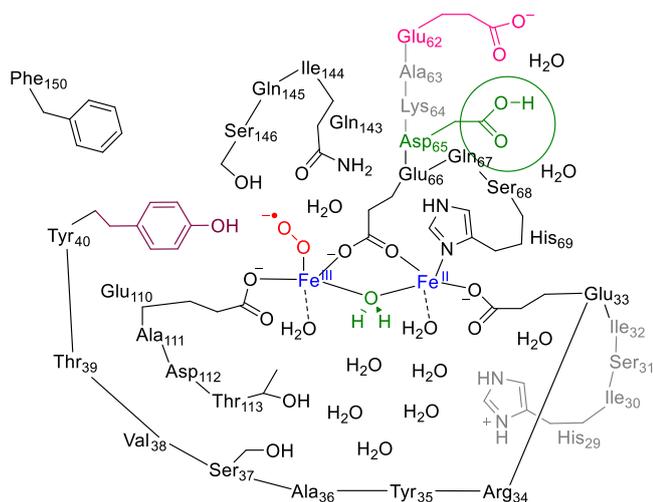
## Results and Discussion

### Model set-up and validation.

A large active-site cluster model was created based on the structure of *SynFtn* as deposited in the 6GKA pdb file, see Figure 2.<sup>[12a,18]</sup> Dioxygen was added to the Fe2 atom and hydrogen atoms and solvent (water) added in Chimera.<sup>[22]</sup> The model includes the 4  $\alpha$ -helical chains around the diiron center with all residues pointing outward as truncated to Gly residues. In addition, a number of polar and charged residues that potentially can induce a local dipole moment or act as part of the hydrogen bonding network have been taken into consideration. The full reaction mechanism was calculated for model **B** and **BP**, which differ in the protonation state of Asp<sub>65</sub>: namely, it is protonated in model **BP** while deprotonated in model **B**. Thus, a  $\text{pK}_a$  analysis in

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PropKa predicts Glu<sub>62</sub> and Asp<sub>65</sub> to have deprotonated amino acid chains. However, based on a visual inspection of the structure, we reasoned that since these anionic groups point toward each other that they may be part of a cation binding site although no cation is found in the crystal structure. Therefore, both models **B** and **BP** were tested here. A further expanded model **C** of 439 atoms was tested with Asp<sub>65</sub> deprotonated, but gave the same structure and electronic configuration as model **B**.



**Figure 2.** Ferritin model **B** and **BP** as investigated in this work. In model **BP** the Asp<sub>65</sub> group is protonated, while it is deprotonated in model **B**.

### Geometry optimization of large cluster models.

Following the manual addition of a dioxygen group bound to one of the Fe<sup>2+</sup> ions (Fe2) of the ferroxidase center (see model setup in Methods section), geometry optimization leads to essentially identical end-on superoxo structures for all residues conserved in each of the large cluster models (**B**, **BP** and **C**). This is due to the presence of a strong hydrogen bonding network included in the models that restrains the systems and maintains the site of iron and oxygen binding in a rigid conformation. An overlay of the optimized geometry of <sup>11</sup>Re<sub>B</sub> with the crystal structure coordinates deposited under 6GKA pdb file puts most protein chains in virtually identical positions and retains the basic features of the biochemical system (Figure S14, Supporting Information). The superoxo group is hydrogen bonded to both the side chain of Gln<sub>143</sub> and the alcohol group of Ser<sub>146</sub>, while the phenol group of Tyr<sub>40</sub> forms a hydrogen bond with the carboxylate of Glu<sub>110</sub>. Overall, the extra-large model **C** resulted in the same fold and electronic configuration and structural features as model **B**. We therefore continued the work with model **B** and **BP** only.

### Calculations on unprotonated Asp model **B**.

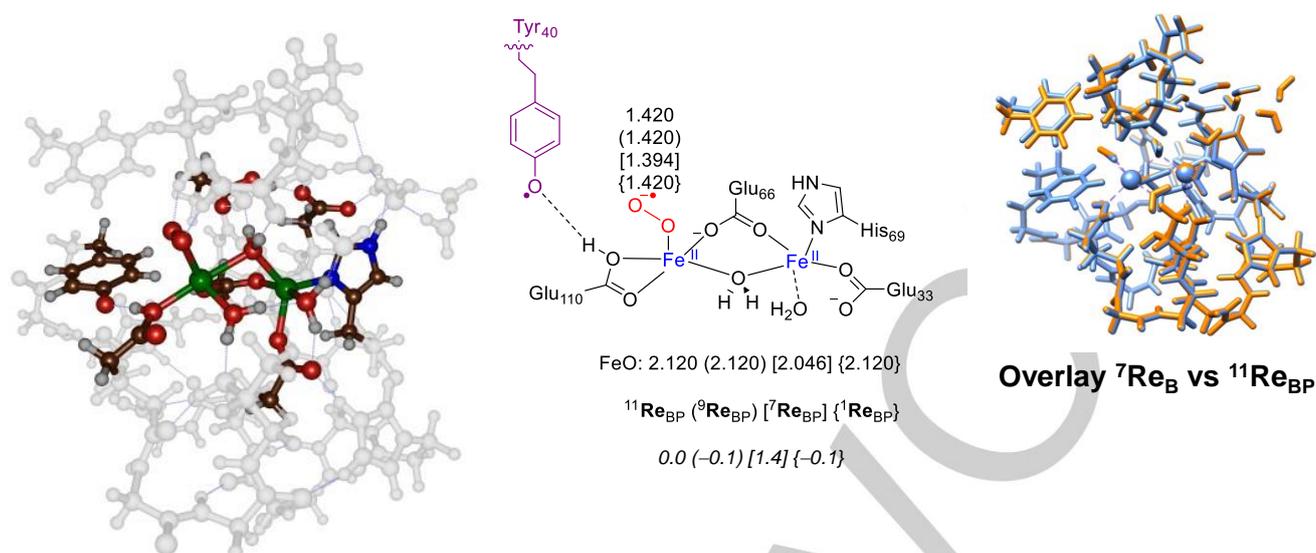
As previous computational studies on diiron complexes showed that they contain many close-lying spin and electronic configurations in the various complexes,<sup>[20,23]</sup> we tested the reaction mechanism on multiple spin state surfaces in various possible configurations for each complex. For all spin states for model **B**, optimization resulted in a small redistribution of electrons, giving a diiron(II)-superoxo complex with an unpaired electron on the superoxo group, as well as an unpaired electron shared between the carboxylate groups of Glu<sub>62</sub> and Asp<sub>65</sub> in the

orbital labeled as  $\pi^*_{\text{Asp}}$ . Note, that in none of the reactant complexes, **Re<sub>B</sub>** or **Re<sub>C</sub>**, was spin density found on the Tyr<sub>40</sub> residue. Attempts were made to swap molecular orbitals and find an iron(III)-superoxo with closed-shell deprotonated Asp<sub>65</sub> instead; however, during the SCF convergence the system returned to an Asp<sub>65</sub> radical instead. Consequently, in our model without cation bound to Glu<sub>62</sub> and Asp<sub>65</sub> and both residues in their deprotonated form, an electron transfer happens from these carboxylates to the iron center that creates an electronic configuration with an Asp radical as the lowest energy structure for model **Re<sub>B</sub>**. This is unusual in bioinorganic chemistry as no experimental evidence for Asp radicals has ever been found in ferritin proteins. As such, model **B** may be a spurious result due to a missing charge and may not be a realistic model of ferritin enzymes. We conclude; therefore, that we expect that in reality Asp<sub>65</sub> is probably protonated or binds a cation.

To find out if system **B** with deprotonated Glu<sub>62</sub> and Asp<sub>65</sub> would be catalytically active, we nevertheless, as a test of principle, investigated the potential energy landscape of dioxygen conversion into H<sub>2</sub>O<sub>2</sub> using ferritin model **B** on the lowest energy singlet, triplet, quintet, septet, nonet and undecaplet spin states. In all spin states there was radical character on Asp<sub>65</sub> (Table S10, Supporting Information) in most configurations along the mechanism with <sup>7</sup>Re<sub>B</sub> as the lowest in energy. The reaction for model **B** starts from a diiron(II)-superoxo structure by proton abstraction from the bridging water molecule to form an iron(II)-iron(III)-hydroperoxo species with barriers below 11 kcal mol<sup>-1</sup>. As the pK<sub>a</sub> of an ionized Tyr residue is low (pK<sub>a</sub> = -2),<sup>[24]</sup> a proton coupled electron transfer from Tyr<sub>40</sub> transfers a proton to Glu<sub>110</sub> while an electron is moved from Tyr<sub>40</sub> to the diiron system to form a diiron(II)-hydroperoxo species with a barrier of 15.2 kcal mol<sup>-1</sup> above reactants (Figure S16, Supporting Information). A final proton transfer from Glu<sub>110</sub> shuttles the proton to the hydroperoxo group to form H<sub>2</sub>O<sub>2</sub> in a slightly overall endothermic reaction by 5.1 kcal mol<sup>-1</sup>. As the highest barrier along the calculated mechanism is only 19.9 kcal mol<sup>-1</sup> this implies that model **B** could be reactive and convert dioxygen to H<sub>2</sub>O<sub>2</sub> on the diiron(II) center of ferritin despite showing an unusual radical center on Asp<sub>65</sub>. However, the structural orientation of the side chains of Asp<sub>65</sub> and Glu<sub>110</sub> imply space for a cation to bind there. As such, we think it more likely that Asp<sub>65</sub> is either protonated or bears a cation during the reaction mechanism. Nevertheless full details of the predicted transition states and intermediates for the unprotonated Asp model **B** and a discussion on the calculations are given in the Supporting Information.

### Calculations on Asp protonated model **BP**.

Upon visual inspection of the model **B** structures, we decided to repeat some of the calculations with a proton added to the Asp<sub>65</sub> residue, i.e., Model **BP**. In particular, the Asp<sub>65</sub> side-chain of each subunit may either bind a cation such as Na<sup>+</sup> or K<sup>+</sup> or even Fe<sup>2+</sup>. Alternatively the side chains of Glu<sub>62</sub> and Asp<sub>65</sub> may share a proton during the catalytic cycle of ferritin. While the overall spin state of the reactant complex is the same as that of model **B**, there is an increased charge of +1. The optimized geometries of structures <sup>11,9,7,1</sup>Re<sub>BP</sub> have no spin density located on the two carboxylate residues. These optimized geometries are shown in Figure 3 and an overlay of the optimized geometries of <sup>7</sup>Re<sub>B</sub> and <sup>11</sup>Re<sub>BP</sub> (right-hand-side of Figure 3) shows them to be almost identical.



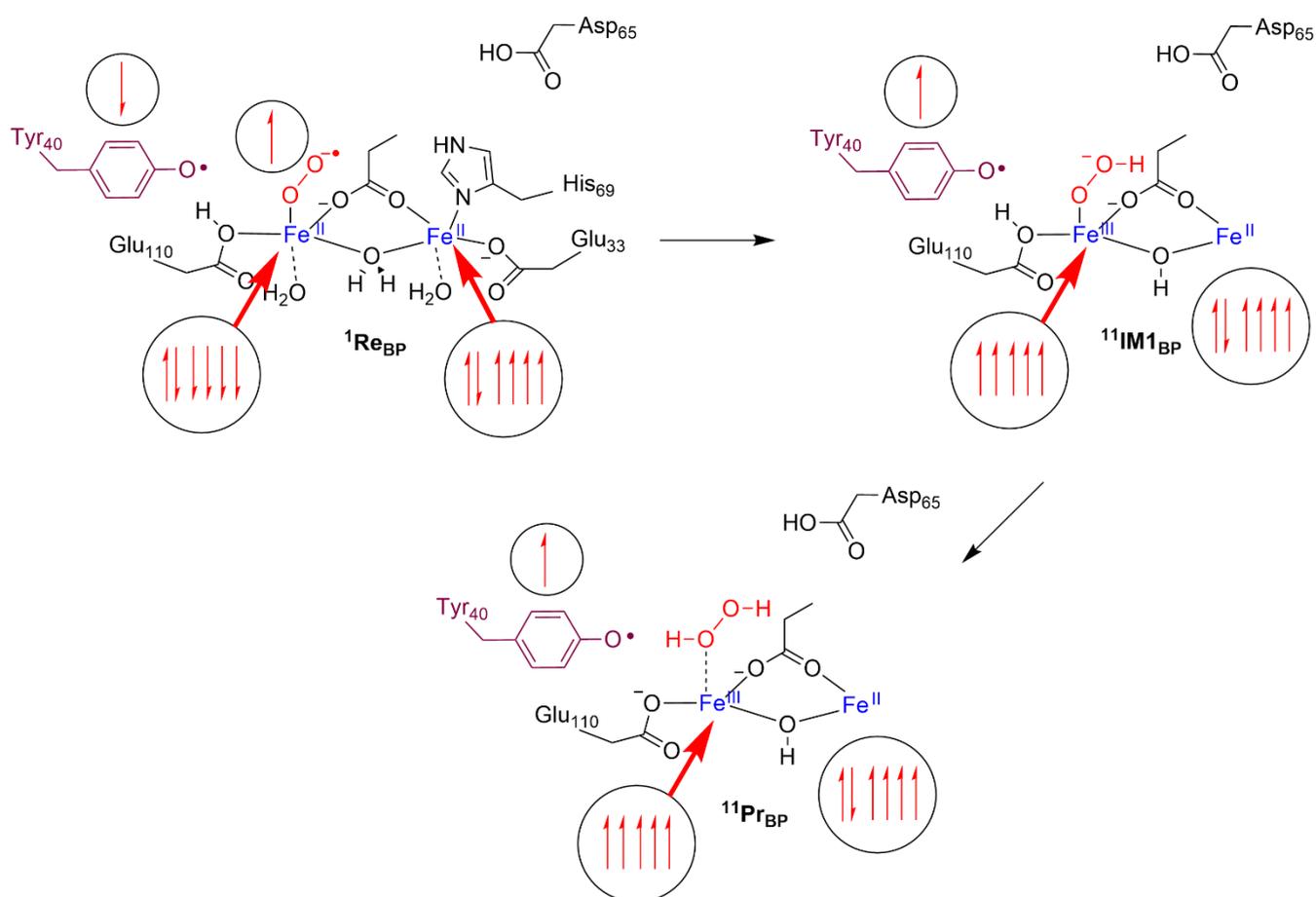
**Figure 3.** UB3LYP/BS1 optimized geometries of the protonated reactant complexes  $^{11,9,7}\text{Re}_{\text{BP}}$  with bond lengths in angstroms. Relative energies ( $\Delta E + \text{ZPE}$  values) are in kcal mol<sup>-1</sup>. The right-hand-side shows an overlay of the  $^{11}\text{Re}_{\text{BP}}$  (light blue) and  $^7\text{Re}_{\text{B}}$  (amber) optimized geometries.

Therefore, as expected, the protein fold and second-coordination sphere are not affected by protonation of Asp<sub>65</sub> and the characteristic features of the enzyme structure stay the same. The major difference between the  $^7\text{Re}_{\text{B}}$  and the  $\text{Re}_{\text{BP}}$  structures relates to Tyr<sub>40</sub>, which is deprotonated in  $\text{Re}_{\text{BP}}$  and bears an unpaired electron, while it is closed-shell and protonated in  $\text{Re}_{\text{B}}$ .

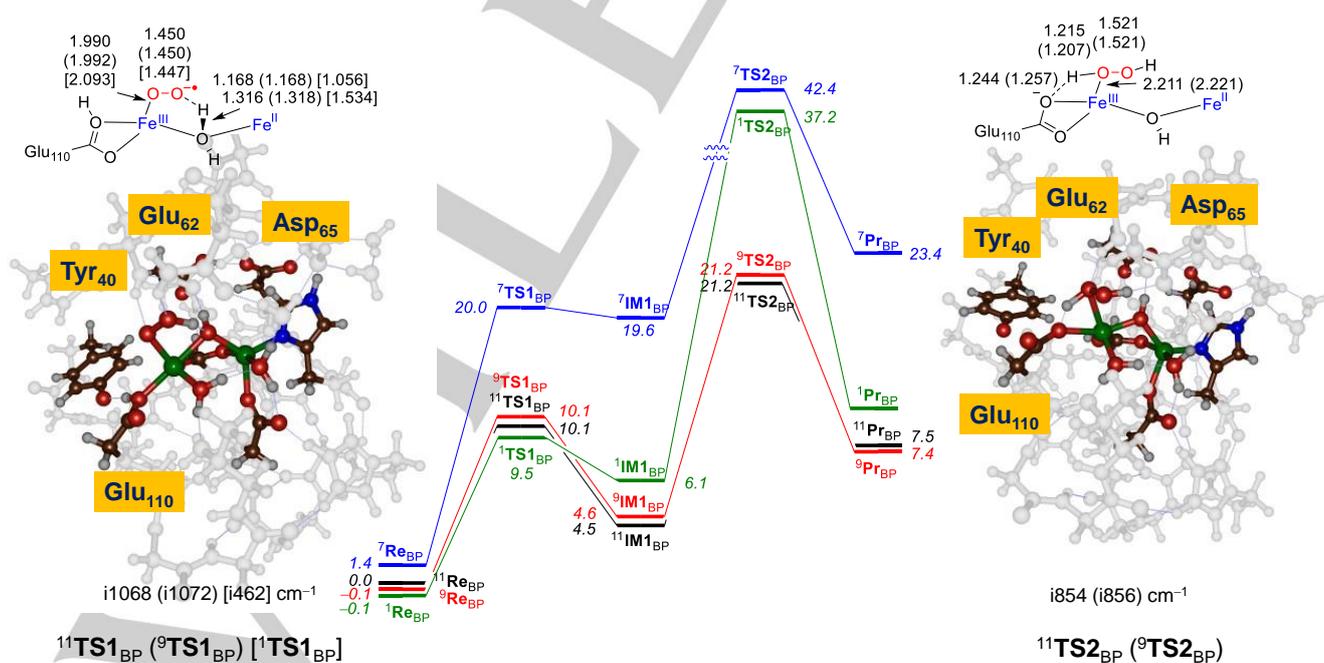
During the geometry optimization of  $\text{Re}_{\text{BP}}$  the proton from the phenol group of Tyr<sub>40</sub> spontaneously moved to Glu<sub>110</sub>. Consequently, Glu<sub>110</sub> in the ferritin environment is much more basic than Tyr<sub>40</sub> and accepts its proton rapidly. Attempts to swap molecular orbitals and create an electronic configuration without the Tyr<sub>40</sub> radical in  $\text{Re}_{\text{BP}}$  failed and did not converge. Hence, the lowest energy state has a radical on Tyr<sub>40</sub>. The molecular orbitals of the reactant complexes are shown in Figure S13, Supporting Information. The relevant molecular orbitals mainly originate from the metal 3d sets of orbitals on Fe1 and Fe2 and their interactions with first-coordination sphere ligands. Overall, all reactant complexes of model BP have an electronic configuration with two iron(II) centers and a radical on superoxo and Tyr<sub>40</sub>, i.e.  $\pi^*_{xy, \text{Fe}1}{}^2 \pi^*_{xz, \text{Fe}1}{}^1 \pi^*_{yz, \text{Fe}1}{}^1 \sigma^*_{z2, \text{Fe}1}{}^1 \sigma^*_{x2-y2, \text{Fe}1}{}^1 \pi^*_{xy, \text{Fe}2}{}^2 \pi^*_{xz, \text{Fe}2}{}^1 \pi^*_{yz, \text{Fe}2}{}^1 \sigma^*_{z2, \text{Fe}2}{}^1 \sigma^*_{x2-y2, \text{Fe}2}{}^1 \pi^*_{\text{OO}}{}^1 \pi^*_{\text{Tyr}1}$ . The eight unpaired electrons on Fe1/Fe2 are ferromagnetically coupled to the unpaired electrons on the superoxo and Tyr<sub>40</sub> units in  $^{11}\text{Re}_{\text{BP}}$ , while they are antiferromagnetically coupled in  $^7\text{Re}_{\text{BP}}$ . In  $^1\text{Re}_{\text{BP}}$  the electrons on Fe1 and superoxo are up-spin, whereas those on Fe2 and Tyr<sub>40</sub> are down-spin. Due to the same electron distribution in  $^1\text{Re}_{\text{BP}}$ ,  $^7\text{Re}_{\text{BP}}$ ,  $^9\text{Re}_{\text{BP}}$  and  $^{11}\text{Re}_{\text{BP}}$  these structures are close in energy and, indeed, we find them all within 1.5 kcal mol<sup>-1</sup>, with  $^1\text{Re}_{\text{BP}}$  lowest in energy. As a result equilibration between the four reactant complexes will be possible and all will have a finite lifetime. Moreover, reactivity patterns originating from all reactant complexes can be expected through multistate reactivity.<sup>[19]</sup> In all structures, the O–O distance is short at 1.420 Å in  $^{11}\text{Re}_{\text{BP}}$ ,  $^9\text{Re}_{\text{BP}}$  and  $^1\text{Re}_{\text{BP}}$  while it is 1.394 Å in  $^7\text{Re}_{\text{BP}}$ .

The mechanism of dioxygen conversion into H<sub>2</sub>O<sub>2</sub> on the diiron center of SynFtn for model BP is illustrated in Scheme 1 and Figure 4. First, proton transfer from the water molecule bridging the two iron ions to the terminal oxygen atom of the iron(II)-superoxo group occurs, along with electron transfer from the iron(II) to the superoxo group via a transition state  $\text{TS1}_{\text{BP}}$ . This forms intermediate  $\text{IM1}_{\text{BP}}$ , which features an iron(II)-iron(III)-hydroperoxo species. Barriers for the proton coupled electron transfer are  $\Delta E + \text{ZPE} = 10.1$  (10.1) kcal mol<sup>-1</sup> on the undecaplet (nonet) spin state surfaces, while the open-shell singlet spin state is at 9.5 kcal mol<sup>-1</sup>. Energetically these barriers are slightly lower than those for  $^{11,9}\text{TS1}_{\text{B}}$  reported in the Supporting Information Figure S16. The transition states are product-like with long H–OH distances on the bridging water of 1.316 (1.318) [1.534] Å for  $^{11}\text{TS1}_{\text{BP}}$  ( $^9\text{TS1}_{\text{BP}}$ ) [ $^1\text{TS1}_{\text{BP}}$ ], while the distance to the terminal oxygen of the superoxo is much shorter: 1.168 Å for both  $^{11,9}\text{TS1}_{\text{BP}}$  and 1.056 Å for  $^1\text{TS1}_{\text{BP}}$ . The nonet and undecaplet transition states also have relatively large imaginary frequencies,  $i1068$  and  $i1072$  cm<sup>-1</sup>, respectively, for the O–H–O stretch vibration between the peroxy group and the  $\mu$ -water ligand, while it is much smaller for the singlet spin structure.

After the transition states, the resulting iron(II)-iron(III)-hydroperoxo complex ( $\text{IM1}_{\text{BP}}$ ) retains the radical on Tyr<sub>40</sub> and a protonated Glu<sub>110</sub> group. The latter releases its proton to the distal oxygen of the iron(III)-hydroperoxo species via barriers of 21.2 kcal mol<sup>-1</sup> on the nonet and undecaplet spin states, while the septet and singlet are much higher lying. The  $^{9,11}\text{TS2}_{\text{BP}}$  structures are shown on the right-hand-side of Figure 4. They have imaginary frequencies of  $i854$  ( $i856$ ) cm<sup>-1</sup> for the undecaplet (nonet) spin states. Both  $\text{TS2}_{\text{BP}}$  structures are relatively central, i.e. neither early nor late, with the transferring hydrogen atom almost midway between the donor and acceptor oxygen atom, namely the distances for the donating O–H interaction is 1.244 (1.257) Å and the distances for the accepting O–H interaction is 1.215 (1.207) Å for  $^{11}\text{TS2}_{\text{BP}}$  ( $^9\text{TS2}_{\text{BP}}$ ).



**Scheme 1.** Lowest energy electronic configuration for each intermediate and electron transfer pathways for  $\text{H}_2\text{O}_2$  production from model  $\text{Re}_{\text{BP}}$ .



**Figure 4.** Potential energy profile ( $\Delta E_{\text{BS}2} + \text{ZPE}_{\text{BS}1}$  data in  $\text{kcal mol}^{-1}$ ) for dioxygen activation by ferritin models  $^{11,9,7,1}\text{Re}_{\text{BP}}$  as calculated in Gaussian. Optimized geometries of key transition states in the mechanism with bond lengths in angstroms and the imaginary frequency in  $\text{cm}^{-1}$ .

The Fe–O distances toward the H<sub>2</sub>O<sub>2</sub> group have elongated to values above 2.2 Å. Interestingly, the <sup>1</sup>TS<sub>2BP</sub> barrier is relatively high in energy and much higher than those on the undecaplet and nonet spin states. The same is true for the singlet spin product complex. This is surprising as the electronic configuration of the singlet, nonet and undecaplet products all have an iron(II)iron(III) complex coupled to a radical on Tyr<sub>40</sub>. However, in the nonet and undecaplet the two iron centers have all metal electrons in up-spin, while they are antiferromagnetically coupled in the singlet spin state. Moreover, in the singlet spin state the iron(II) center ferromagnetically couples with the tyrosinyl radical, while it is antiferromagnetic with the iron(III) center. Since, most metal-type orbitals are smeared out over the full diiron system this results in a higher singlet spin product complex than the higher-spin orientations. Several attempts were made to swap orbitals to find a lower energy singlet spin product state, but all converged to the same state shown in Figure 4.

Overall, the potential energy landscapes for dioxygen activation by **Re<sub>B</sub>** and **Re<sub>BP</sub>** show similar patterns with a small initial proton coupled electron transfer event with barriers of 9 – 11 kcal mol<sup>-1</sup>, followed by a larger second barrier of around 20 kcal mol<sup>-1</sup> for H<sub>2</sub>O<sub>2</sub> formation. The details of the electronic changes during the reaction mechanism of oxygen activation by the protonated Asp<sub>65</sub> model **Re<sub>BP</sub>** are given in Scheme 1. As discussed above, the singlet spin state reactant is the ground state with two antiferromagnetically coupled iron(II) centers coupled to a superoxo and a tyrosyl radical. Upon proton transfer from the μ-H<sub>2</sub>O group to superoxo a high-spin state is formed (<sup>11</sup>IM<sub>1BP</sub>) that is built up from an iron(III)-hydroperoxo group in sextet spin coupled to a quintet spin iron(II) ion and a tyrosyl radical. A final proton transfer from Glu<sub>110</sub> to iron(III)-hydroperoxo generates the H<sub>2</sub>O<sub>2</sub> product and leaves a mixed-valent Fe(III)-Fe(II) center with a nearby tyrosyl radical.

The mechanism described for model **Re<sub>BP</sub>** closely matches experimental data for *SynFtn*. Thus, Bradley et al.<sup>[12a]</sup> detected both a mixed valent iron(III)-iron(II) ferroxidase center and a tyrosyl radical during the catalytic cycle, consistent with our findings for model **Re<sub>BP</sub>** and its reaction mechanism. In conclusion, our work points to the mechanism initiating from **Re<sub>BP</sub>** as the one that best links to experimental work. Even though the mechanism and barriers for the unprotonated model **Re<sub>B</sub>** are similar to those for model **Re<sub>BP</sub>**, a radical on Tyr<sub>40</sub> is formed only at the final step leading to a mixed-valent intermediate and product structure. As such, we believe the Asp<sub>65</sub> group is either protonated or binds a cation and thereby guides the reaction to mechanism **Re<sub>BP</sub>**.

Interestingly, a recent study of a D65A variant of *SynFtn* revealed a significant, unexplained effect on the mechanism of iron(II) oxidation,<sup>[24]</sup> consistent with the results reported here suggesting an important functional role for this residue in determining the mechanistic path of iron oxidation/O<sub>2</sub> activation. A survey of ferritin sequences reveals that neither Glu<sub>62</sub> nor Ser<sub>146</sub> are conserved within H-chain peptides (Figure S1, Supporting Information). There is considerable variation in the residues at the position equivalent to 146 whilst that equivalent to position 62 is typically Gln, His or occasionally Ala. The residue equivalent to position 65 is invariably a Glu in the other proteins surveyed. Comparison between coordinate sets deposited in the PDB shows Asp<sub>65</sub> of *SynFtn* to adopt a conformation distinct from the Glu of other ferritins (Figure S1, Supporting Information). The results of our calculations therefore provide a rationale for the unique reactivity reported for *SynFtn* with other ferritins utilizing

an alternative pathway to O<sub>2</sub> activation due to the absence of a hydroxyl on residue 146 and a different electronic potential in the region of residues 62 and 65 of the *SynFtn* peptide chain.

We also explored possible reaction mechanisms that proceed via a μ-1,2-peroxo intermediate. In particular, for the deprotonated Asp<sub>65</sub> model we located its optimized geometry and found it higher in energy than the lowest reactant complex by ΔE+ZPE = 8.5 kcal mol<sup>-1</sup>. Moreover, its formation barrier from a diiron(II)-peroxo reactant complex was calculated to be well over 25 kcal mol<sup>-1</sup>. This barrier is far higher than the alternative proton coupled electron transfer to form the mixed valent iron(II)-iron(III)-hydroperoxo species. Therefore, the μ-1,2-peroxo diiron complex will not take part in the catalytic reaction mechanism of *SynFtn* and consequently is a side-product from an alternative higher energy pathway. Our studies match previous computational studies on benzoyl coenzyme A epoxidase,<sup>[23b]</sup> that also assigned the μ-1,2-peroxo diiron(III) complex as a side reaction channel of a higher energy pathway.

## Conclusion

A series of density functional theory calculations on the catalytic cycle of ferritin based on a model of *SynFtn* are reported using large cluster models of well over 300 atoms that contain the first- and second-coordination sphere around the diiron center. The calculations suggest that formation of the first experimentally observed reaction intermediate is preceded by several rapid electron and proton transfer events offering new insight into the mechanism of O<sub>2</sub> activation by ferritin enzymes specifically, and diiron proteins more broadly. Specifically our calculations show that rather than oxidation of Tyr<sub>40</sub> by an iron(II)-iron(III)-superoxo species, as was originally proposed,<sup>[10]</sup> *SynFtn* first forms a diiron(II)-superoxo species and a tyrosyl radical. The iron(II)-iron(III)-hydroperoxo form of the diiron site is then generated via subsequent facile electron and proton transfer processes.

The end point of our calculations here is an iron(II)-iron(III)-hydrogen peroxide complex and a Tyr radical. Experimental evidence indicates that the hydrogen peroxide readily dissociates from the ferroxidase center, and that the Tyr radical is rapidly quenched. According to the scheme of Bradley et al, the radical centered on Tyr<sub>40</sub> is translocated to a remote diiron(II) catalytic center, which has not encountered O<sub>2</sub>, by a series of electron transfer steps, resulting in an iron(III)-iron(II) ferroxidase center with no hydroperoxo group bound. In this way, the overall reaction of 24 diiron(II) ferroxidase centers with 12 molecules of O<sub>2</sub> results in 12 molecules of H<sub>2</sub>O<sub>2</sub> and 24 iron(III)-iron(II) ferroxidase centers.<sup>[12a]</sup>

The calculations shown in this work highlight a strong hydrogen-bonding network in the active site that positions the diiron center and dioxygen and enables facile proton transfer. In particular, geometry optimizations indicate that the alcohol group of Ser<sub>146</sub> stabilizes the superoxo species bound to Fe<sub>2</sub> via a hydrogen bonding interaction. We also propose that protonation of, or cation binding between, the carboxylates of Asp<sub>65</sub> and Glu<sub>62</sub> is key to determining *SynFtn* reactivity. With a proton/cation located on the Asp<sub>65</sub> group, a mechanism of O<sub>2</sub> activation is found in which the first proton is delivered by a bridging water molecule, while the second one is shuttled in from Tyr<sub>40</sub> via Glu<sub>110</sub>. The mechanism produces a mixed-valent iron(III)-iron(II) product complex and relies on the nearby Tyr<sub>40</sub> that forms a tyrosyl radical, in excellent

agreement with experimental observation. Overall our studies highlight the importance of second-coordination effects in the catalytic mechanism of nonheme diiron enzymes, where hydrogen bonding interactions position polar residues and assist with proton-relay mechanisms. In addition, long-range electrostatic interactions influence electronic configurations and spin-state stabilities that are essential for chemical catalysis.

## Experimental Section

### Model Set-Up.

We initially ran a 500 ns molecular dynamics (MD) simulation on the ferritin structure starting from the crystal structure coordinates (6GKA pdb file) and with hydrogen atoms added under pH 7 conditions as well as a water layer. The MD simulation (Supporting Information) shows that the structure is highly rigid throughout with a very stable fold and second-coordination environment. Based on these results, models of the active site of ferritin were created and validated as described previously and followed standard procedures for the set-up of cluster models.<sup>[26]</sup> The cluster models were created from the 6GKA pdb file as retrieved from the protein databank.<sup>[12a,18]</sup> The pdb file describes a monomer with both iron atoms included but without dioxygen. Hydrogen atoms were added in Chimera using pH 7 conditions,<sup>[22]</sup> which assumed all Arg and Lys side-chains were protonated and all Asp and Glu side-chains taken to be in their deprotonated forms. The protonation states of all acidic and basic residues were further confirmed with PropKa that also assigned the Glu<sub>62</sub> and Asp<sub>65</sub> residues to be deprotonated. However, upon visual inspection of the protein structure we decided to investigate models with Asp<sub>65</sub> in its deprotonated form (models **A**, **B**, **C**) and in its protonated form (model **BP**). His<sub>29</sub> was assumed to be doubly protonated, while all other histidine residues were singly protonated. Large active site cluster models were generated that incorporate the first-, second- and third-coordination sphere of the catalytic center and further studied with density functional theory methods. These models often describe short- and long-range electrostatic interactions well and are the method of choice for enzymatic reaction mechanisms.<sup>[26,27]</sup>

To gain experience with the chemical system, we first explored a small cluster model of 121 atoms (model **A**), see Supporting Information for details. Subsequently, we investigated more realistic models with a large number of second- and third-coordination sphere residues included: Model **B** with 340 atoms and Model **BP** with 341 atoms. Model **B** included the two iron atoms bridged by a water molecule and the carboxylate of Glu<sub>66</sub> which is part of the peptide chain Glu<sub>62</sub>-Ala<sub>63</sub>-Lys<sub>64</sub>-Asp<sub>65</sub>-Glu<sub>66</sub>-Gln<sub>67</sub>-Ser<sub>68</sub>-His<sub>69</sub>, whereby the Ala<sub>63</sub>, Lys<sub>64</sub>, Gln<sub>67</sub> and Ser<sub>68</sub> residues were truncated to Gly. Another peptide chain incorporated in the model covered the sequence from His<sub>29</sub> to Tyr<sub>40</sub>. Again, the amino acid side chains pointing away from the active site were truncated to Gly, i.e. Ile<sub>30</sub>, Ser<sub>31</sub>, Ile<sub>32</sub>, Arg<sub>34</sub>, Tyr<sub>35</sub>, Val<sub>38</sub> and Thr<sub>39</sub>. The Glu<sub>110</sub> ligand of one of the iron atoms was included as part of the short peptide chain Glu<sub>110</sub>-Ala<sub>111</sub>-Asp<sub>112</sub>-Thr<sub>113</sub> with the Ala and Asp residues shortened to Gly. A final peptide chain Gln<sub>143</sub>-Ile<sub>144</sub>-Gln<sub>145</sub>-Ser<sub>146</sub>, with the two middle residues without side chains donating hydrogen bonding interactions into the active site. Lastly, the model contains the side chain of Phe<sub>150</sub> as toluene and eleven water molecules. A dioxygen group was manually added to one of the iron(II) atoms (Fe2) in an end-on conformation. Our model is overall charge neutral and was investigated with odd spin multiplicity ( $M = 1, 3, 5, 7, 9, 11$ ). To test the reproducibility of the model, we expanded model **B** further by adding Ala<sub>70</sub>-Val<sub>71</sub>-His<sub>72</sub>-Phe<sub>73</sub>, Phe<sub>107</sub>-Gln<sub>108</sub>-Met<sub>109</sub> and Thr<sub>114</sub> with the side chains of Ala<sub>70</sub>, Val<sub>71</sub>, Phe<sub>107</sub> and, Gln<sub>108</sub> truncated to Gly to get model **C** of 439 atoms and minimized its geometry <sup>11</sup>Rec. The structure and electronic configuration of <sup>11</sup>Rec showed little difference to that of <sup>11</sup>Re<sub>B</sub>; hence the work was continued with model **B** and **BP** only.

Despite the fact that the Glu<sub>62</sub> and Asp<sub>65</sub> side chains are considered to have low pK<sub>a</sub> values, actually their local environment on visual inspection does not show a nearby positive counter charge. Therefore, we investigated a protonated model whereby a proton was added between the two carboxylate side chains of Glu<sub>62</sub> and Asp<sub>65</sub>: Re<sub>BP</sub>. The full mechanism of dioxygen activation on several spin states surfaces was also investigated for model Re<sub>BP</sub>. All models were run without geometric constraints and a comparison of optimized geometries with crystal structure coordinates only showed minor deviations.

### Procedures.

Density Functional Theory (DFT) calculations were performed on the ferritin models using the Gaussian-09 software package,<sup>[28]</sup> and utilized the unrestricted B3LYP hybrid density functional method.<sup>[29]</sup> Geometry optimizations, frequencies, intrinsic reaction coordinate scans and geometry scans were performed with an LANL2DZ basis set on iron with core potential and 6-31G on the rest of the atoms: basis set BS1.<sup>[30]</sup> To correct the energies, single point calculations on the optimized geometries were performed with the LACV3P+ basis set on iron with core potential and 6-311+G\* on the rest of the atoms: basis set BS2. All calculations were run with the continuum polarized conductor model (CPCM) included with a dielectric constant mimicking chlorobenzene.<sup>[31]</sup> For a number of structures, dispersion corrected DFT was tested, but resulted in energies very similar to those obtained without dispersion, in agreement with previous work.<sup>[32]</sup> The methods used for this work have been extensively tested for analogous systems and reproduce spin-state orderings, product distributions and free energies of activation well.<sup>[33]</sup>

Transition states were obtained through full geometry optimizations and were characterized with a single imaginary mode for the correct transition. For a selection of transition states we ran intrinsic reaction coordinate scans (IRCs) that confirmed the connection of the TS with the two local minima, see Supporting Information. Free energies were obtained from unscaled vibrational frequencies and entropies at 298 K. All energies reported in this work were obtained using UB3LYP/BS2 with solvent and zero-point corrections included.

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## Data sharing statement

All relevant data are presented in the main paper or in the Supporting Information, and are available from the authors upon reasonable request.

**Keywords:** Density functional theory • enzyme mechanism • enzyme catalysis • inorganic reaction mechanisms • oxygen activation

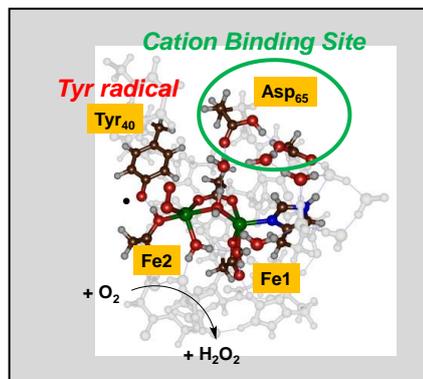
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## Entry for the Table of Contents

Table of Contents graphic:



**Insert text for Table of Contents here:** Density functional theory calculations on ferritin implicate novel features in the catalytic cycle for H<sub>2</sub>O<sub>2</sub> synthesis from dioxygen on a diiron center including a cation-binding site held by Glu<sub>62</sub> and Asp<sub>65</sub>. The reaction is stepwise with the involvement of an active site Tyr residue that delivers a hydrogen atom through proton-coupled-electron-transfer.

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