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A Water Dimer Shift Activates a Proton Pumping Pathway in the $P_R \rightarrow F$ Transition of ba_3 Cytochrome *c* Oxidase

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Abstract

Broken-symmetry density functional calculations have been performed on the $[Fe_{a3}^{4+}, Cu_B^{2+}]$ state of the dinuclear center (DNC) for the $P_R \rightarrow F$ part of the catalytic cycle of ba_3 cytochrome *c* oxidase (CcO) from *Thermus thermophilus* (*Tt*), using OLYP-D3-BJ functional. The calculations show that the movement of the H_2O molecules in the DNC affects the pK_a values of the residue side chains of Tyr237 and His376⁺, which are crucial for proton transfer/pumping in ba_3 CcO from *Tt*. The calculated lowest energy structure of the DNC in the $[Fe_{a3}^{4+}, Cu_B^{2+}]$ state (state **F**) is of the form $Fe_{a3}^{4+}=O^{2-}\cdots Cu_B^{2+}$, in which the H_2O ligand that resulted from protonation of the OH^- ligand in the P_R state is dissociated from the Cu_B^{2+} site. The calculated $Fe_{a3}^{4+}=O^{2-}$ distance in **F** (1.68 Å) is by 0.03 Å longer than that in P_R (1.65 Å), which can explain the different $Fe_{a3}^{4+}=O^{2-}$ stretching modes in **P** (804 cm^{-1}) and **F** (785 cm^{-1}) identified by resonance Raman experiments. In this **F** state, the $Cu_B^{2+}\cdots O^{2-}$ (ferryl-oxygen) distance is only around 2.4 Å. Hence the subsequent **O_H** state $[Fe_{a3}^{3+}-OH^--Cu_B^{2+}]$ with a μ -hydroxo bridge can be easily formed as shown by our calculations.

Graphical Abstract

The $P_R \rightarrow F$ process and further the state **O_H** beyond **F** have been calculated for the dinuclear center (DNC) of ba_3 cytochrome *c* oxidase. Calculations show that the movement of the H_2O molecules in the DNC affect the proton transfer/pumping process. State **F** is likely in the $Fe_{a3}^{4+}=O^{2-}\cdots Cu_B^{2+}$ form, in which the H_2O ligand dissociates from the Cu_B^{2+} site.

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Supporting Information

The detailed calculation method and the Cartesian coordinates of several optimized DNC clusters discussed in Tables 1–3 and Table S1 are given as supporting information.

1. Introduction

Cytochrome *c* oxidase (CcO) is the terminal enzyme in the respiratory electron transport chain located in the mitochondrial or bacteria membrane. It reduces O_2 to H_2O and pumps protons across the membrane to create the chemiosmotic proton gradient used by ATP synthase to synthesize ATP.^{1–4} The catalytic site of CcO which binds and reduces O_2 by $4e^-/4H^+$ transfer is called the dinuclear (or binuclear) center (DNC or BNC), which contains a heme a_3 (Fe_{a3}) and a Cu ion (Cu_B). Cu_B is structurally very close to Fe_{a3} ($\sim 5 \text{ \AA}$).^{5–15} Two other redox centers also exist in CcO's: a homodinuclear Cu dimer (Cu_A) which serves as the initial site of electron entry to CcO,^{16,17} and another heme, which is heme A (Fe_a) in the case of aa_3 type of CcO, or heme B (Fe_b) in ba_3 type of CcO.

The structures and residues of the DNC's in aa_3 and ba_3 types of CcO's are very similar.^{10–15} In general, The Fe_{a3} site has one histidine ligand (His384, residue numbers in this paper are by default for ba_3 CcO from *Thermus thermophilus* (*Tt*)) and Cu_B has three histidine ligands: His233, His282, and His283. His233 covalently links with the Tyr237 side chain. This linkage is common to all CcO's but otherwise unknown in metalloenzymes. This unique cross-linked tyrosine residue takes an important role in the processes of electron/proton transfer in CcO. The oxidation, spin, and ligation states of the Fe_{a3} and Cu_B sites change during the catalytic cycle. In the fully reduced and relaxed state (state **R**), both Fe_{a3}^{2+} and Cu_B^+ sites do not have an oxygen coordinating species. Then molecular O_2 binds with Fe_{a3}^{2+} and forms a formally $Fe_{a3}^{3+}-O_2^--\cdots Cu_B^+$ state,^{18–20} which is called state (intermediate) **A**.²¹ The next observed state in the cycle is called **P**. This state is, however, not a peroxide-containing compound (as implied in the notation), but one in which the dioxygen O-O bond has already been cleaved.^{22–26} Four electrons need to transfer to O_2 for the O-O bond cleavage. It is well established that, among the four electrons, two are from the Fe_{a3} site ($Fe_{a3}^{2+} \rightarrow Fe_{a3}^{4+}$) and one is from Cu_B ($Cu_B^+ \rightarrow Cu_B^{2+}$). Experiments starting from the mixed valence state ($Cu_A^{2+}, Fe_{a,b}^{3+}, Fe_{a3}^{2+}, Cu_B^+$)^{27–29} show that the 4th electron originates from the unique cross-linked tyrosine ($Tyr237^- \rightarrow Tyr237^{\bullet}$ radical).^{30,31} The **P** state obtained this way is called **P_M**, which can be represented as $[Fe_{a3}^{4+}=O^{2-}, OH^--Cu_B^{2+}, Tyr237^{\bullet}]$. Although no stable intermediate states between **A** and **P_M** are observed, it is generally believed that a bridging ferric-hydroperoxide state $[Fe_{a3}^{3+}-(O_2H)^--Cu_B^{2+}, Tyr237^-]$ has to be formed before the O-O bond cleavage, and the proton in the bridging OOH^- originates from the unique cross-linked tyrosine.^{2,32} Our recent broken-

symmetry^{33–35} density functional theory (DFT) calculations have shown that the O-O bond breaking energy barrier in the transition of $[\text{Fe}_{\text{a}3}^{3+}(\text{O}-\text{OH})^{-}\text{Cu}_{\text{B}}^{2+}, \text{Tyr237}^{-}] \rightarrow [\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{HO}^{-}\text{Cu}_{\text{B}}^{2+}, \text{Tyr237}^{\bullet}]$ is very small (less than 3.0/2.0 kcal mol⁻¹ in PW91-D3/OLYP-D3^{36–39} calculations).⁴⁰ These calculations have also shown that the His376 side chain, which is above propionate-A (Prop-A), favors the cationic protonated state His376⁺ when the DNC is in the $[\text{Fe}_{\text{a}3}^{3+}(\text{O}-\text{OH})^{-}\text{Cu}_{\text{B}}^{2+}, \text{Tyr237}^{-}]$ and $[\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{HO}^{-}\text{Cu}_{\text{B}}^{2+}, \text{Tyr237}^{\bullet}]$ states.⁴⁰ A histidine residue at the position of His376 is totally conserved in all B-type CcO's.⁴¹ Based on site-directed mutagenesis experiments on *ba*₃ CcO from *Tt*, His376, Prop-A, and the nearby water molecules play an important role in proton pumping.^{41,42} The mutants His376Asn and Asp372Ile (Asp 372 H-bonds with Prop-A) fail to pump protons.^{41,42} Our recent molecular dynamics simulations show that the imidazole group of His376 can easily rotate around the C_β-C_γ bond.⁴³ This function is important for His376 to take up protons from Prop-A and possibly to serve as the proton loading site (PLS).

When the Tyr237[•] in **P_M** receives an electron, or when the O₂ binding starts from the fully reduced enzyme, state **P_R** $[\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{HO}^{-}\text{Cu}_{\text{B}}^{2+}, \text{Tyr237}^{-}]$ is then formed.²⁹ The Fe⁴⁺=O²⁻ stretching mode of the **P_M** intermediate was identified at 804 cm⁻¹ by resonance Raman measurements.^{20,25,44–46} The optical spectrum of **P_R** was found similar or identical to the spectrum of the **P_M** intermediate.²⁹ Further, another intermediate state **F** was detected in *aa*₃ oxidase with the Fe⁴⁺=O²⁻ stretching mode at 785 cm⁻¹.^{25,45,47–50} **F** differs from **P_R** by an additional proton.^{2,29,51,52} The state **F**, however, is not populated to a significant extent in *ba*₃ oxidase at neutral pH because **F** is formed and decays (to the oxidized state **O_H**) over the same time scale.^{4,53} The observed EPR spectra for **P_R** and **F** are very different.²⁹ The magnetic coupling between the two ion centers in **F** was much larger than in **P_R**.²⁹ Hansson *et al.*²⁷ showed that, the copper hyperfine lines of the unique EPR signal of the **P_R** intermediate were broadened when ¹⁷O₂ was used as oxidant, and the broadening was consistent with bonding of one of the two oxygen atoms as an OH⁻ ligand of Cu_B. Morgan *et al.*²⁹ supported this proposal and further suggested that, in **F**, the copper OH⁻ ligand may have been protonated to water, allowing for strong hydrogen bonding and exchange coupling between the Fe_{a3} and Cu_B sites. Therefore, it is usually modeled that the Cu_B site has an OH⁻ ligand in **P_M**/**P_R**, and an H₂O ligand in **F**.^{51,54} Sharma *et al.* have proposed that the water ligand in **F** likely dissociates from the copper (they call the state **F_H**).⁵² However, the **F_H** state they obtained from their DFT calculations has higher energy than the prior state with the H₂O ligand on Cu_B.⁵² The shift of the Fe⁴⁺=O²⁻ stretching mode from **P_R** to **F** was also suggested due to the hydrogen bonding of the distal histidine ligand of Fe_{a3}.⁵⁵ However, forming 1 hydrogen bond to the N_δ-proton of the distal histidine has been shown to shift the Fe⁴⁺=O stretching mode by 6–8 cm⁻¹.^{46,55} The ~20 cm⁻¹ shift can only be obtained by forming 2 hydrogen bonds connected to the N_δ-proton of the distal histidine.⁴⁶ Further, the very similar Fe⁴⁺=O²⁻...HO⁻-Cu²⁺ DNC structures⁴⁶ with various distal histidine H-bonds may not explain the very different EPR spectra of the **P_R** and **F** intermediates.²⁹

In the current paper, starting from our DNC structure of state **P_M**,⁴⁰ which was geometry optimized building from the X-ray crystal structure of *ba*₃ CcO from *Tt* (pdb code: 3S8G, 1.7 Å resolution, in the radiolytically reduced state),¹⁵ we study feasible structural changes of the DNC from state **P_R** to **F**, as well as the protonation states of Tyr237 and His376. In the

X-ray crystal structure 3S8G, two water molecules are detected (HOH604 and HOH608 H-bonding with each other) close to the Cu_B site in the DNC (see Figure S1 in Supporting Information). We have included these two water molecules in the DNC models in our previous studies for the DNC structures,⁵⁶ ⁵⁷Fe_{a3}^{2+/3+} Mössbauer properties,⁵⁷ the reaction cycle,⁵⁸ and the O-O bond cleavage calculations.⁴⁰ In the geometry optimized structure of state P_M, HOH604 H-bonds to HOH608, which also has H-bonding interactions with both the carbonyl of Gly232 and the OH⁻ ligand of Cu_B (see Figure 1.C). It is not certain in the real catalytic cycle when these two water molecules enter the DNC and when and how they exit the DNC. Our recent molecular dynamics simulation studies on the water exit pathways in *Tt* ba₃ CcO have shown that the extensive H-bonding network found in the water exit pathways can be also strong candidates for proton exit pathways.⁴³ Here we will study how shifting these water molecules (HOH604, HOH608 and the H₂O ligand of Cu_B) affects the calculated p*K*_a's of Tyr237 and His376⁺ side chains, and consequently affects the proton uptake and proton pumping in CcO.

2. [Fea₃⁴⁺, Cu_B²⁺] DNC Models, Results and Discussion

The detailed method for calculations is given in the Supporting Information. Briefly, all calculations are performed using the Amsterdam Density Functional Package (ADF2016.104).^{59–61} All DNC model clusters are geometry optimized in the broken-symmetry state,^{33–35} in which the Fe_{a3}⁴⁺ site is in intermediate spin state (*S* = 1) antiferromagnetically (AF) coupling with the Cu_B²⁺ (*S* = 1/2) site, using the OLYP functional plus Grimme's latest dispersion corrections (D3-BJ)⁶² within the conductor like screening (COSMO) solvation model.^{63–66} The triple- ζ plus polarization (TZP) Slater-type basis set is applied to the Fe and Cu atoms and double- ζ plus polarization (DZP) basis set to other atoms. The inner cores of C(1s), N(1s), O(1s), Fe(1s,2s,2p), and Cu(1s,2s,2p) are treated by frozen core approximation.

Three [Fe_{a3}⁴⁺, Cu_B²⁺] DNC Models (Models 1–3) are studied here (see Figures 1–3 and their captions). The models differ in the different locations of selected water molecules (HOH604, HOH608, and the H₂O formed from the OH⁻ ligand of Cu_B). In each model, different protonation states of the Tyr237 and His376 side chains are studied and the p*K*_a's are calculated. In Models 1 and 2, whether the Cu_B²⁺ site energetically favors an OH⁻ or an H₂O as a ligand is also studied and the p*K*_a of the H₂O ligand is calculated. Our models contain 204–207 atoms, depending on the protonation state of His376, Tyr237, and the presence of OH⁻/H₂O ligand to Cu_B. In addition, a model in which two additional water molecules are added into Model 1 is also studied in section 2.2 in order to investigate the reasons for the p*K*_a change of His376⁺ from Model 1 to Model 2.

2.1. Model 1: HOH604 and HOH608 Close to the Fe_{a3}⁴⁺ and Cu_B²⁺ Sites

The whole DNC structure of Model 1 is shown in Figure 1.A. For clarity, the top portion of the cluster is shown in Figure 1.B, which includes Prop-A and Prop-D of heme-a₃, the side chains of Arg449, His376⁺ (or His376, if the proton on the top N_ε atom is gone), Asp372 and His283, and five water molecules observed in the X-ray crystal structure 3S8G.¹⁵ The two water molecules HOH604 and HOH608 are also taken from 3S8G, and they are in

positions just above the OH⁻/H₂O ligand (the oxygen atom is labeled as O2) of Cu_B. The detailed structure and the H-bonding interactions of Fe_{a3}⁴⁺=O²⁻ (labeled as O1), OH⁻/H₂O-Cu_B²⁺, HOH608, HOH604, and Gly232 are shown in Figures 1.C and 1.D. The lower part of Figure 1.A shows the unique covalent linkage between His233 and Tyr237.

In our previous calculations,⁴⁰ the **P_M** state is obtained in the structural form of Fe_{a3}⁴⁺=O²⁻...HO⁻-Cu_B²⁺-Y237[•]-H376⁺ with Tyr237[•] radical and cationic protonated His376⁺. The initial **P_R** state is then in the form of Fe_{a3}⁴⁺=O²⁻...HO⁻-Cu_B²⁺-Y237⁻-H376⁺ with an electron added to give Tyr237⁻. The following steps can include the Tyr237⁻ receiving a proton (from the K-path in *Tt ba3*) and further transferring the proton to the OH⁻ ligand of Cu_B. In this part of the reaction cycle, proton pumping has been observed in *aa3 CcO*.^{4,67} In *ba3*, however, proton pumping is proposed to occur only after protonation of HO⁻-Cu_B²⁺.⁴ Further, the *ba3*-type pump efficiency may be underestimated due to difficulties with the measuring technique together with a higher sensitivity of the B-type enzyme to the protonmotive force that opposes pumping.⁶⁸

In order to understand the proton uptake/pumping steps, we calculate the p*K_a*'s of His376⁺, Tyr237, and the H₂O ligand with the energies obtained by optimizing the geometry of the DNC cluster with the combination of different protonation states for His376/His376⁺, Tyr237/Tyr237⁻, and OH⁻/H₂O ligand of Cu_B (see Supporting Information for p*K_a* calculations). When Tyr237 is in the anionic deprotonated Tyr237⁻ state, the proton on the heme-a₃ farnesyl hydroxyl group is rotated to have H-bonding interaction with the Tyr237⁻ side chain. The main calculated geometric, energetic, p*K_a*, and Mulliken net spin population properties of seven optimized DNC clusters of Model 1 are given in Table 1.

Because the Mulliken unpaired spin population is measured in unpaired electron spin units, in the ideal ionic limit, the net unpaired spin population for an intermediate-spin Fe⁴⁺ site is 2 (i.e. *S* = 1). The calculated net-spins on Fe_{a3}⁴⁺ in Table 1 are all smaller than 2. Also, the sum of the net-spins on Fe_{a3}⁴⁺ (1.28–1.38) and O1 (0.66–0.77) for each DNC cluster is about 2, indicative of substantial Fe_{a3}⁴⁺=O²⁻ covalency. The negative sign of the net spins on the Cu_B site indicate that the spins of the Fe_{a3} and Cu_B sites are AF-coupled.

The calculated p*K_a*'s of Tyr237 (13.4) and His376⁺ (9.2) in Fe_{a3}⁴⁺=O²⁻...HO⁻-Cu_B²⁺-Y237-H376⁺-M1 (M1 represents Model 1) show that, when the Tyr237[•] radical in state **P_M** receives an electron to form the **P_R** state, the Tyr237⁻ side chain will also uptake a proton, and meanwhile, the His376⁺ side chain remains in the cationic protonated state (no proton pumping). Next, the very high calculated p*K_a* (15.4) of the H₂O ligand in Fe_{a3}⁴⁺=O²⁻...H₂O-Cu_B²⁺-Y237⁻-H376⁺-M1 shows that, the proton on Tyr237 (p*K_a* = 13.4) in state Fe_{a3}⁴⁺=O²⁻...HO⁻-Cu_B²⁺-Y237-H376⁺-M1 would preferentially transfer to the HO⁻ ligand. From Fe_{a3}⁴⁺=O²⁻...HO⁻-Cu_B²⁺-Y237-H376⁺-M1 to Fe_{a3}⁴⁺=O²⁻...H₂O-Cu_B²⁺-Y237⁻-H376⁺-M1, the net spin on Cu_B is decreased (from 0.5 to 0.4), and the net spin on the Tyr237 side chain is increased (from 0 to 0.3 with ~1/3 of that spin on the O atom). Therefore, in Fe_{a3}⁴⁺=O²⁻...H₂O-Cu_B²⁺-Y237⁻-H376⁺-M1, the Cu_B and Tyr237 are in a mixed valence state between Cu_B²⁺-Tyr237⁻ and Cu_B⁺-Tyr237[•]. Finally, the calculated p*K_a*'s of the H₂O ligand (12.8), neutral Tyr237 (11.0) and His376⁺ (12.2) indicate that at this step the Tyr237⁻ would further take up a proton from the K-path, which would produce the

F state in the form of $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}-\text{Y237}-\text{H376}^+-\text{M1}$. However, early Fourier transform infrared (FTIR) spectroscopy experiments⁶⁹ indicated that the cross-linked tyrosine side chain is deprotonated in state **F**, which implicates a more complicated pathway.

2.2. Model 2: HOH604 and HOH608 Shifted up

To see whether the two water molecules HOH604 and HOH608 would shift up in the DNC during the **P_R** → **F** transition, and how the $\text{p}K_{\text{a}}$'s of Tyr237 and His376⁺ will be affected by the movement of these two water molecules, in Model 2, we have moved HOH604 and HOH608 to the upper region of the DNC (upper water cluster) to H-bond with HOH607 and His376/His376⁺. See the H-bonding patterns around HOH604 and HOH608 of Model 2 with His376 and His376⁺ in Figures 2.A and 2.B, respectively. In this way, Model 2 has the same number of waters as Model 1, so that the calculated energies can be compared directly between Models 1 and 2. The comparable calculated results for Model 2 in $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{HO}^--\text{Cu}_{\text{B}}^{2+}$ and $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}$ states with different protonation forms of Tyr237/Tyr237[−] and His376/His376⁺ are given in Table 2. To follow the complex issues presented below, the reader can refer also to Figures 3, 4, and 5.

After Tyr237[•] radical in state **P_M** receives an electron, the $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{HO}^--\text{Cu}_{\text{B}}^{2+}-\text{Y237}^--\text{H376}^+$ state of Model 1 is 3.8 kcal mol^{−1} lower in energy than the corresponding state in Model 2 (Figure 5). Further, when Tyr237[−] receives a proton, the $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{HO}^--\text{Cu}_{\text{B}}^{2+}-\text{Y237}-\text{H376}^+$ state of Model 1 remains lower in energy than Model 2 by 7 kcal mol^{−1}. Therefore, HOH604 and HOH608 directly above the OH[−] ligand stabilize the $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{HO}^--\text{Cu}_{\text{B}}^{2+}$ state (Figure 1). However, after the OH[−] ligand becomes H₂O, our model calculations predict that HOH604 and HOH608 will shift to the upper water cluster of the DNC, since the $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}-\text{Y237}^--\text{H376}^+-\text{M2}$ (M2 stands for Model 2) state is significantly lower in energy than $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}-\text{Y237}^--\text{H376}^+-\text{M1}$. There are two different reaction pathways resulting in this Model 2 state, as discussed later.

A major change of the calculated $\text{p}K_{\text{a}}$'s of Tyr237 and His376⁺ comparing Model 1 to Model 2 happens in the $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}-\text{Y237}-\text{H376}^+$ state. In Model 1, the calculated $\text{p}K_{\text{a}}$'s of Tyr237 and His376⁺ in this state are 11.0 and 12.2, which indicate that, after Tyr237 transfers the proton to OH[−] on Cu_B (Figure 4, Panels b → c), it would receive a proton from the K-path and both Tyr237 and His376⁺ would stay protonated. However, after the water shift generating Model 2, the calculated $\text{p}K_{\text{a}}$'s of Tyr237 and His376⁺ for the corresponding state decrease to 5.5 and 6.5, respectively. In order to be sure that the $\text{p}K_{\text{a}}$ change of His376⁺ from Model 1 to Model 2 is related to the two water molecules shifting up to the top of the DNC and is not just a consequence of the addition of the H-bonding interactions to His376 in Model 2, we added two additional water molecules to Model 1 (M1+2H₂O) at the positions of HOH604 and HOH608 in Model 2 (Figures 2A and 2B), and geometry optimized the following two states: $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}-\text{Y237}-\text{H376}^-(\text{M1}+2\text{H}_2\text{O})$ and $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}-\text{Y237}-\text{H376}^+(\text{M1}+2\text{H}_2\text{O})$. The DNC structures of these two clusters are very similar to the corresponding states of Model 1 and are given in the Supporting Information (Table S1). The calculated $\text{p}K_{\text{a}}$ of the side chain of His376⁺ in $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}-\text{Y237}-\text{H376}^-(\text{M1}+2\text{H}_2\text{O})$ is 10.7, which is 1.5 pH unit lower than that (12.2) in $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}-\text{Y237}-\text{H376}^+-\text{M1}$, but this $\text{p}K_{\text{a}}$ is also 4.2 pH unit

higher than the corresponding value (6.5) in $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}-\text{Y237}^--\text{H376}^+-\text{M2}$. Therefore, the decrease of the calculated $\text{p}K_{\text{a}}$ ($12.2 \rightarrow 6.5$) of His376^+ from Model 1 to Model 2 is mainly caused by the two water molecules (HOH604 and HOH608) shifting away from $\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}$ to the upper water cluster of the DNC, which descreens the local $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}$ charge distribution.

More globally, Figure 4 shows that there is a branchpoint at Panel c ($\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}-\text{Y237}^--\text{H376}^+-\text{M1}$). One branched pathway proceeds from $c \rightarrow d \rightarrow e \rightarrow f$, while the other proceeds directly from $c \rightarrow f$, so both pathways merge at f. We will analyze these alternative pathways in more detail in Section 2.4. In the next section, we will follow the reaction path from Panel f onward.

2.3. Model 2 $\rightarrow$ Model 3 Transition: H_2O ligand Dissociates from $\text{Cu}_{\text{B}}^{2+}$

Note that the state $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}-\text{Y237}^--\text{H376}^+-\text{M2}$ (Figure 4, Panel f) has more $\text{Cu}_{\text{B}}^{2+}-\text{Tyr237}^*$ character⁵² and much longer $\text{Cu}_{\text{B}}-\text{O2}$ distance (2.26 Å) than the corresponding Model 1 (2.10 Å) state, it is highly likely that the Cu_{B} -bound H_2O ligand will dissociate from Cu_{B} as Sharma *et al.* suggested (Figure 4, Panel f $\rightarrow$ g).⁵² We then moved the H_2O ligand away from the Cu_{B} site, and optimized the cluster with this H_2O molecule in different places. In the lowest-energy structure we obtained, the H_2O molecule still H-bonds to the O^{2-} , and the two metal sites move closer to each other with the $\text{Fe}\cdots\text{Cu}_{\text{B}}$ distance 3.8 Å and the $\text{O1}\cdots\text{Cu}_{\text{B}}$ distance only 2.4 Å. We then use $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{Cu}_{\text{B}}^{2+}$ to represent this model and call it Model 3. The major DNC components of Model 3 are shown in Figure 3. The relative positions of HOH604 and HOH608 in Model 3 are the same as in model 2 (Figures 2.A and 2.B). The calculated results for Model 3 with different protonation forms of $\text{Tyr273}/\text{Tyr237}^-$ and $\text{His376}/\text{His376}^+$ are given in Table 3.

Since Model 3 does not have states in which Cu_{B} has the OH^- ligand, the four states in Table 3 correspond more closely to the last four states in Tables 1 and 2. Dissociation of the H_2O ligand from the Cu_{B} site in state $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}-\text{Y237}^--\text{H376}^+-\text{M2}$ (Table 2, earlier form of **F**) leads to formation of $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{Cu}_{\text{B}}^{2+}-\text{Y237}^--\text{H376}^+-\text{M3}$ (state **F**), which is 6 kcal mol⁻¹ lower in energy. Although Sharma *et al.* suggested that the H_2O ligand may well dissociate from the Cu_{B} site in state **F**, in their model calculations, the H_2O -dissociated **F** state (called **F_H** in their paper) is 5 kcal mol⁻¹ higher in energy than its corresponding H_2O -bound structure.⁵² By contrast, our current calculations support the proposal that the H_2O ligand dissociates from the Cu_{B} site in state **F** based on the energy change. The Tyr237 side chain remains in the deprotonated Tyr237^- form, since now even when His376 is in the neutral state, Tyr237 has a small calculated $\text{p}K_{\text{a}}$ (4.0), and when His376 is in its protonated His376^+ form, the $\text{p}K_{\text{a}}$ of Tyr237 is even smaller (1.8). Further, regardless of the protonation state of Tyr237 , His376 will stay in the cationic His376^+ form, since its calculated $\text{p}K_{\text{a}}$'s are all above 10 (see Table 3). In short, starting from state $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}-\text{Y237}^--\text{H376}^+-\text{M2}$, when the H_2O ligand dissociates from Cu_{B} and moves to the position shown in Figure 3, the protonation states of Tyr237^- and His376^+ side chains will remain unchanged.

Note that in $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{Cu}_{\text{B}}^{2+}\text{-Y237}^{-}\text{-H376}^{+}\text{-M3 (F)}$, the O^{2-} (labeled as O1) is only 2.4 Å away from Cu_{B} ; therefore, the O^{2-} weakly binds with Cu_{B} . As a result, in the transition from $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O-Cu}_{\text{B}}^{2+}\text{-Y237}^{-}\text{-H376}^{+}\text{-M2}$ to $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{Cu}_{\text{B}}^{2+}\text{-Y237}^{-}\text{-H376}^{+}\text{-M3}$, the binding between $\text{Fe}_{\text{a}3}^{4+}$ and O^{2-} is weakened and the Fe-O1 distance is elongated from 1.66 Å to 1.68 Å. In the initial P_{R} state where OH^{-} binds with Cu_{B} ($\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{HO}^{-}\text{-Cu}_{\text{B}}^{2+}\text{-Y237}^{-}\text{-H376}^{+}\text{-M1/M2}$), the Fe-O1 distance is even shorter (1.65 Å). The elongation of the Fe-O1 distance from $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{HO}^{-}\text{-Cu}_{\text{B}}^{2+}\text{-Y237}^{-}\text{-H376}^{+}\text{-M1/M2}$ (P_{R}) to $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{Cu}_{\text{B}}^{2+}\text{-Y237}^{-}\text{-H376}^{+}\text{-M3 (F)}$ is consistent with the frequency shift from resonance Raman measurements, in which the $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}$ stretching mode was identified at 804 cm^{-1} for state $\text{P}_{\text{M/R}}$ and at 785 cm^{-1} for state F .^{44,47-50} Therefore it is likely that our $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{Cu}_{\text{B}}^{2+}\text{-Y237}^{-}\text{-H376}^{+}\text{-M3}$ structure (Figure 3) represents state F of the DNC. The deprotonated state of Tyr237⁻ in $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{Cu}_{\text{B}}^{2+}\text{-Y237}^{-}\text{-H376}^{+}\text{-M3}$ is also in agreement with FTIR data⁶⁹ which indicated that the cross-linked tyrosine side chain is deprotonated in state F . Further, the different DNC structures of P_{R} and F states presented here may also explain the very different EPR spectra observed in experiment.²⁹ It has been suggested that the EPR spectrum of the P_{R} intermediate is due to a coupled $\text{Fe}^{4+}=\text{O}^{2-}\cdots\text{HO}^{-}\text{-Cu}^{2+}$ spin systems,^{27,29} which is consistent with our current model for P_{R} . The magnetic coupling between the two centers in F was found much larger than in P_{R} , which is consistent with our calculated much shorter $\text{Fe}_{\text{a}3}^{4+}\cdots\text{Cu}_{\text{B}}^{2+}$ distance in F (3.8 Å) than in P_{R} (4.3 Å).

2.4. $\text{P}_{\text{R}} \rightarrow \text{F}$ Transition and Proton Pumping

Based on the above calculations of Models 1–3, the $\text{P}_{\text{R}} \rightarrow \text{F}$ transition of the DNC is summarized in Figure 4. The $2\text{H}_2\text{O}$ in each state of this figure represent HOH604 and HOH608 starting from the X-ray crystal structure 3S8G (see Figures 1 and 2). Our calculations show that the shift in positions of these two water molecules affect the pK_{a} 's of the Tyr237 and His376⁺ side chains. The changes of protonation state $\text{Tyr237-O}^{-} \rightarrow \text{Tyr237-OH}$ and $\text{His376-H}^{+} \rightarrow \text{His376}$ can be related to proton uptake and proton pumping. Depending on the sequence and branch along the reaction path ($\text{P}_{\text{R}} \rightarrow \text{F}$ or $\text{a} \rightarrow \text{g}$) where the $2\text{H}_2\text{O}$ molecules shift up within the DNC, there can be $2\text{H}^{+}\text{-uptake(a} \rightarrow \text{b,c} \rightarrow \text{d)}/1\text{H}^{+}\text{-pumping(d} \rightarrow \text{e)}$ in the path $\text{a} \rightarrow \text{b} \rightarrow \text{c} \rightarrow \text{d} \rightarrow \text{e} \rightarrow \text{f} \rightarrow \text{g}$ or alternatively $1\text{H}^{+}\text{-uptake(a} \rightarrow \text{b)}/\text{zero-H}^{+}\text{-pumping in a} \rightarrow \text{b} \rightarrow \text{c} \rightarrow \text{f} \rightarrow \text{g}$. The calculated G (kcal mol⁻¹) change at pH=7 [$G = 1.37(\text{pK}_{\text{a}} - 7)$] between two different protonation states as well as the energy difference between different tautomers are also given in the figure. And the energies are corrected with the zero point energy (ZPE) differences (see the Supporting Information).

The relative fluxes through the two different reaction pathway branches are important factors in determining the efficiency of proton pumping in the $\text{P}_{\text{R}} \rightarrow \text{F}$ transition. The protonation of Tyr237, which is strongly favored (Figure 4, Panels c $\rightarrow$ d) ($G = -5.5\text{ kcal mol}^{-1}$) within Model 1, leads after a shift of $2\text{H}_2\text{O}$ (endergonic by $G = +4.2\text{ kcal mol}^{-1}$) to a large change in pK_{a} for His376⁺ from 12.2 to 6.5 (see the last rows of Tables 1 and 2). The G for proton pumping at pH = 7 becomes $G = -0.7\text{ kcal mol}^{-1}$ after the $2\text{H}_2\text{O}$ shift, compared to $G = +7.1\text{ kcal mol}^{-1}$ before the $2\text{H}_2\text{O}$ shift. The combination of the $2\text{H}_2\text{O}$ shift and proton pumping is then only moderately endergonic ($G = +3.5\text{ kcal mol}^{-1}$, Panels d $\rightarrow$ e). The following tautomeric proton shift from Tyr237 to neutral His376 (Panels e $\rightarrow$ f) is also

exergonic by about $-1.4 \text{ kcal mol}^{-1}$. Further calculations and analysis of reaction path barriers using quantum chemistry and molecular dynamics will be needed to properly compare the relative rates for $c \rightarrow d \rightarrow e \rightarrow f$ versus the other branch $c \rightarrow f$ without proton pumping. At $\text{pH} = 7$, both branches have the same net $G = -3.4 \text{ kcal mol}^{-1}$.

There is also a 3rd reaction path branch to consider starting at Figure 4, Panel d. After the $2\text{H}_2\text{O}$ shift, the $\text{p}K_a$ of Tyr237 is lowered from 12.8 to 5.5, so there is a possible loss of the Tyr237 proton. The proton on Tyr237 was originally delivered by the ba_3 K-path.⁷⁰ This pathway contains a hydrogen bonded sequence of protein residue side chains and waters that can deliver a single proton to Tyr237. This set of side chains starts with Glu15B and includes the important side chain of Tyr244 partway up the chain (see Figure 1 in Ref. ⁷⁰). According to the electrostatics and MD calculations,⁷⁰ after a proton is passed from Glu15B $\rightarrow$ Tyr244 $\rightarrow$ Tyr237, the deprotonated Tyr244⁻ shifts position which breaks the H-bonded pathway from Glu15B to Tyr237. This process then acts to block proton backflow from Tyr237 to Glu15B, and should be effective even at the low $\text{p}K_a$ for Tyr237. Also, there is no apparent proton acceptor in the DNC cluster until His376⁺ loses its proton, so deprotonation of Tyr237 should be inhibited at $d \rightarrow e$ (Figure 4), occurring only later at $e \rightarrow f$. The final step $f \rightarrow g$ involves the water ligand to Cu_B dissociation analyzed in Section 2.3.

Figure 5 displays the comparative energies for a sequence of Model 2 states with Model 1 analogs. The main point is that Model 1 states are consistently lower in energy than the analogous Model 2 states for the $^-\text{HO-Cu}_B^{2+}$ type metal site (+3.8 and +7.0 kcal mol^{-1} energy differences between Model 2 and Model 1). Therefore, the water dimer shift can occur only after protonation of the hydroxyl on Cu_B^{2+} to give $\text{H}_2\text{O-Cu}_B^{2+}$ (Panel f, $-3.4 \text{ kcal mol}^{-1}$).

2.5. The Oxidized State beyond F — State O_H

Although it is not the principal focus of our current study, a very important activated oxidized $\text{Fe}_{a3}^{3+} \cdots \text{Cu}_B^{2+}$ state beyond state **F**, which is called **O_H**, has been identified with a high-spin Fe_{a3}^{3+} and with the $\text{Fe}_{a3}^{3+}\text{-OH}^-$ stretching mode obtained in aa_3 CcO at 450 cm^{-1} .⁴⁸ Such a stretching frequency is very low with respect to other hydroxide bound heme species.⁵⁴ Therefore, it has been suggested that there is a strong H-bond to the oxygen atom of the heme-bound hydroxide, thereby weakening the Fe-O bond and generating a high-spin configuration.⁵⁴ However, a feasible alternative is the **O_H** state which has a bridging OH^- in the $\text{Fe}_{a3}^{3+}\text{-OH}^-\text{-Cu}_B^{2+}$ form, as shown in both Figures 4 and 5. Since the O^{2-} ligand already bridges the two metal sites in **F**, the **O_H** state with bridging OH^- is readily formed upon one electron reduction and proton transfer. Our recent Mössbauer calculations (geometry optimized the DNC of the ba_3 1EHK¹⁰ X-ray crystal structure using OLYP functional)⁵⁷ have shown that such a DNC structure $\text{Fe}_{a3}^{3+,\text{HS}}\text{-OH}^-\text{-Cu}^{2+}\text{-Y237}^-$, in which the high-spin Fe_{a3}^{3+} and the Cu_B^{2+} sites are AF-coupled, yields very similar Mössbauer properties ($\delta = 0.38 \text{ mm s}^{-1}$, $E_Q = 0.51 \text{ mm s}^{-1}$) to the experimentally observed $^{57}\text{Fe}_{a3}^{3+}$ high-spin species with ($\delta = 0.41 \text{ mm s}^{-1}$, $E_Q = 0.7 \text{ mm s}^{-1}$) in *Tt* ba_3 in the temperature range of 4.2 – 245 K.⁷¹ Now, starting from the structure of $\text{Fe}_{a3}^{4+}=\text{O}^{2-} \cdots \text{Cu}_B^{2+}\text{-Y237}^-\text{-H376}^+\text{-M3}$ (**F**), supposing an electron is transferred to the Fe_{a3}^{4+} site and a proton is taken up from the K-path and is transferred to the O^{2-} , we then performed geometry optimization with high-spin

$\text{Fe}_{\text{a}3}^{3+}$ AF-coupled to $\text{Cu}_{\text{B}}^{2+}$ and obtained the $\text{Fe}_{\text{a}3}^{3+}\text{-OH}^{-}\text{-Cu}_{\text{B}}^{2+}\text{-Y237}^{-}\text{-H376}^{+}$ state. During the geometry optimization, the $\text{Fe}_{\text{a}3}^{3+}$ and the $\text{Cu}_{\text{B}}^{2+}$ sites move closer to each other with the bridging hydroxide (see Figure 6B), and the water molecule HOH610 also move closer to HOH608 (see Figure 6A and Figure 2B) and form an H-bonding interaction. Meanwhile, since the position of the H_{link} atom (replacing C_{α}) on the His384 is fixed, the Fe-N distance between Fe and His384 is increased (to 2.43 Å). We have given the calculated properties of this structure (as $\mathbf{O}_{\text{H}}$) at the bottom of Table 3. The G^0 from state $\mathbf{F}$ to state $\mathbf{O}_{\text{H}}$ (in Figures 4 and 5) is estimated at $-14.6 \text{ kcal mol}^{-1}$, which corresponds to the redox potential $E^0 = +0.63 \text{ eV}$ with respect to the standard hydrogen electrode. This is equivalent to $G = -9.5 \text{ kcal mol}^{-1}$ with respect to a typical cytochrome *c* redox potential of $E^0 = +0.22 \text{ eV}$. The procedure for calculating the G^0 is given in the Supporting Information. It is worth noting that in our $\mathbf{O}_{\text{H}}$ state geometry optimization trajectory, there is a local minimum which is 5 kcal mol^{-1} higher in energy than the lowest-energy structure we obtained. The $\text{Fe}_{\text{a}3}^{3+}\text{-OH}^{-}\text{-Cu}_{\text{B}}^{2+}$ DNC geometry of that local-minimum structure is very similar to the lowest-energy structure, however, the water molecule HOH610 in the local-minimum structure does not have H-bonding interaction with HOH608 (instead relative positions are similar as in Figure 2B). Therefore, the rearrangement of the water molecules and the formation of an extra H-bonding interaction lowers the energy of the $\mathbf{O}_{\text{H}}$ state by $\sim 5 \text{ kcal mol}^{-1}$.

3. Conclusions

A specific sequence of transitions between intermediates is required to activate a proton pumping pathway during the $\mathbf{P}_{\text{R}} \rightarrow \mathbf{F}$ part of the catalytic cycle of *ba*₃ cytochrome *c* oxidase. Broken-symmetry DFT calculations on a large active site model of the dinuclear complex (DNC) show that the following events are essential: 1) Proton uptake by Tyr237[−] and subsequent proton transfer to generate H_2O bound to $\text{Cu}_{\text{B}}^{2+}$ from $\text{OH}^{-}\text{-Cu}_{\text{B}}^{2+}$; 2) A second proton uptake from the K-pathway to regenerate neutral protonated Tyr237. Both of these steps are strongly exergonic from the DFT model calculations; 3) A water dimer ($2\text{H}_2\text{O}$) shift away from the $\text{H}_2\text{O-Cu}_{\text{B}}^{2+}$ and into the vicinity of His376- H^{+} completes the sequence of steps needed to strongly lower the $\text{p}K_{\text{a}}$ of His376- H^{+} by about $\text{p}K_{\text{a}} = 5.7 \text{ pH}$ units (approximate change in $G = -7.8 \text{ kcal mol}^{-1}$) for deprotonation of His376- H^{+} . In earlier work, we have addressed the probable proton exit pathways from His376 toward the outside (p side) of the membrane. The $2\text{H}_2\text{O}$ shift is itself endergonic (approximate $G = +4.2 \text{ kcal mol}^{-1}$). It activates the large $\text{p}K_{\text{a}}$ change by descreening the local $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O-Cu}_{\text{B}}^{2+}$ charge distribution (see Figure 4, Panels c,d,e). The effect of the neutral Tyr237 compared to Tyr237[−] (deprotonated anion) is also strongly felt (see Table 2, $\text{p}K_{\text{a}}$ of H376⁺), and acts in conjunction with the $2\text{H}_2\text{O}$ shift (see Table 1).

The alternative non-pumping pathway (Figure 4, $\text{c} \rightarrow \text{f}$) proceeds with a similar $2\text{H}_2\text{O}$ shift, but without protonating Tyr237[−] first. The G is less negative for $\text{c} \rightarrow \text{f}$ compared to $\text{c} \rightarrow \text{d}$. However, additional analysis of these different kinetic pathways is planned to properly analyze the comparative fluxes for proton pumping *vs.* non-pumping paths. The remaining steps lock-in the directionality from $\text{f} \rightarrow \text{g} \rightarrow \text{h}$, $\mathbf{P}_{\text{R}}(\text{endpoint}) \rightarrow \mathbf{F} \rightarrow \mathbf{O}_{\text{H}}$.

Since the A- and B-type CcO's have nearly identical DNC structures, this water shifting effect on the proton uptake/pumping processes studied here for *ba*₃ CcO may also apply to the *aa*₃ enzymes. Very recently, Supekar *et al.*⁷² have found using multi-scale simulations of CcO that a protonated water cluster above the DNC can transiently store a proton during the pumping process. Therefore, the water molecules in/above the DNC of CcO play an important role in helping proton translocation.

Our calculations also suggest that, in state **F**, the H₂O ligand is energetically favored to dissociate from the Cu_B²⁺ site, and the O²⁻ ligand of Fe_{a3}⁴⁺ becomes the only oxygen species bridging the two metal sites. Our calculated Fe_{a3}⁴⁺=O²⁻ distance in **F** (1.68 Å) is by 0.03 Å longer than that in **P_R** (1.65 Å), which can explain the different Fe_{a3}⁴⁺=O²⁻ stretching modes in **P** (804 cm⁻¹) and **F** (785 cm⁻¹) identified by resonance Raman experiments.^{20,54} The very short calculated 3.8 Å Fe_{a3}⁴⁺...Cu_B²⁺ distance in **F** vs. 4.3 Å in **P_R** may also explain the very different EPR spectra in the two intermediates and the much larger magnetic coupling between the two ion centers in **F** than in **P_R**.²⁹ Further, by receiving an electron and a proton, such an Fe_{a3}⁴⁺=O²⁻...Cu_B²⁺ DNC structure of state **F** would probably lead to the so called **O_H** state with a μ-hydroxo bridge: Fe_{a3}³⁺-OH⁻-Cu_B²⁺. The DNC structure of such an Fe_{a3}³⁺-OH⁻-Cu_B²⁺ **O_H** state structure was also calculated in this paper, and the *G*⁰ of **F** → **O_H** was estimated at about -14.6 kcal mol⁻¹ for the redox half-reaction with respect to the standard H electrode. Equivalently, *E*⁰ = +0.63 V (see Supporting Information). The *G* of **F** + 1e⁻ + 1H⁺ → **O_H** in Figure 4 was computed with respect to a typical cytochrome *c* redox potential *E*⁰ = +0.22 V, which should be close to that of the heme *b* electron donor center.¹⁶

Further frequency calculations for the Fe-O stretching modes on the proposed **P_R**, **F**, and **O_H** states are planned in the near future to compare with the corresponding results obtained from Resonance Raman experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structure of Model 1 of the $[\text{Fe}_{\text{a}3}^{4+}, \text{Cu}_{\text{B}}^{2+}]$ dinuclear center (DNC) cluster. In this model, the two water molecules HOH604 and HOH608 found in the X-ray crystal structure 3S8G^{15} are in the positions above the ligand $\text{OH}^-/\text{H}_2\text{O}$ within the DNC. Figure A shows the complete DNC model of our calculation. The cluster is geometry optimized with the combination of different protonation states of His376/His376^+ , Tyr237/Tyr237^- , and $\text{OH}^-/\text{H}_2\text{O}$ ligand of Cu_{B} . When Tyr237 is in ionic deprotonated Tyr237^- state, the proton on the heme- a_3 farnesyl hydroxyl group is rotated to have H-bonding interaction with the Tyr237^- side chain. When His376 is in the neutral form, the proton on the top N atom (N_{e}) of His376^+ is removed. For clarity, the top portion of the cluster with the H-bonding interactions is shown in Figure B. The detailed H-bonding interactions around HOH604 and HOH608 in the centers of states $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{HO}^- - \text{Cu}_{\text{B}}^{2+}$ and $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O} - \text{Cu}_{\text{B}}^{2+}$ are shown in Figures C and D, respectively.

Figure 2.

Structure of Model 2 of the $[\text{Fe}_{\text{a}3}^{4+}, \text{Cu}_{\text{B}}^{2+}]$ DNC cluster. In this model, the two water molecules HOH604 and HOH608 are shifted up (from the positions in Model 1) to the top region of the DNC to H-bond with HOH607 and His376 (A) or His376⁺ (B). The centers of the $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{HO}^{-}\text{-Cu}_{\text{B}}^{2+}$ and $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O-Cu}_{\text{B}}^{2+}$ states are shown in C and D, respectively. The rest of the Model is the same as in Model 1.

Figure 3.

The major components at the center of the DNC of Model 3 of the $[\text{Fe}_{\text{a3}}^{4+}, \text{Cu}_{\text{B}}^{2+}]$ cluster. In this model, the water molecule (with the oxygen atom named O2), which is a ligand of $\text{Cu}_{\text{B}}^{2+}$ in Model 2, now dissociates from $\text{Cu}_{\text{B}}^{2+}$ but still has H-bonding interaction with the oxygen atom O1. The relative positions of HOH604 and HOH608 in Model 3 are the same as in Model 2 (Figures 2A and 2B). The cluster is also geometry optimized with different protonation states of His376/His376⁺ and Tyr237/Tyr237⁻, and the pK_{a} calculations show that the DNC cluster in this state would energetically prefer the Tyr237⁻ and His376⁺ forms.

Figure 4.

Feasible proton uptake/pumping processes connecting with the movement of water molecules in the DNC during the $\text{P}_R \rightarrow \text{F}$ transition based on our current Models 1–3 calculations. The $2\text{H}_2\text{O}$ represents HOH604 and HOH608. The G (kcal mol^{-1}) change at $\text{pH}=7$ [$G = 1.37(\text{p}K_a - 7)$] between two different protonation states and the energy difference corrected by zero point energy (ZPE, see the Supporting Information) between two tautomers are also given in the figure. The state $d \rightarrow e$ transition has two parts. The $2\text{H}_2\text{O}$ shift has approximate $G = +4.2 \text{ kcal mol}^{-1}$, and the proton pump step after has $G = -0.7 \text{ kcal mol}^{-1}$ (at $\text{pH}=7$), giving total $G = +3.5 \text{ kcal mol}^{-1}$. We note that the following internal proton transfer $e \rightarrow f$ also has a negative $G = -1.4 \text{ kcal mol}^{-1}$.

Figure 5.

Feasible **P_R** → **F** transition process starting from Model 2. The approximate free energy difference (ΔG , kcal mol⁻¹) of each state compared to the corresponding Model 1 analog is given in this figure. It is clear that Model 1 states are consistently lower in energy than the analogous Model 2 states for the ⁻HO-Cu_B²⁺ type metal site.

Figure 6.

The major components of the geometry optimized $\text{Fe}_{\text{a}3}^{3+}\text{-OH}^{-}\text{-Cu}_{\text{B}}^{2+}\text{-Y237}^{-}\text{-H376}^{+}$ (O_{H}) state DNC cluster. “A” shows the upper water cluster and the H-bonding interactions, in which the water molecule HOH610 also forms an H-bonding interaction with HOH608 during the geometry optimization. “B” shows the major $\text{Fe}_{\text{a}3}^{3+}\text{-OH}^{-}\text{-Cu}_{\text{B}}^{2+}\text{-Y237}^{-}$ portion of the cluster. Calculated properties are given in the last line of Table 3, and the Cartesian coordinates are given in the Supporting Information.

Table 1

OLYP-D3-BJ Calculated Properties for the Optimized DNC Geometries of Model **1**^a in $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{HO}^{-}-\text{Cu}_{\text{B}}^{2+}$ and $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}$ States with Different Protonation Forms of the Tyr273 and His376 Side Chains.^b

State	Geometry					<i>E</i>	<i>Q</i>	<i>pK_a</i> (H ₂ O)	<i>pK_a</i> (Y237)	<i>pK_a</i> (H376 ⁺)	Net Spin				
	Fe-N(H384)	Fe-O1	O1...O2	Cu-O2	Fe...Cu						Fe _{a3}	O1	O2	Cu _B	Y237
Fe _{a3} ⁴⁺ =O ²⁺ ...HO ⁻ -Cu _B ²⁺ -Y237 ⁻ -H376 ⁺ -M1	2.09	1.65	2.67	1.91	4.42	0.0	0				1.28	0.77	-0.21	-0.50	0.0
Fe _{a3} ⁴⁺ =O ²⁺ ...HO ⁻ -Cu _B ²⁺ -Y237 ⁻ -H376-M1	2.08	1.65	2.68	1.89	4.29	-5.8	0				1.28	0.77	-0.22	-0.50	0.0
Fe _{a3} ⁴⁺ =O ²⁺ ...HO ⁻ -Cu _B ²⁺ -Y237 ⁻ -H376 ⁺ -M1	2.09	1.65	2.67	1.90	4.33	-3.9	1		13.4	9.2	1.29	0.77	-0.21	-0.50	0.0
Fe _{a3} ⁴⁺ =O ²⁺ ...H ₂ O-Cu _B ²⁺ -Y237 ⁻ -H376 ⁺ -M1	2.10	1.66	2.55	2.10	4.79	-6.0	1	15.4		9.5	1.34	0.72	-0.03	-0.40	-0.31
Fe _{a3} ⁴⁺ =O ²⁺ ...H ₂ O-Cu _B ²⁺ -Y237 ⁻ -H376-M1	2.09	1.66	2.52	2.11	4.63	-7.7	0				1.35	0.71	-0.03	-0.41	-0.31
Fe _{a3} ⁴⁺ =O ²⁺ ...H ₂ O-Cu _B ²⁺ -Y237 ⁻ -H376-M1	2.08	1.66	2.49	2.01	4.59	-4.2	1	9.9	8.2		1.38	0.67	-0.09	-0.51	0.0
Fe _{a3} ⁴⁺ =O ²⁺ ...H ₂ O-Cu _B ²⁺ -Y237 ⁻ -H376 ⁺ -M1	2.08	1.66	2.48	2.01	4.54	-6.4	2	12.8	11.0	12.2	1.38	0.66	-0.08	-0.51	0.0

^aIn Model **1** (M1), the water molecules HOH604 and HOH608 are within the DNC (see Figure 1) as observed in the X-ray crystal structure 3S8G.¹⁵

^bThe calculated properties include geometries (Å), broken-symmetry state energies (*E*, offset by -28844.1 kcal mol⁻¹), the net charge (*Q*) of the model cluster, the *pK_a* values of the ligand H₂O and the Tyr237 and His376⁺ side chains, and the Mulliken net spin populations on Fe_{a3}, O1, O2, and Cu_B, and on the heavy atoms of the Tyr237 side chain (the sum total).

Table 2

OLYP-D3-BJ Calculated Properties for the Optimized DNC Geometries of Model **2^a** in $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{HO}^--\text{Cu}_{\text{B}}^{2+}$ and $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{H}_2\text{O}-\text{Cu}_{\text{B}}^{2+}$ States with Different Protonation Forms of the Tyr273 and His376 Side Chains.^b

State	Geometry						<i>E</i>	<i>Q</i>	<i>pK_a</i> (H ₂ O)	<i>pK_a</i> (Y237)	<i>pK_a</i> (H376 ⁺)	Net Spin				
	Fe-	Fe-O1	O1...	O2	Cu-O2	Fe...Cu						Fe _{a3}	O1	O2	Cu _B	Y237
Fe _{a3} ⁴⁺ =O ²⁻ ...HO ⁻ -Cu _B ²⁺ -Y237 ⁻ -H376 ⁺ -M2	2.09	1.65	2.68	1.89	1.89	4.21	3.8	0				1.27	0.77	-0.27	-0.48	0.0
Fe _{a3} ⁴⁺ =O ²⁻ ...HO ⁻ -Cu _B ²⁺ -Y237 ⁻ -H376-M2	2.08	1.65	2.73	1.89	1.89	4.26	1.2	0				1.27	0.77	-0.27	-0.49	0.0
Fe _{a3} ⁴⁺ =O ²⁻ ...HO ⁻ -Cu _B ²⁺ -Y237 ⁻ -H376 ⁺ -M2	2.09	1.65	2.71	1.90	1.90	4.29	3.1	1		11.2	9.2	1.27	0.78	-0.27	-0.48	0.0
Fe _{a3} ⁴⁺ =O ²⁻ ...H ₂ O-Cu _B ²⁺ -Y237 ⁻ -H376 ⁺ -M2	2.10	1.66	2.52	2.26	2.26	4.58	-9.4	1	20.7		11.5	1.37	0.69	-0.01	-0.36	-0.41
Fe _{a3} ⁴⁺ =O ²⁻ ...H ₂ O-Cu _B ²⁺ -Y237 ⁻ -H376-M2	2.08	1.66	2.51	2.23	2.23	4.58	-8.1	0				1.37	0.68	-0.01	-0.37	-0.38
Fe _{a3} ⁴⁺ =O ²⁻ ...H ₂ O-Cu _B ²⁺ -Y237 ⁻ -H376-M2	2.07	1.67	2.46	2.06	2.06	4.46	-7.8	1	17.6	10.5		1.43	0.61	-0.06	-0.51	-0.01
Fe _{a3} ⁴⁺ =O ²⁻ ...H ₂ O-Cu _B ²⁺ -Y237 ⁻ -H376 ⁺ -M2	2.08	1.67	2.45	2.05	2.05	4.51	-2.2	2	14.9	5.5	6.5	1.43	0.61	-0.07	-0.51	-0.01

^aIn Model **2** (M2), the water molecules HOH604 and HOH608 are moved above the DNC (see Figures 2A and 2B) to have H-bonding interactions with His376 and HOH607.

^bThe calculated properties include geometries (Å), broken-symmetry state energies (*E*, offset by $-28844.1 \text{ kcal mol}^{-1}$), the total charge (*Q*) of the model cluster, the *pK_a* values of the ligand H₂O and the Tyr237 and His376⁺ side chains, and the Mulliken net spin populations on Fe_{a3}, O1, O2, and Cu_B, and on the heavy atoms of the Tyr237 side chain (the sum total).

Table 3

OLYP-D3-BJ Calculated Properties for the Optimized DNC Geometries of Model **3^a** in $\text{Fe}_{\text{a}3}^{4+}=\text{O}^{2-}\cdots\text{Cu}_{\text{B}}^{2+}$ States with Different Protonation Forms of the Tyr273 and His376 Side Chains.^b Also Given in the Table is the Calculated Subsequent Oxidized **O_H** State with a Bridging Hydroxide.

State	Geometry				<i>E</i>	<i>Q</i>	<i>pK_a</i> (Y237)	<i>pK_a</i> (H376 ⁺)	Net Spin			
	Fe-N(H384)	Fe-O1	Cu···O1	Fe···Cu					Fe _{a3}	O1	Cu _B	
Model 3:												
Fe _{a3} ⁴⁺ =O ²⁺ ···Cu _B ²⁺ -Y237-H376 ⁺ -M3 (F)	2.15	1.68	2.40	3.81	-15.5	1		12.5	1.37	0.67	-0.22	-0.59
Fe _{a3} ⁴⁺ =O ²⁺ ···Cu _B ²⁺ -Y237-H376-M3	2.15	1.68	2.44	3.86	-12.9	0			1.36	0.68	-0.23	-0.59
Fe _{a3} ⁴⁺ =O ²⁺ ···Cu _B ²⁺ -Y237-H376-M3	2.14	1.67	2.35	3.78	-3.7	1	4.0		1.37	0.56	-0.40	-0.10
Fe _{a3} ⁴⁺ =O ²⁺ ···Cu _B ²⁺ -Y237-H376 ⁺ -M3	2.14	1.67	2.41	3.85	-3.2	2	1.8	10.2	1.38	0.62	-0.38	-0.13
Oxidized State O _H :												
Fe _{a3} ³⁺ -OH ⁻ -Cu _B ²⁺ -Y237-H376 ⁺	2.43	1.98	1.99	3.58	-124.7	1			4.02	0.13	-0.37	-0.25

^aIn Model **3 (M3)**, the water molecule (with the oxygen atom named O2), which is a ligand of $\text{Cu}_{\text{B}}^{2+}$ in Model **2**, now dissociates from $\text{Cu}_{\text{B}}^{2+}$ but still has H-bonding interaction with the oxygen atom O1 (see Figure 3). The relative positions of HOH604 and HOH608 in Model **3** are the same as in model **2** (Figures 2A and 2B).

^bThe calculated properties include geometries (Å), broken-symmetry state energies (*E*, offset by $-28844.1 \text{ kcal mol}^{-1}$), the total charge (*Q*) of the model cluster, the *pK_a* values of the the Tyr237 and His376⁺ side chains, and the Mulliken net spin populations on Fe_{a3}, O1, and Cu_B, and on the heavy atoms of the Tyr237 side chain (the sum total).