Research Article
B3LYP Study on Reduction Mechanisms from \( \text{O}_2 \) to \( \text{H}_2\text{O} \) at the Catalytic Sites of Fully Reduced and Mixed-Valence Bovine Cytochrome \( \text{c} \) Oxidases

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Reduction mechanisms of oxygen molecule to water molecules in the fully reduced (FR) and mixed-valence (MV) bovine cytochrome \( \text{c} \) oxidases (CcO) have been systematically examined based on the B3LYP calculations. The catalytic cycle using four electrons and four protons has been also shown consistently. The MV CcO catalyses reduction to produce one water molecule, while the FR CcO catalyses to produce two water molecules. One water molecule is added into vacant space between His240 and His290 in the catalytic site. This water molecule constructs the network of hydrogen bonds of Tyr244, farnesyl ethyl, and Thr316 that is a terminal residue of the K-pathway. It plays crucial roles for the proton transfer to the dioxygen to produce the water molecules in both MV and FR CcOs. Tyr244 functions as a relay of the proton transfer from the K-pathway to the added water molecule, not as donors of a proton and an electron to the dioxygen. The reduction mechanisms of MV and FR CcOs are strictly distinguished. In the FR CcO, the Cu atom at the CuB site maintains the reduced state Cu(I) during the process of formation of first water molecule and plays an electron storage. At the final stage of formation of first water molecule, the Cu(I) atom releases an electron to Fe-O. During the process of formation of second water molecule, the Cu atom maintains the oxidized state Cu(II). In contrast with experimental proposals, the K-pathway functions for formation of first water molecule, while the D-pathway functions for second water molecule. The intermediates, \( P_M, P_R, F, \) and \( O \), obtained in this work are compared with those proposed experimentally.

1. Introduction

Cytochrome \( \text{c} \) oxidase (CcO) is known to be a terminal oxidase of cellular respiration system and/or electron-transportation system in aerobic organism and to be also a metalloenzyme in inner membrane of mitochondria. It catalyzes the reduction of oxygen molecule to water molecules with the sequential four-electron transfer from cytochrome \( \text{c} \) through heme \( \alpha \) and it also moves four protons from the matrix side (N-side) of mitochondrial membrane toward the cytosolic side (P-side) (so-called proton pumping) [1–4].

\[
\text{O}_2 + 4e^- + 8H^+ \xrightarrow{\text{CcO}} 2\text{H}_2\text{O} + 4H^+ \quad \text{(proton pumping).} \quad (1)
\]
uniquely cross-linked to C6 of phenol of tyrosine (Tyr244) with single covalent bond. The heme-copper oxidase oxidations which have been determined by the X-ray crystallographic analyses [7, 8, 14–16] have a common structure to the bovine CcO. This superfamily has been classified into A1, A2, B, and C families by amino acid sequence analyses [17, 18]. The bovine heart CcO, which belongs to the A1 family, has two distinct proton pathways, K-pathway and D-pathway [5, 8, 17–23]. The K-pathway begins from Lys319 and ends at Glu242. The K-pathway is used to transfer two electrons at Thr316, while the D-pathway begins from Asp91 and ends at Thr316, while the D-pathway begins from Asp91 and ends at Glu242. The K-pathway is used to transfer two protons toward the catalytic site, while the D-pathway is used to transfer the remaining six protons. Thus, the D-pathway transfers two substrate protons to reduce the O2 molecule and four protons that are pumped across the membrane [24, 25]. It is presumed that the branching point is located at Glu242.

As can be seen in Table 1, the mixed-valence (MV) CcO and FR CcO should be strictly distinguished. The FR state has four electrons to produce two H2O molecules due to the reduced valence state of CuA, heme a, heme a3, and CuB, while the MV state has only two electrons in the catalytic site of heme a3 and CuB. Thus, the MV state has possibility to produce only one H2O molecule. Although both MV and FR states have two electrons in the catalytic site of heme a3 and CuB, it is expected that the reduction mechanisms of the O2 molecule for MV and FR CcOs should be different after the [Fe(III)-O2 Cu(I)] intermediate was formed. The [Fe(III)-O2 Cu(I)] intermediate of FR CcO have possibility to receive an electron from heme a, while an electron in Cu(I) should be used to reduce the O2 molecule in MV CcO.

The numerous mechanisms of the reduction of O2 molecule to H2O molecule have been proposed experimentally [2–4, 13, 17, 19–22, 26–55]. There is now consensus that O2 molecule in the triplet state is initially bound to Fe a in the reduced state [Fe(II) Cu(I)] (R) to yield the ferric peroxide intermediate [Fe(III)-O2 Cu(I)] (A), as shown in Scheme 1. The intermediate A has the optical absorbance at 595 nm and a mode of 568 cm−1 due to the Fe-O2 vibration was detected by resonance Raman studies [2, 3]. The subsequent intermediate [Fe(IV)=O2− H2O Cu(II)], which includes Fe(IV)=O2− in heme a, as shown in Scheme 1, has also been experimentally observed [3, 19, 20, 26–28, 31–40]. [Fe(IV)=O2− H2O Cu(I)] is usually designated by the symbols, PM, PR, and F [2, 17, 19, 33–36]. It is considered that the differences of these intermediates are due to the protonation state of a nearby protonable center or number of electrons in the catalytic site. For MV CcO, a mode of 804 cm−1 due to Fe=O vibration has been observed by resonance Raman spectroscopy [31], while for FR CcO a mode of 786 cm−1 has been observed [41]. Before formation of the intermediate F, it was shown from both optical and EPR spectroscopy that the PR intermediate exists and exhibits spectroscopic properties quite distinct from F [30, 32, 42].

However, the reaction mechanism from the [Fe(III)-O2 Cu(I)] (A) to [Fe(IV)=O2− H2O Cu(II)] (P or F) is not conclusive yet. Although the hydrogenated/protonated Fe-OOH, which will be considered as the intermediacy from A to P or F, has been discussed in numerous proposals based on the experimental results, it is not beyond the region of speculation. In addition, the geometrical and electronic structures of [Fe(IV)=O2− H2O Cu(II)] and Fe-OOH have not been entirely elucidated yet. Yoshikawa and coworkers have proposed the mechanism that the proton transfer is induced from Tyr244 to FeOO to yield hydroperoxide and subsequently one electron transfer from CuB in its oxidation state [36]. It has been proposed for FR CcO that the oxidation states of Cu and Fe atoms in heme a and heme a3 change through the reaction without generation of oxoferryl-tyrosine radical intermediate which was formed in the MV CcO [21, 22]. It has been also proposed by Wikström that phenol of tyrosine dose not affect the reaction [32]. In FR CcO, Fe of heme a (Fea3) is initially in the ferrous state. From optical experiments, Fea3 is oxidized at the same time that the [Fe(III)-O2− Cu(I)] intermediate disappeared [45, 46]. This observation is also supported from resonance Raman experiments and it was concluded that the electron transfers from Fea3 to binuclear center [47]. Several groups have speculated that the crosslinked tyrosine plays roles for a hydrogen atom donor [2, 21, 29, 31, 48] to molecular oxygen bound to heme a3 in order to activate O−O bond. It has been proposed from recent experimental studies that a tyrosyl radical is formed in the [Fe(IV)=O2− H2O Cu(II)] intermediate [1–4, 31, 49, 50]. Direct evidences are not,
however, observed. The mutation of histidine coordinated to Cu induced the damage of catalytic effects with retaining the electron transfer between heme $a_3$ and heme $a$ [51–54]. The reaction mechanisms proposed by several groups are still controversial and the structures of the [Fe(IV)−O$^2^-$ H$_2$O Cu(II)] intermediates, $P_M$, $P_S$, and $F$ and the intermediacy Fe-OOH are still unknown and their changes through O$_2$-reduction are also unknown.

The reaction proceeds in a stepwise manner by the transfer of four electrons and four protons. There are K- and D-pathways for the proton transfer [44, 56–63]. The D-pathway that ends at Glu242 near to the catalytic site has been experimentally and theoretically studied. The molecular dynamic simulations have shown that the conformational switch of Glu242 functions the proton pumping through H$_2$O network connecting to the D-propionate group of heme $a_3$ and transfer of two protons through H$_2$O network connecting to the catalytic site [57, 58]. The FTIR measurement has shown that the reduction of O$_2$ molecule stops at the $P_R$ intermediate in the Glu278Glh mutant enzyme from *paracoccus denitrificant* [59]. It was also proposed that the K-pathway is catalytic only in the last steps of the catalytic cycle [44, 60, 61]. It was, however, proposed that the K-pathway is used for the uptake of two substrate protons upon reduction of catalytic site [23, 24, 60]. Recently, Lepp et al. showed that mutations in the K-pathway of proton transfer slowed down formation of the $P_R$ intermediate [64]. Thus, the sequential uptakes of four substrate protons from the K- and D-pathways are still unclear.

On the other hand, theoretical studies on the reduction mechanism and the proton pathways in CcO have been extensively performed [65–92]. “Splitting the Water Molecule” mechanism has been proposed based on the hybrid density functional calculations [65–68]. The water molecule is initially located in the vicinity of the Cu$_B$ center in their mechanism. This water molecule provides a proton to oxy intermediate [Fe(III)−O$_2^-$ Cu(I)] and at the same time copper atom provides an electron. The products of this mechanism would be [Fe(III)−OOH HO$^-$−Cu(I)]. The reaction systems were calculated on the potential energy surface of the triplet state. For MV CcO in which an electron cannot transfer from heme $a$ to heme $a_3$, the density functional theory (B3LYP) has been applied to examine O−O bond cleavage using a large model of Fe(II)−Cu(I) binuclear site [69–71]. They have proposed that it is necessary to add two water molecules in the catalytic site in order to form hydrogen bonds connecting between Fe-OO and Tyr244. It was also proposed that the proton transfer from the K-pathway to the catalytic site enhances the proton transfer from Tyr244 to FeOO. In recent works [71, 72], they employed the bigger model that contains the Cu$_B$ moiety, Thr244 and protonated lysine. It was proposed that the protonation of the FeOO proceeds the OO bond cleavage with higher activation energy of 18.6 kcal/mol than the experimental value and yields the [Fe=O, HO−Cu] intermediate where the tyrosine is a neutral radical. Namely they showed that the additions of two electrons and single proton to the OO bond induce the OO bond cleavage to yield the $P_M$ intermediate.

In our previous works [76, 77], we have pointed out the possibility of existence of single water molecule between His290 and Tyr244 with the hydrogen bonds. For the hem dioxygen complex [78, 80], we have shown that the OO bond cleavage occurs when the OO bond receives two electrons and two protons. For the H$_2$O formation in FR CcO [79], we showed that the H$_2$O molecule on the Cu atom plays crucial roles. This H$_2$O molecule provides a proton to Fe(IV)−O$^2^-$ to yield [Fe(III)OH HOCu(II)] with the electron transfer from Cu$_B$ to heme $a_3$, so called the proton-coupled electron transfer. From these results, we have also speculated the mechanism of the H$_2$O formation for FR and MV CcO. However, the reduction mechanism and catalytic cycle were not studied systematically and concretely.

In this article, we propose new reduction mechanisms from O$_2$ molecule to H$_2$O molecules by MV and FR CcOs from theoretical viewpoints. The intermediates, and their electronic structures obtained by the sequential additions of electrons and protons are thoroughly examined. The functions of Tyr244 in our mechanism are distinguishably different from those proposed from experiments as a proton and an electron donor. This paper is composed of as follows: (1) possibility of H$_2$O coordination to Cu of the Cu$_B$ site is examined, (2) formation mechanism of single H$_2$O molecule from [Fe(III)−O$_2^-$ Cu(I)] (A) in MV CcO is examined, (3) formation mechanism of two H$_2$O molecules from [Fe(III)−O$_2^-$ Cu(I)] (A) in FR CcO is examined, (4) the reduction mechanisms for MV and FR CcO are summarized. Our reaction scheme is compared with other mechanisms proposed previously from experimental and theoretical viewpoints.

### 2. Computational Details

#### 2.1. Model of a Catalytic Site for Calculations

The model of the catalytic site of CcO to examine the O$_2$ reduction mechanism was constructed from geometry based on the X-ray crystallographic study for FR CcO of bovine heart muscle (1OCR in PDB) [9]. As shown in Figure 1, all histidine residues, His240, His290, His291, and His376, were replaced by imidazoles. Tyr244, which is covalently bonded to His240, functions of Tyr244 in our mechanism are distinguishably different from those proposed from experiments as a proton and an electron donor. This paper is composed of as follows: (1) possibility of H$_2$O coordination to Cu of the Cu$_B$ site is examined, (2) formation mechanism of single H$_2$O molecule from [Fe(III)−O$_2^-$ Cu(I)] (A) in MV CcO is examined, (3) formation mechanism of two H$_2$O molecules from [Fe(III)−O$_2^-$ Cu(I)] (A) in FR CcO is examined, (4) the reduction mechanisms for MV and FR CcO are summarized. Our reaction scheme is compared with other mechanisms proposed previously from experimental and theoretical viewpoints.

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Cu atoms \[93\]. The 6-31G basis set was employed for Fe and SCF solutions with the broken symmetry (BS) procedure.

The all-electron DZ basis set was used for key O atoms of reacting O2 molecule, phenol and H2O molecules W1 and W2 are detailed in text.

Four electrons transfer from CuA to the active site through heme a1. Thus the examination of the reaction mechanism is equivalent to determining the pathway to provide the protons to the dioxygen on heme a3. The conformation of the catalytic site in the FR CcO is similar to that in the FO state [7–12]. Heme a3, Cu, His240, His290, His291, and Tyr244 have same geometrical configurations for both FR and FO CcO. The Cu atom is fixed by coordination of three histidine residues, His240, His290 and His291. Tyr244 makes the hydrogen bond to the farnesylthyl, group in heme a3. Tyr244 is fixed by its hydrogen bond and the cross-linked single covalent bond with His240 that coordinates to the Cu atom. Heme a3 is also fixed by the axial coordination of His376 and the hydrogen bond with Tyr244. Accordingly the essential change for the structure of the active site is not expected in the reduction of the dioxygen on heme a3. However, the pathway of the proton transfer plays crucial roles. The proton pathway must approach to the dioxygen bound to heme a3 in order to provide protons. The water molecule W1 connects with the K-pathway through the hydrogen bonds of W1, Tyr244, farnesylthyl and W2. In fact, in our previous work [76], the hydronium ion W1H+, where a proton is added to W1, approaches to the dioxygen to give the proton, yielding the bond FeOOH on the heme a3. Thus it is expected that a remarkable change is found in the proton pathway of the hydrogen bond network. The fragments of reacting O2, H2O molecules, and H atoms of –CH2OH and OH in phenol, which are directly connected with the hydrogen bond network, were optimized. Since our optimizations were performed for limited parameters, our discussion will be qualitative not quantitative. We draw the potential energy surfaces along the path of the proton transfer in a stepwise manner. It could be confirmed form the potential energy surfaces that the optimized intermediates are local minimums. The point with maximum energy is assigned to the transition state.

Since correlation effects are important to elucidate the transition-metal systems, the usual Hartree-Fock methods lead to poor estimations for the binuclear systems. The hybrid exchange-correlation functional B3LYP method [94–97] was most widely used for the transition-metal system. Since the B3LYP method contains the moderate static correlation effects, it provided the suitable results for the desired d-electron configurations in good agreement with experiments [80, 98, 99]. Thus, we employed the B3LYP method to estimate the electronic structures of the reaction systems.

The dioxygen binds to Fe in heme a3 at an initial stage of the reaction. The bound dioxygen is reduced by sequential additions of four protons and four electrons on heme a3.

\[ \langle \hat{S}_i^Z \rangle_{SC} = \langle \hat{S}_i^Z \rangle_{BS} - \langle \hat{S}_i^Z \rangle_{Pure} \approx \sum_{i=1}^{N_p} n_{-i} n_{+i} = \sum_{i=1}^{N_p} n_{-i} n_{+i}, \]  

\[ n_{-i} + n_{+i} = 2, \quad 1 \leq n_{-i} \leq 2. \]  

2.2. Theoretical Examination. The Fe and Cu atoms have possibilities of unpaired electron spins for the oxidation states of Fe(III) and Cu(II) in the course of reduction of the O2 molecule, respectively. Their unpaired spins will be ferromagnetically coupled in the high-spin (HS) state, while they are antiferromagnetically coupled in the low-spin (LS) state. The electronic structure of the HS state can be well presented by a usual unrestricted molecular orbital method. The LS states can be presented by the unrestricted SCF solutions with the broken symmetry (BS) procedure. The all-electron DZ basis set was employed for Fe and Cu atoms [93]. The 6-31G* basis set was used for key O atoms of reacting O2 molecule, phenol and H2O molecules and the 3-21G basis set for C, H, N, and other O atoms. Although the 3-21G basis set is tight for the transition-metal complexes in some cases, these combined basis sets used here reproduced reasonably the electronic structures of heme-oxygen complexes estimated by using more flexible basis sets [80].

The dioxygen binds to Fe in heme a3 at an initial stage of the reaction. The bound dioxygen is reduced by sequential additions of four protons and four electrons on heme a3.
Here, $n_{-i}$ and $n_{+i}$ are the occupation numbers of the bonding natural orbitals $\phi_{-i}$ and antibonding $\phi_{+i}$, respectively. $N_\beta$ is the number of $\beta$-electron. $\langle \hat{S}_2^2 \rangle_{BS}$ is the expectation value of square of the spin angular momentum for the BS solution, while $\langle \hat{S}_2^2 \rangle_{pure}$ is one of the corresponding pure spin state. When $\phi_{-i}$ is an doubly occupied orbital, it does not contribute to the spin contamination because of $n_{-i} = 2$ and $n_{+i} = 0$. For the BS solution where $\phi_{-i}$ and $\phi_{+i}$ are coupled antiferromagnetically, the spin contamination $\langle \hat{S}_2^2 \rangle_{SC}$ is increased by unity because of $n_{-i} \approx n_{+i} \approx 1$. 

**Figure 2:** Geometries optimized on the pathways of the $\text{O}_2$ reduction at the catalytic sites in MV and FR $\text{CcOs}$, and the reaction paths of MV and FR $\text{CcOs}$ examined in this study.
and \( n_i \cdot n_j = 1 \). In the BS calculation of the singlet state, if the single pair of the antiferromagnetic spin coupling exists in the system, the \( \langle S^2 \rangle_{BS} \)-value will be nearly equal to unity with \( \langle S^2 \rangle_{\text{pure}} = 0 \). For the BS calculation of the doublet state, \( \langle S^2 \rangle_{BS} \approx 1.75 \) with \( \langle S^2 \rangle_{\text{pure}} = 0.75 \). In other word, the spin contamination \( \langle S^2 \rangle_{SC} \), the deviation of \( \langle S^2 \rangle_{BS} \) from \( \langle S^2 \rangle_{\text{pure}} \) represents the numbers of pairs of the antiferromagnetically coupled spins \( (N_p) \) in the system under examination. Although the spin contamination \( \langle S^2 \rangle_{SC} \) gives valuable information, the coupled spin-site in the system must be identified by the spin population.

The spin contamination is a serious problem in the BS solution. No exact procedure to remove the spin contamination is proposed at the B3LYP level. All procedures proposed currently are approximate. In this paper, we employed the energies for discussion without projection to the pure spin state.

3. Results and Discussion

Figure 2 shows nineteen intermediates optimized in this study. The reduction pathways of \( \text{O}_2 \) molecule at the catalytic sites of the MV and FR CuOs are also shown to make easy understanding our reaction scheme estimated in this study. Their total energies, expectation values of square of spin angular momentums, and relative energies are summarized in Table 2. The Mulliken charge and spin populations of atoms and groups are summarized in Tables S1–S6 in Supplementary material available at doi: 10.1155/2010/182804. The atomic distances between key atoms are also tabulated in Table 3.

3.1. Early Stage of the \( \text{O}_2 \) Reduction (1–2)

3.1.1. On \( \text{H}_2\text{O} \) Coordination to Cu in the CuB Site. When the catalytic site is an oxidized state [Fe(III) Cu(II)], it was shown that the \( \text{H}_2\text{O} \) molecule coordinated to the Cu atom of the CuB site plays a crucial role for the formation of second \( \text{H}_2\text{O} \) molecule from Fe=O of the heme \( \alpha_3 \) site [79]. However, it is not clear whether the \( \text{H}_2\text{O} \) molecule coordinates to the Cu(I) atom in the reduced [Fe(II) Cu(I)] catalytic site at an early stage of the reduction or not. In order to account for possibility of coordination of \( \text{H}_2\text{O} \), the full geometry optimizations of the CuB site with and without \( \text{H}_2\text{O} \) were carried out. The optimized geometries with and without \( \text{H}_2\text{O} \) are shown, respectively, in Figure 3 and Supplementary Figure S1.

It is apparent from Figure 3 that the geometry of Cu(II) is different from that of Cu(I). For the oxidized Cu(II), the distances between Cu and N of His290, His291, and His240 are, respectively, 1.982, 1.984, and 1.972 Å, comparable with 1.957, 1.913, and 2.162 Å of the reduced 1OCR and 1.914, 1.920, and 2.194 Å of the oxidized 1OCC. The distance of \( \text{H}_2\text{O} \) toward Cu atom is given by 2.062 Å, showing that the \( \text{H}_2\text{O} \) molecule coordinates to the Cu atom as a fourth ligand. Thus, the optimized geometry is in reasonable agreement with the X-ray structures of the reduced 1OCR and oxidized 1OCC. However, the optimized geometry of the reduced Cu(I) deviates remarkably from the X-ray structures. Three histidines are rotated around the N–Cu bond. The distance between Cu and N of His290 is 2.611 Å, being remarkably longer by 0.654 Å than 1.957 Å of 1OCR. A notable distance is 3.623 Å between \( \text{H}_2\text{O} \) and Cu, being remarkably longer than 2.062 Å of the Cu(II) geometry. However, the O atom of \( \text{H}_2\text{O} \) has the distances of 2.514 and 2.233 Å toward the H atoms of His240 and His291, respectively, indicating that the \( \text{H}_2\text{O} \) molecule is weakly bound to His240 and His291 by the hydrogen bonds rather than the coordination to the Cu atom. Accordingly, it is probable that the \( \text{H}_2\text{O} \) molecule is not bound to the Cu(I) atom in the reduced catalytic site [Fe(II), Cu(I)]. On the other hand, the optimized geometries of the CuB site without \( \text{H}_2\text{O} \) molecule are shown in Supplementary Figure S1. Both geometries of Cu(II) and Cu(I) are almost similar to those with the \( \text{H}_2\text{O} \) molecule shown in Figure 3. It is found that the CuB sites of the reduced and oxidized CuOs have similar geometries to those examined here, if they do not have any constraints such as the surrounding peptide bonds and amino acid residues. Therefore, it can be considered that the CuB site in the reduced 1OCR observed by the X-ray crystallographic study is energetically activated by the steric hindrance, while the oxidized CuB site is energetically stable with release from the steric hindrance.
3.1.2. FeOO in Heme a3 Site. It is reasonable to begin the examination of the O2-reduction path from the reduced catalytic site, [Fe(II)Cu(I)] shown in Scheme 1, since [Fe(II) Cu(I)] is a common state for both MV and FR catalytic sites. Figure 2 shows the geometry of [Fe(II)Cu(I)] (1) (same as 1 shown in Figure 1). The distances between H(W1) and O(Tyr244), between O(W1) and H(His290), and between H(−CH3OH) and O(W1) are estimated to be 1.918, 2.117, and 1.770 Å, respectively, showing that W1 is hydrogen-bonded to both His290 and Tyr244, and W2 is also hydrogen-bonded to the farnesylethyl group. It is, thus, apparent that the network of the hydrogen bonds from W2 to W1 through the farnesylethyl and Tyr244 is constructed. Since the spin density of Fe atom in heme a3 is 2.151e (Supplementary Table S1), I is a triplet spin-state and has two unpaired spins localized on the Fe atom.

Since the electronic structures of [FeOO] in the intermediate 2 have been well characterized [77, 78, 102–105], those are briefly commented here. The intermediate 2 is a singlet state where an O2 molecule is bound to the Fe atom of heme a3 and is 3.5 kcal/mol lower in energy than the triplet state (Table 1). It is found from spin populations shown in Supplementary Table S1 that the FeOO moiety has the antiferromagnetically coupled spins localized on the Fe atom and OO bond, consistent with the (S2) value of 0.9297 larger than (S2) = 0.0 of the pure singlet spin-state, as shown in (2).

Two unpaired spins occupy the bonding and antibonding orbitals of 3d on Fe and π∗ on O2, such as 3d_{yz} + π∗ and 3d_{zx} − π∗. The spin population of the Fe atom is 1.062e, indicating that the Fe atom is oxidized from Fe(II) of I to Fe(III) with one electron transfer from the Fe atom to the OO bond. Therefore, at this stage of the reduction, the OO bond receives one electron necessary to reduce the OO bond from the reduced Fe atom.

3.2. Reduction Mechanism of MV CcO (2–7 in Figure 2). It was shown in the previous work [80] that the cleavage of the OO bond occurs when FeOO on porphyrin ring receives two electrons and two protons. The OO moiety in FeOO receives already one electron from the Fe atom to give the electronic structure of Fe(III)-OO−. Accordingly, in the case of MV CrO, the OO moiety has to receive sequentially one electron from the Cu atom of the CuB site and two protons from the outside of the catalytic site.

It is reasonable to suppose that the proton transfers to OO to yield FeOOH through the hydrogen-bond network from W2 to W1, since W2 hydrogen-bonds to the terminal Thr316 residue of the K-pathway. The intermediate 3 shown in Figure 2 corresponds to the geometry where a proton from the K-pathway is trapped on −CH2OH. It can be easily seen from Tables S1 that the electronic structures of Cu and FeOO portions do not change from those of the unprotonated state 2. Interestingly, it is found from Table 3 that W1 approaches to the proximal O_b atom of FeOO with shortening the distance of O_b−O(W1) from 3.652 to 2.801 Å. The distance between the O_a atom of phenol and W1 is also made shorter from 2.871 to 2.561 Å, indicating that the hydrogen bond of phenol and W1 is made stronger. Thus, it is apparent that the addition of the proton to the catalytic site from the K-pathway induces formation of the stronger network of the hydrogen bonds in order to open a pathway of the proton transfer from −CH2OH to FeOO through Tyr244 and W1.

In the intermediate 4 shown in Figure 2, FeOOH is formed. In the change from 3 to 4, protons move simultaneously from −CH2OH to Tyr244, from Tyr244 to W1, and from W1 to FeOO. From Table 2, 4 is 33.5 kcal/mole more stable than 3. From Supplementary Table S1, the protonated FeOOH has negative charge of −0.561e not nearly equal to zero, similar to −0.599e of the OO moiety in 3. The spin population of OOH disappears to 0.071e from −0.959e of OO in 3, while the spin population of the Cu atom grows up from −0.039e to −0.507e. In the formation of 4 from 3, the antiferromagnetic spin coupling shifts from between Fe and OO in 3 to between Fe and Cu in 4. These indicate that one electron transfers from the Cu atom to the OOH moiety with changing the oxidation state of the Cu atom from Cu(I) to Cu(II), consistent with the increase of the bond distance of OOO from 1.307 Å to 1.449 Å. It should be noted here that at this stage of the reaction the OOH moiety receives two electrons from the reduced Fe and Cu atoms and one proton from the K-pathway.

In order to explore the formation of 4 from 3 in more details, the H-atom on −CH3OH+ moved toward the O_b-atom of Tyr244 in a stepwise manner. Supplementary Figure S2 shows the change of the relative energy, the variations of charge and spin populations for the key atoms and OO (OOH) moiety and the atomic distances. The relative energy rapidly decreases from 7.4 to −14.4 kcal/mol in range of 1.4 and 1.35 Å. Supplementary Figure S3 shows the geometries 3a and 4a at R_{OHH} = 1.4 and 1.35 Å, respectively. It is found that the proton of Tyr244 transfers simultaneously to W1 in the geometry 3a with the proton transfer from −CH2OH to Tyr244. At 1.35 Å, the proton of Tyr244 has transferred to W1, and simultaneously the other proton of W1 has transferred to FeOO to yield the FeOOH moiety. From simple insight, it seems that W1H+, which is formed by receiving a proton from Tyr244, blows off the other proton to FeOO. However, W1H+ is a transient state on the potential energy surface [78]. With decreasing the energy without barrier, W1H+ moves to approach to proximal O_b of FeOO, and at about 2.6 Å a proton shifts from W1H+ to FeOO to give FeOOH, and remainder W1 switches back to the original position to give the state 4a. It can be seen from the change of the spin populations that the electron transfers from Cu to FeOOH at the same time of formation of FeOOH. Apparently the structural change from 1.4 to 1.35 Å is continuous. Accordingly, the reaction from 3 to 4 proceeds in mechanism of the proton-coupled electron transfer (PCET) with the activation energy of about 7.4 kcal/mol.

The intermediate 5 corresponds to the geometry that a proton from the K-pathway is captured on −CH2OH of farnesylethyl group in 4. 5 is a singlet spin-state that the antiferromagnetic spin coupling exists on Fe(III) and Cu(II). Similar to the formation of FeOOH from 3 to 4, the H-atom on −CH2OH+ moved toward O_a (Tyr244) from the geometry of 5. A proton of Tyr244 moves simultaneously to W1. The formed W1H+ does not move toward FeOOH to yield FeOOH2 or FeO + H2O, in contrast to the case
Table 2: Total energies (au), expectation values of square of spin angular momentums (au), and relative energies (kcal/mol) of optimized intermediates.

<table>
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<th>Intermediates</th>
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<th>(E_{\text{total}})</th>
<th>(\langle S^2 \rangle)</th>
<th>(\Delta E_{\text{rel}})</th>
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^a(C, 2S + 1) means (total charge, spin multiplicity).  
^b\(\Delta E_{\text{rel}} = E(2) - E(1) - E(O_2)\).  
^c\(\Delta E_{\text{rel}} = E(2) - E(\text{W}1)\).  
^d\(\Delta E_{\text{rel}} = E(2) - E(O_2^-)\).  

Table 3: Interatomic distances (Å) of key atoms in the optimized intermediates^a.

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<th>Oa-O (W2)</th>
<th>Cu-O (W3)</th>
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^aOxygen symbols, Oa, Ob, Oc, and Od, are shown in Figure 2. Oa and Ob are oxygen atoms to be reduced to 2H2O. Oc and Od are oxygen atoms of phenol (Tyr244) and −CH2OH, respectively.
from 3 to 4. The geometry 6 with W1H+ was obtained. The variations of energy and spin populations with moving the proton to O of Tyr244 is shown in Supplementary Figure S4. The activation energy of the proton transfer is about 9.3 kcal/mol, slightly higher by 1.9 kcal/mol than that from 3 to 4. The geometry 6 is 5.4 kcal/mol lower than 5, smaller than 33.5 kcal/mol from 3 to 4. From Supplementary Table S2, the change from 5 to 6 proceeds in the proton transfer without the electron transfer from the Cu(II) atom.

The geometry 7 corresponds to the intermediate where the H2O molecule is formed by cleaving the OO bond and moving the proton from W1H+ to separated OH−. The 7 is 13.4 kcal/mol lower than 6, and is a singlet spin-state with the expectation value of the squared spin angular momentum of 2.0580, indicating existence of two pairs of the antiferromagnetic spin couplings in 7. The spin population of Fe=O is given by 2.078e with two parallel unpaired spins distributed over Fe=O, showing Fe(IV)=O2−. The molecular orbitals corresponding to Fe=O are composed of two antibonding orbitals of d_{xz} (Fe)-p_y (O) and d_{yz} (Fe)-p_x (O), which are the same as those of the naked heme Fe=O [78, 80]. The Cu atom has the spin population of −0.590e (−1.001e for the CuB site), showing that the oxidation state of the Cu atom does not change from Cu(II), compared with those of 6. However, the spin population of the porphyrin ring decreases in negative value from −0.350 to −1.089e, and the charge population increases from −0.729 to −0.173e. This indicates that the porphyrin ring loses one electron and has single unpaired electron of the antiparallel spin to the Fe=O. Accordingly the heme a1 site is thought to be the compound I with the radical cation of the porphyrin ring [78, 80, 106–108], consistent with the experimental results of the time resolved Raman spectroscopy [109, 110]. The Fe−O2− distance is estimated to be 1.658 Å, comparable with 1.64–1.70 Å determined by experiments [111, 112], and with 1.669 Å of theoretical value [106].

In order to confirm the connection from the state 6 to 7, the O−O distance of FeOOH is increased. Supplementary Figure S5 shows the energy change with increasing the O−O distance from the state 6. The energy increases and has a maximum of 4.9 kcal/mol at R_{OO} = 1.8 Å. Supplementary Figure S6 shows the geometries at R_{OO} = 1.6 and 1.7 Å. It is easily found that the proton moves from W1H+ to FeOOH− to yield the H2O molecule at the early stage of the OO-bond cleavage. After passing R_{OO} = 1.8 Å, the energy decreases gradually and crosses to the potential energy surface connecting to the state 7.

For MV CcO, only one water molecule was produced by two-electron reduction of the oxygen molecule. Two electrons are provided from the reduced Fe(II) of heme a3 and Cu(I) of the CuB site, while two protons are provided from the network of hydrogen bonds including W1, Tyr244, −CH2OH, and W2 connecting to the terminal Thr316 of the K-pathway. When Fe(III)OOH− (4) is formed from Fe(III)OO− (2) by the proton transfer, the electron transfers from Cu(I) to Fe(III)OO− in manner of PCET. On the pathway from 6 to 7 where the H2O molecule is produced, the recombination of the electronic structure occurs at the catalytic site, in good agreement with observation that the reduction in MV CcO is 5–6 times slower than in FR CcO [2, 3, 32].

3.3. Reduction Mechanism of FR CcO

3.3.1. First H2O Formation (2, 8–13 in Figure 2). On the contrary to MV CcO, in FR CcO, there are two more electrons to reduce the O2 molecule in the reaction system, heme a and CuA site. It is, thus, expected that one electron is put into the catalytic site from heme a after the intermediate 2 is formed. The geometry 8 in Figure 2 is a one-electron reduced state of 2.

The 8 is 81.0 kcal/mol lower than 2, indicating the possibility that the O2-adduct to heme a1 can receive easily an electron, 8 is also the bound state with 60.5 kcal/mol lower than the dissociation state of 1 and O2−. It is found from Supplementary Table S3 that the unpaired spin is localized on the OO moiety with small distribution of 0.157e on the Fe atom, showing that 8 is reduced by the addition of one electron with changing the oxidation state from Fe(III) to Fe(II). The charge populations on the porphyrin ring and OO moiety are increased in negative values, showing that the paired electrons are delocalized to the porphyrin ring and OO moiety. These features are consistent with the results for the reduced heme [113, 114].

It is reasonable to consider that the increases of the electron-negative characteristic on the porphyrin ring and OO moiety enhance the possibility of receipt of a proton. It is expected that the proton is provided to the catalytic site through the K pathway similar to the MV CcO. The intermediate 9 is an optimized geometry where the proton is trapped on −CH2OH with decreasing the energy by 0.394 au from 8, compared with 0.299 au from 2 to 3. It is found from Table 3 that W1 approaches to the proximal O8 atom of FeOO with shortening the distance of O8−O(W1) from 3.073 to 2.593 Å. The distance of O2−O(W1) is also shortened from 2.891 to 2.481 Å. These show that the hydrogen bonds are made stronger among phenol, W1, and O8 of FeOO.

Similar to the variation from 3 to 4 in MV CcO, the H atom of Tyr244 was moved toward O of W1 from b. It is found from Supplementary Figure S7 that the relative energy is rapidly decreased in the region from 1.3 Å to 1.2 Å, being similar behavior to the rapid decrease from 1.4 Å to 1.3 Å to yield FeOOH (4) from FeOO (3) in the MV CcO. In this region, the H atom of W1 transfers as a proton to FeOO (9) to give FeOOH (10). This structural change is fairly similar to that found in MV CcO. The spin populations of Fe and OO are rapidly increased from 0.3 to 0.95e and decreased from 0.9 to 0.1e, respectively. On the contrary, the oxidation state of the Cu atom maintains Cu(I). This shows that an electron of Fe transfers to the OO moiety to make a paired spin with an unpaired electron of OO. Namely, the proton transfer occurs concerted with the electron transfer from Fe to OO, being different from the electron transfer from Cu to OO in MV CcO. From the small activation energy, the proton from K-pathway transfers to Tyr244 without the capture on −CH2OH. The formed 10 is 55.3 kcal/mole lower than 9 and has an unpaired spin localized on the Fe atom in 10. At this stage of the reaction, the OOH moiety receives two electrons.
and one proton necessary for performing the reduction of the O₂ molecule. (Supplementary Figure S7).

In order to yield the FeOOH (10) from FeOO (2), the electron and proton were sequentially added. However, the alternative path to obtain 10 can be considered, as shown in Scheme 2. The path from 2 to 10 through 8 and 9 has been mentioned in this section. The path from 2 to 4 through 3 was also mentioned as a path of the MV CcO in the preceding section. The catalytic site of 4 is simply presented by \([\text{Fe(III)}\cdot\text{OOH}^-\cdot\text{Cu(II)}]\). The \([\text{Fe(III)}-\text{OOH}^-\cdot\text{Cu(I)}]\) (10) can be easily obtained by addition of an electron to the catalytic site of 4 from the heme a site, if the added electron occupies the 3d orbital of the Cu atom. Actually, in our calculation, the addition of an electron to 4 gave 10 with decrease of the energy by 0.2588 au, as found from Table 2. We would like to discuss later which path is favorable.

The geometry 11 is an intermediate where a proton from the K-pathway is trapped on \(\text{CH}_2\text{OH}\), corresponding to 5 in formation from FeOOH to FeO + H₂O. Similar to the proton transfer from 3 to 4, the H-atom on \(\text{CH}_2\text{OH}^+\) was shifted toward O(Tyr244) from the geometry of 11. Supplementary Figure S8 shows the change of the relative energy. In contrast with the rapid decrease of energy from 3 to 4, the change of energy shows the smooth curve to connect continuously to the state 12 with the activation energy of about 6.4 kcal/mol.

The intermediate 12 is 14.6 kcal/mol lower than 11, which is remarkably smaller than 33.5 kcal/mol from 3 to 4. (Supplementary Figure S8)

The 12 has the structure of FeOOH₂ where H is added to FeOOH of 11. The charge population of the porphyrin ring and OOH moiety changes from −0.413 to −1.035e and from −0.583 to −0.065e, respectively. The spin populations of the porphyrin ring and OOH moiety do no change. This shows that the added H to FeOOH is a proton without any electron transfer. The OO scission does not occur with the OO distance of 1.484 Å which is slightly longer than 1.453 Å in the state 11, in contrast with the OO-bond cleavage on the naked heme by receiving two electrons and two protons [80]. This might be due to the hydrogen bond to W₁ which is hydrogen-bonded to His290 and Tyr244.

We cleave the OO bond from the geometry of 12. Supplementary Figure S9 shows the changes of the relative energy. The energy gradually increases with breaking the OO bond and decreases through the maximum point at \(R_{OO} = 1.9\) Å. On the optimization at \(R_{OO} = 2.0\) Å using the geometry optimized at \(R_{OO} = 1.9\) Å, the energy was rapidly decreased.

As can be seen in Supplementary Figure S10, the difference in two geometries at \(R_{OO} = 1.9\) and 2.0 Å is found in directions of OH bond in OOH₂. The OH of H₂O faces to the Cuₙ site at \(R_{OO} = 1.9\) Å, while the OH faces to the porphyrin ring at \(R_{OO} = 2.0\) Å. Using the optimized geometry and the molecular orbital at \(R_{OO} = 2.0\) Å, we carried out again the geometry optimization at \(R_{OO} = 1.8\) Å. The geometry where the OH faces to the porphyrin ring was obtained. It possesses 5.1 kcal/mol lower in energy than the original geometry where OH faces to the Cuₙ site. Decreasing the OO distance from geometry at \(R_{OO} = 1.8\) Å, the potential energy curve crosses with the original curve at \(R_{OO} = 1.7\) Å and has a maximum at \(R_{OO} = 1.65\) Å. Finally the minimum energy point, 12a, was obtained with the OO distance of 1.489 Å, comparable with 1.484 Å of 12. The 12a is only 2.4 kcal/mol higher than 12, showing that 12a has higher possibility to cleave the OO bond because of lower activation energy of 2.3 kcal/mol than 7.6 kcal/mol from 12. From Tables S4, the charge and spin populations of 12a are the same as those of 12. The change of direction of the OH bond from 12 to 12a has small activation energy of 3 kcal/mol, showing the possibility to easily convert from 12 to 12a before the OO bond breaking.

On the other hand, the increase of the OO bond length leads to the monotonous decrease of the total energy and finally the intermediate 13 was obtained as a minimum geometry. The 13 is 25.1 kcal/mol lower in energy than 12a. Interestingly, it is apparent from the geometry shown in Figure 2 that the first H₂O molecule is formed with small activation energy of 2.3 kcal/mol. The spin populations of the FeO moiety formed are 2.081e (= 1.304e(Fe) + 0.774e(O)), showing that the Fe=O moiety has two unpaired spins with parallel direction. The spin populations of the Cuₙ site grow up from zero value to −0.711e in negative value. The spins of Fe \((S = 1)\) and Cu \((S = 1/2)\) are antiferromagnetically coupled, in agreement with experimental proposal [32]. This shows that the oxidation state of the Cu atom alters from Cu(I) to Cu(II) with loss of one electron, consistent with change of the charge population of the Cuₙ site from 1.095e to 1.536e. Therefore, it is formally considered that one electron of Fe(III) transfers to the O atom and one electron of Cu(I) also transfers to the O atom to yield the Fe(IV)=O²⁻ bond. Accordingly, the heme a₃ of 13 is a compound II, even that the porphyrin ring has small spin population.

Figure 4 summarizes schematically the energy variations for the formation of first H₂O molecule from 11 to 13. At the early stage of the reaction from 11, the proton transfers to FeOOH to yield 12 (FeOOH₂) through the K-pathway with the activation energy of 5.4 kcal/mol and exothermic energy of 14.6 kcal/mol. After 12 was formed, 12 is converted to 12a with the rotation of OH in FeOOH₂ in order to connect smoothly to 13. The rotation barrier is estimated to be 3.3 kcal/mol, showing that the transition state is extremely lower than 11. Consequently, the OO bond cleavage is induced to produce first H₂O molecule with small activation energy of 2.3 kcal/mol and exothermic energy of 25.1 kcal/mol. The rate determining step of the reaction from 11 to 13 is the first one from 11 to 12. Since 12, 12a,
and 13 are lower in energy than 11, this reaction easily proceeds when the catalytic site captures one proton from the K-pathway. It is probably considered that the rotation of OH and cleavage of the OO bond occurs concertedly without forming 12 and 12a to yield the desired H₂O molecule.

3.3.2. Second H₂O Formation (14–17 in Figure 2). As mentioned above, three electrons from heme a, Cu, and Fe and two protons from the K-pathway have been used to produce a first H₂O molecule. Thus, second H₂O molecule should be produced by remain one electron and two protons. It has been shown in a recent study [57, 58] that the D-pathway links to the catalytic site through the hydrogen-bond network of water molecules. The oxidation state of Cu in 13 is an oxidized Cu(II). As discussed in Section 3.1.1, the oxidized Cu(II) has possibility of fourth ligand of the H₂O molecule. Thus it is reasonable to consider that the D-pathway is open for the hydrogen-bond pathway connecting to the Cu atom in the CuB site to make coordination of H₂O to Cu after the intermediate 13 was formed.

The structure where the H₂O molecule (W₃) coordinates to the Cu atom is shown as 14 in Figure 2. From charge and spin populations shown in Supplementary Table S5, it is found that the electronic structure of 14 is similar to that of 13, even though the spin population on the Cu atom is slightly enhanced. The distance between Cu and O of the coordinating H₂O (W₃) is estimated to be 2.037 Å, in good agreement with 2.062 Å of the Cu₉₃ site model in Figure 3. The distance between H of W₃ and Oₕ of Fe=Oₕ in heme a₃ is estimated to be 1.592 Å, longer than 1.435 Å given in the previous work [79]. However, 1.592 Å is slightly shorter than the standard hydrogen-bond distance. Thus, the added W₃ coordinates to Cu in the CuB site and makes the hydrogen bonding to Fe=O in heme a₃, simultaneously. The Fe=O distance of 1.676 Å is unchanged from 1.660 Å in 13 upon the addition of H₂O molecule.

The last one electron of four electrons necessary for the reduction of O₂ was added to 14 without changing the geometry of 14. The subsequent geometry optimization induces the proton transfer from W₃ on Cu to Oₕ of Fe=Oₕ, giving 15 with FeOH in heme a₃. Obviously, the one-electron reduced 14⁻ is a transient state on the potential energy surface. The Fe=O and O–H distances of FeOH are given to be 1.841 Å and 0.991 Å, respectively, showing formation of a strong OH bond on Fe. The ⟨S²⟩ value of 15 is found to be 2.0192, being close to 2.0 of the pure triplet spin-state. The spin population of Fe plus OH(Fe) is 1.047e, while that of the CuB site (CuB plus OH(W₃)) is 1.001e, indicating that two up-spins are localized on Fe and Cu, respectively. Thus, the oxidation state of the Fe atom changes from Fe(IV) of 14 to Fe(III) with keeping the oxidation state of Cu(II). However, the spin population on Cu is discontinuous because of the change from negative value of 14 to positive one of 15, implying the spin-flip on Cu from 14 to 15. In the 14⁻ state, the added electron occupies 3d orbital of the Cu atom to change the oxidation state from Cu(II) to Cu(I), while the Fe=O moiety keeps two parallel up-spins. With the proton transfer from W₃, an electron of the down-spin in the Cu atom simultaneously transfers to Fe=O, yielding the Fe(III)-OH⁻ and Cu(II)-OH⁻ of 15. This concerted proton-electron transfer gives the continuous change from the 14⁻ state to 15.

Two protons remain to produce a second H₂O molecule from the intermediate 15. One proton was added to OH⁻ on Cu in 15 under the assumption that the proton enters the catalytic site through the D-pathway. This 15H⁺ is also a transient state on the potential energy surface. The geometry optimization leads to the proton transfer from the formed H₂O (W₃) to FeOH, yielding the H₂O molecule on the Fe atom as a second productive H₂O, as shown in 16. Compared the charge and the spin populations of Cu and Fe in 15 and 16, those stay invariant through the proton transfer, showing that the oxidation states of Cu and Fe remain unchanged from Cu(II) and Fe(III). Accordingly, these features show that the change from 15 to 16 is a simple proton transfer without electron transfer, being different from the concerted proton-electron transfer from 14⁻ to 15.

In the structure of 16, the distance of Fe and the formed H₂O is given by 1.928 Å, showing that the formed H₂O is weakly bound to heme a₃. This is due to the strong attraction of the hydrogen bond to OH⁻ coordinating to the Cu atom.

At this stage of the reaction, the aimed second H₂O molecule has been produced, although the fourth proton still remains unused for the reduction in the catalytic site. It is reasonable to consider that the fourth proton enters to neutralize the OH⁻ on Cu through the D-pathway. The neutralized geometry is shown as 17. The second H₂O molecule is slightly separated from Fe with changing the distance from 1.928 Å in 16 to 2.057 Å in 17. The Fe and Cu atoms are oxidized with the oxidation states of Fe(III) and Cu(II), compared with reduced states of Fe(II) and Cu(I) at the starting point of 1. The state 17 is a triplet spin-state with the ferromagnetic coupling of two unpaired spins on Fe and Cu. At this stage of the reaction, the O₂ molecule is
reduced to two H$_2$O molecules by four electrons and four protons.

3.3.3. Catalytic Cycle (17–19, 1). Both the Fe and Cu atoms should be reduced to complete the catalytic cycle, since the Fe and Cu atom in 17 are oxidized. At this stage of the reaction, the formed two water molecules will be excluded from the catalytic site. The geometry without two H$_2$O molecules is shown as 18 in Figure 2. The Cu-O(W$_3$) distance does not alter from 2.068 Å of 17 to 2.042 Å of 18. After exclusion of two water molecules, it is expected that two electrons are sequentially put into the catalytic site from heme $a$ in order to reduce both Fe and Cu atoms. The first electron occupies the 3d$_z$ orbital of the Fe atom not the 3d$_{yz}$ orbital, giving the intermediate 19 with three parallel spins localized on Fe and Cu. 19 is a quartet spin-state.

The second electron occupies 3d orbital to reduce the Cu atom and simultaneously the W$_3$ coordinated to the Cu atom is released because of Cu(I), leading to the closure of the D-pathway. Finally, the reduced catalytic site of 1 reverts to perform the next reduction of the O$_2$ molecule. At this stage, the catalytic cycle of FR CcO is completed.

4. Summary of Reduction Mechanism

As shown in Scheme 1, several intermediates have been experimentally observed in the reduction of O$_2$ molecule. Their intermediates have been assigned by R, A, and PM in reduction by MV CcO, while R, A, PR, F, and O have been assigned in this order through the catalytic reaction by FR CcO. There is consensus that the intermediate R is composed of the reduced Fe(II) and Cu(I) and A has the structure where the O$_2$ molecule is bound to Fe in heme $a_3$. However, the proposals for structures of PR, F, and O are in debate.

Summarized in Figure 5 are the schematic structures which have been proposed by experimental studies up to now. HOY shows a neutralized Tyr244, while ·OH and ·O$_2$ shows a deprotonated Tyr244 and a neutralized Tyr244 radical, respectively. Also shown in the parenthesis are total charge and spin multiplicity that are estimated from the proposed structure. For each of PR, F, and O, those are apparently conflicting in the state of the Cu$_B$ site including Tyr244. These might be from speculation due to the fact that the Cu$_B$ site is silent for observations of EPR and spectroscopy and the phenol has properties of a proton and electron donors. Several points are, however, common for PM, PR, F, and O. The heme $a_3$ moiety has the electronic structure of Fe(IV)·O$_2$– and compound II where the porphyrin ring is neutral. In the bond of Fe(IV)·O$_2$–, two spins are coupled ferromagnetically.

In this work, we theoretically examined the reduction mechanisms of O$_2$ molecule at the catalytic sites of MV and FR CcOs. Our mechanisms are summarized in Scheme 3. The intermediate A (2) is produced by binding O$_2$ on Fe of heme $a_3$ in the reduced state R (1). The reduced Fe atom is oxidized and an electron of Fe transfers to dioxygen, yielding Fe(III)-OO$^-$ in the singlet biradical state. For MV CcO, after A is formed, two protons are sequentially added to the catalytic site through the K-pathway. The first proton transfer provides the Fe(III)OOH$^-$ (4) with simultaneous electron transfer from Cu(I) to FeOOH. The activation energy for the proton transfer was estimated to be 7.4 kcal/mol. The addition of the second proton leads to the OO bond cleavage to produce the H$_2$O molecule (7). As shown in Supplementary Figure S5, the recombination of the electronic structure occurs at the catalytic site, in good agreement with the observation that the reduction in MV CcO is 5-6 times slower than in FR CcO [2, 3, 32]. The heme $a_3$ is a compound I with a radical cation of the porphyrin ring, consistent with the experimental result of the time resolved Raman spectroscopy [109, 110]. We assign the intermediate 7 as PM, being in conflict with PM1 [22, 32, 43, 44, 74, 83, 115] shown in Figure 5. The intermediate PM1 is obtained under the consideration that the proton transfers from Tyr244 to FeOO to yield hydroperoxide and subsequently one electron transfer from Cu$_B$ is induced to cleave O–O bond [3]. Then third electron transfers from Tyr244 to FeOO to yield Fe(IV)·O$_2$– and tyrosyl radical. As shown in 4, on the pathway that the Fe(III)OOH$^-$ is formed, an electron certainly transfers from Cu(I) to FeOOH. However, the cleavage of the OO bond does not occur in 4. The OO bond breaking necessitates the addition of one proton to Fe(III)OOH$^-$ in the reaction from A to PM1 is endothermic. The distance between H of phenol and O$_b$ of FeOO is estimated to be 4.697 Å, which is too far to perform the proton transfer. The reasonable distances for the proton transfer are in the range of 1.4–1.8 Å. At least
one more H\textsubscript{2}O molecule is necessary to induce the proton transfer between FeOO and Tyr244 [70, 71]. Even the H\textsubscript{2}O molecule(s) are added, the endothermicity of the reaction will not be changed. In addition, the phenoxy radical has π-character, not σ-character. The pathway interacting with the π-orbital of the phenoxy radical is necessary to induce the smooth electron transfer from Tyr244 to FeO. However, it is not expected from the structure of the catalytic site.

It can be thought in our examinations that the intermediate A is a branching point to divide mechanisms of MV and FR C\textsuperscript{c}Os. For FR C\textsuperscript{c}O, before two protons transfer from the K-pathway, an electron transfers from heme \textit{a} to the catalytic site with changing the oxidation state of the Fe atom from Fe(III) to Fe(II). After the state \textit{8} was formed, two protons sequentially transfer from the K-pathway to reduce the dioxygen of FeOO. As discussed in Scheme 2, there is an alternative pathway that the order of the electron and proton transfer is reversed. The addition of an electron to \textit{4} leads to \textit{10}. The path from \textit{2} to \textit{10} through \textit{4} has the proton acceptability (proton affinity) of 0.2989 au (Table 2) from the K-pathway and the activation energy of 7.4 kcal/mol for the proton transfer in the catalytic site. The addition of an electron to the intermediate A to provide the state \textit{8} induces decreasing the energy by 0.1291 au (positive electron affinity). The state \textit{8} has the higher proton acceptability of 0.3938 than 0.2989 au from A to 3. There is no activation energy (~0.2 kcal/mol) for the proton transfer to produce the state \textit{10}. Thus, the path A–8–10 is preferable to the path A–4–10, in agreement with the experimental proposal that an electron transfers from heme \textit{a} to heme \textit{a}\textsubscript{3} after the intermediate A is formed [61, 64].

We assign the intermediate \textit{13} as P\textsubscript{R}, being in conflict with P\textsubscript{R}1 [32, 43, 74] and P\textsubscript{R}2 [22, 44, 56, 83, 115] except for Fe(IV)\textsuperscript{=}O\textsuperscript{2–} in compound II. In the Cu\textsubscript{B} site, the \textit{13} has the oxidized Cu(II) without any ligand, while both P\textsubscript{R}1 and P\textsubscript{R}2 have a hydroxy anion. P\textsubscript{R}2 has the −OY anion at the site of Tyr244 and is connected by addition of one electron to PM1. As mentioned above, these conflicts are originated by differences of the procedure of the proton donation to the dioxygen. HOY plays roles of the donations of a proton and an electron to FeOO in P\textsubscript{R}1 and P\textsubscript{R}2, while HOY transfers the proton from the K-pathway without any electron transfer in our PR. When \textit{8} changes to \textit{9} in which the proton was trapped on –CH\textsubscript{2}OH of heme \textit{a}\textsubscript{3}, W\textsubscript{1} approaches to both FeOO and HOY to make the strong network of the hydrogen bonds from the K-pathway to FeOO through W\textsubscript{2}, –CH\textsubscript{2}OH, HOY, and W\textsubscript{1}. Thus, W\textsubscript{1} moves to help the transportation of the proton from the K-pathway to the dioxygen moiety.
In the process of formation of first H$_2$O molecule (2, 8–13), since $W_1$ walks around the space composed of FeOO, HOY, and His290, $W_1$ will not be detectable in the X-ray crystallographic measurement [7–12]. It is thought that the first H$_2$O molecule cannot be formed without $W_1$, being consistent with the observation that the reduction does not proceed by the mutation of His290 [51–54]. Therefore, $W_1$ plays a crucial role for the formation of the first H$_2$O molecule in the reduction of O$_2$ molecule, while HOY plays a role of a relay point for the proton transfer from the K-pathway to the dioxygen.

The oxidation state of the Cu atom maintains the reduced Cu(I) on the path from the intermediate A to PR, and Cu is oxidized to Cu(II) at the formation of the intermediate PR. During this process, the Fe atom receives an electron from heme $a$ and delivers the electron to the OO moiety. The intermediate PR is stabilized by the release of the energy of the steric hindrance with the change of the oxidation state of the Cu atom from Cu(I) to Cu(II), as can be seen from Supplementary Figure S1. Then the Cu atom has high potentiality of the coordination of the H$_2$O molecule, as shown in Figure 3. At this stage, the D-pathway is open. An H$_2$O molecule coordinates to the Cu atom, giving 14. We assign the intermediate 14 as F.

For F, F1 [32], F2 [22], F3 [19, 44, 83], and F4 [43, 74] are proposed based on the spectroscopic observation. These proposed four structures have a common Fe(IV)=O$^{2-}$, being coincident with our F. The Cu$_B$ and tyrosine sites are, however, different. F4 is a transient state that is on the path from 14 to 15 in our reduction process. When the Cu atom is reduced, immediately the proton transfers from the coordinating H$_2$O to FeO with the simultaneous electron transfer from Cu(I) to FeO, giving 15. It is surprising that F3 is similar to PR. We could not make a comment which is assigned to PR or F. F2 is obtained by the addition of the proton to PR [22]. Our F has a neutralized HOY with the total charge of two, which is different from $^-YO$ of F2. F1 coincides with our F. We would like to consider that this is an accidental agreement, because F1 is thought to be obtained by addition of the proton to PR. However, it might be concluded that the intermediate F has the structure such as F1 and our F. Our assigned F has the same oxidation state as PR, in agreement with the proposal that the PR $\rightarrow$ F transition is not coupled with the electron transfer [61].

Sequential additions of one electron and two protons lead to the intermediate 17 through 15 and 16. Interestingly, 15 is the same as O2 shown in Figure 5 [44, 115]. Also 16 is the same as O3 [43, 74, 83]. Similar to the sequential addition of protons in the process from 8 to 10, 15 and 16 will not be detectable. Since it could be considered that 17 is stabilized, we assign 17 as O. For the process of the formation of the second H$_2$O molecule (14–17), the Cu atom is maintained the oxidized Cu(II), in interest contrast with the process of the formation of the first H$_2$O molecule (8–13) that the Cu atom is maintained the reduced Cu(I). Further, through the formation of two water molecules, the Fe atom is always the oxidized Fe(III) except for the intermediate 8 of Fe(II), and the intermediates 13 and 14 of Fe(IV)=O$^{2-}$. The 18 is obtained by removing two produced H$_2$O molecules from 17 to the outside of the catalytic site. However, the possibility that 18 is assigned as O is left.

Four electrons and four protons are used to produce two water molecules for the reduction of the oxygen molecule in the catalytic site of FR CcO. Two of four protons are provided from the K-pathway to produce the first water molecule, while the remainder two protons are from the D-pathway to produce the second water molecule. In our reduction mechanism, the K-pathway is ahead of the D-pathway, in conflict with the reversed order proposed from the experiments [44, 60, 61]. Our mechanism is, however, consistent with the recent observation that mutations in the K-pathway slowed down the formation of the PR intermediate [64].

5. Concluding Remarks

We have examined systematically the reduction mechanisms of the oxygen molecule in the mixed-valence and fully reduced CcOs and shown consistently the catalytic cycle based on the theoretical calculations. The $W_1$ added to the catalytic site plays crucial roles for the production of the first water molecule. However, $W_1$ is not observable for the X-ray crystallographic measurement due to the rapid motion in the catalytic site. The $W_3$ coordinated to Cu also plays crucial roles for the production of the second water molecule. The Cu atom is an electron storage during the formation of the first water molecule, while the Cu atom keeps the oxidized state of Cu(II) during the formation of the second water molecule. Some aspects of our mechanism are in good agreement with the experimental proposals, but some aspects are in disagreement. In our mechanism, Tyr244 plays a relay for the proton transfer from the K-pathway to the dioxygen moiety. It is unreasonable that Tyr244 is the proton and electron donors, since the distance between Tyr244 and the dioxygen moiety is too long. The K-pathway functions for the formation of the first water molecule, while the D-pathway functions for the second molecule. This order is reversed in the experimental proposal.

We have examined the bovine CcO which belongs to the A1 family $aa_1$ [17, 18]. The A1 family has the K- and D-pathways for the proton channels, while the B family $ba_1$ has only the K-pathway. Actually, in our trial calculation for 1XME of the B family (not shown here), the proton does not easily transfer from H$_2$O of the Cu$_B$ site to Fe=O on the way from 14 to 15 [74]. The reduced 14$^-$ is not a transient state on the potential energy surface. This may indicate that the D-pathway proposed by us is not available for the second water molecule, consistent with characteristics in the B family. It is probable that the reduction mechanisms of the A1 and B families are different. Thus, it can be thought that the A1 family should be at least distinguished from the B family.

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