The Protein Effect in the Structure of Two Ferryl-Oxo Intermediates at the Same Oxidation Level in the Heme Copper Binuclear Center of Cytochrome c Oxidase*

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Identification of the intermediates and determination of their structures in the reduction of dioxygen to water by cytochrome c oxidase (CcO) are of fundamental importance to understanding both O2 activation and proton pumping by the enzyme. In this work, we report the products of the rapid reaction of O2 with the mixed valence form (Cu2+, heme a3+, heme a32+-Cu4+) of the enzyme. The resonance Raman results show the formation of two ferryl-oxo species with characteristic Fe(IV)=O stretching modes at 790 and 804 cm−1 at the peroxy oxidation level (P_M). Density functional theory calculations show that the protein environment of the proximal H-bonded His-411 determines the strength of the distal Fe(IV)=O bond. In contrast to previous proposals, the P_M intermediate is also formed in the reaction of Y167F with O2. These results suggest that in the fully reduced enzyme, the proton pumping P → F transition is triggered not only by electron transfer from heme a to heme a3 but also by the formation of the H-bonded form of the His-411-Fe(IV)=O conformer in the proximal site of heme a3. The implications of these results with respect to the role of an O=Fe(IV)-His-411-H-bonded form to the ring A propionate of heme a3-Asp-399-H2O site and, thus, to the exit/output proton channel (H2O) pool during the proton pumping P → F transition are discussed. We propose that the environment proximal to the heme a3 controls the spectroscopic properties of the ferryl intermediates in cytochrome oxidases.

Dioxygen activation and reduction by the respiratory enzyme cytochrome c oxidase (CcO) are of fundamental importance in bioenergetics and cell respiration (1–26). The enzyme uses four redox-active centers, CuA, heme a, heme a3, and CuB, to sustain mitochondrial electron transport by reducing O2 to H2O. This catalytic strategy provides an effective means by which to couple the free energy available in late oxygen intermediates to the proton pumping of the enzyme (27–31). Establishing the structures of the intermediates subsequent to O=O bond cleavage is essential to understanding the linkage of these events with proton transfer. The O=O bond cleavage mechanism by CcO and the structures of the intermediates have been a matter of considerable debate (11–29).

Resonance Raman (RR) spectroscopy has been considered as a reliable technique for identifying the structure of the intermediate species (11–29), and time-resolved resonance Raman has been used to monitor the kinetics of the formation and decay of the various intermediates (18, 19, 24, 25). This way, the relationships between the electron transfer events and the formation of each intermediate, which are required to clarify the molecular mechanisms of the O2 reduction, was monitored in the ns-ms time scale. Some of the structures were inferred based on observed kinetic changes, and others were proposed based on spectroscopic properties. Since the characterization of the P (607 nm) intermediate in the reduction of O2 to H2O by CcO as a ferryl-oxo species, several structures and reaction mechanisms have been proposed (20–29). There is a consensus now that the O=O bond cleavage takes place when the binuclear heme Fe-CuB center is reduced, and recently, it has been

* This work was supported by funds from the Cyprus Research Promotion Foundation (to C. V.) (TEKOLOGIA/THESIPS/0609/BE/05), the Minister of Science, Sports and Culture, Japan (Grant 14001004) (to T. K.), and Deutsche Forschungsgemeinschaft (DFG) (Grant SFB472) (to B. L.).
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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 28, pp. 20261–20266, July 12, 2013 © 2013 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
suggested that the additional electron, which is needed to produce the so-called P (607 nm), Fe(IV)=O intermediate, is provided by either a tyrosine or a tryptophan residue (29−31). The determination of the structure(s) of the final product of the reaction was provided by either a tyrosine or a tryptophan residue (29−31). The determination of the structure(s) of the final product of the reaction was provided by either a tyrosine or a tryptophan residue (29−31). The determination of the structure(s) of the final product of the reaction was provided by either a tyrosine or a tryptophan residue (29−31). The determination of the structure(s) of the final product of the reaction was provided by either a tyrosine or a tryptophan residue (29−31). The determination of the structure(s) of the final product of the reaction was provided by either a tyrosine or a tryptophan residue (29−31).

EXPERIMENTAL PROCEDURES
Cytochrome c oxidase was purified from P. denitrificans according to published procedures (27, 28, 31). The enzyme was concentrated to 150 mM with O2, which is linked to the proton pumping function of the enzyme, may resolve controversial aspects of the O2 mechanism. We have studied the reaction of mixed valence MV-CN (Cu2+, heme a3+, heme a3+, CuB+) from Paracoccus denitrificans with O2 by RR. Our results show, for the first time, the presence of two ferryl-oxo intermediates with characteristic frequencies at 790 and 804 cm−1 at the same oxidation state of the heme Fe-CuB center. We attribute the 14 cm−1 frequency difference between the ferryl-oxo species to variations in the basicity (H-bonded versus non-H-bonded conformers) of the His-411 ligand proximal to the heme a3 (Fig. 1), induced at the point of the O–O bond cleavage process. Density functional theory calculations showed that the H-bonded properties of the proximal His-411 control the strength the Fe(IV)=O bond, and the strength of the H-bonding is controlled by the distance between the heme Fe(IV)=O and CuB. In addition, the DFT data excluded the formation of a His-Fe(IV)=O species or that of a heme macrocycle π cation radical Fe(IV)=O structure for the P4 (804 cm−1) intermediate.

Density Functional Theory (DFT)-based Geometry Optimizations and Frequency Calculations were performed for all models by the TURBOMOLE software package on the same level of theory to derive vibrational frequencies (32). A force module of TURBOMOLE used calculated analytically harmonic vibrational frequencies within the Hartree–Fock (HF) or (RI)DFT methods for closed shell and spin-unrestricted open shell systems. RI was only used partially, which means that the resulting Hessian is only a (very good) approximation to exact second derivatives of the (RI)DFT energy expression. A standard force constant calculation predicted all allowed and forbidden vibrational transitions.

In our DFT studies, we used a CuB(II) and a Fea3(IV) site, giving a (+1) overall charge. A doublet state was assumed for the binuclear ferryl Fe(IV)-Cu(II) models. It should be noted that a quartet state leads to a bridged Fe-O-CuB species following a histidine dissociation. For each structure considered, a full geometry optimization was performed using the density functional BLYP method (33, 34). The TZVP triple-ζ valence basis set augmented with polarization functions p on hydrogen and d on first and second row atoms, as implemented in the TURBOMOLE version 5-8-0 software package, was used (32). For the iron and copper metals, an effective core potential (ECP) was used. It is named “ecp-10-mdl” for iron and “ecp-18 arep” for copper. We implemented the computational advantage of the RI-J (Resolution of Identity) approximation as defined in the TURBOMOLE software package with default parameterization.

RESULTS AND DISCUSSION
Mixing of oxygen with the CO-bound MV oxidase yields long-lived oxygenated intermediate(s) that occur after the decay of the oxy intermediate (26). The visible region optical absorption spectra of the wild-type oxygenated species formed this way, and their time evolution is shown in Fig. 2A. The optical data demonstrate that incubation of the oxidized enzyme with CO in the presence of O2 generated the P inter-
mediate (607 nm), but not the F intermediate (580 nm). It also demonstrates that the 607 nm species was generated first and that the heme \( a_3 \) returned to the oxidized form without forming the 580 nm (F) species. It should be noted that in the reaction of oxidized CCo with \( H_2O_2 \), the decay of the 607 nm species to the oxidized form occurs through the 580 nm species (23, 28).

We have used continuous wave 428.7 nm RR excitation to detect the long-lived oxygenated intermediates. In Fig. 2B, we present low frequency RR spectra of the MV-CCo/\(^{18}O_2\) and MV-CCo/\(^{16}O_2\) reactions at 0–5 min at pH 7.5. The \( \text{inset} \) shows the difference a-b spectrum. Several 0–5 min spectra were collected and added for the \(^{16}O_2\) and \(^{18}O_2\) experiments. The enzyme concentration was 50 \( \mu M \), pH 7.5. The excitation wavelength was 428.7 nm, and the incident power was 1 milliwatt.

FIGURE 3. A, optical absorption difference spectra of the reaction products by direct mixing of \( O_2 \) with CO-MV (mixed valence) \( Y167F\ a_3\ oxidase from P. denitrificans \) minus the oxidized form of the enzyme at the indicated times subsequent to mixing, pH 7.5. The enzyme concentration was 5 \( \mu M \), and the path length of the cell was 0.5 cm. B, resonance Raman spectra of CO-MV \( Y167F\) \( a_3\ oxidase from P. denitrificans \) minus the oxidized form of the enzyme at the indicated times subsequent to mixing, pH 7.5. The enzyme concentration was 5 \( \mu M \), and the path length of the cell was 0.5 cm. The \( \text{inset} \) shows the difference a-b spectrum. Several 0–5 min spectra were collected and added for the \(^{16}O_2\) and \(^{18}O_2\) experiments. The enzyme concentration was 50 \( \mu M \), pH 7.5. The excitation wavelength was 428.7 nm, and the incident power was 1 milliwatt.

In the DFT calculations, the binuclear center was composed of \( \text{Cu}_{\mu}(\text{II}) \) and His heme Fe(IV)\( =O \) (Fig. 4). Due to the nature of these large sites, theoretical calculations were performed on simplified models with a restricted number of atoms. In these models, the \( \text{Cu}_{\mu} \) metal site was represented by imidazole ligands instead of histidines, and the cross-linked His-Tyr residue was represented by a cross-linked imidazole phenol unit. In the heme iron, the proximal histidine was represented by an imidazole ligand, and the heme was represented by a simplified porphyrin substituted with a \( \text{CH}_2\text{CH}_2(\text{OH})- \) group to account for the hydroxyethylfarnesyl interactions in the active site of CCo. We investigated the effect of H-bonding to the ferryl-oxo species that is provided by the distal environment of \( \text{Cu}_{\mu} \) and also by the H-bonding interaction of the proximal histidine with the protein environment. Binuclear models with zero (A1), one (A2), or two hydrogen bonds (A3) to the proximal histidine were treated along with a \( \text{Cu}_{\mu}(\text{II}) \) site coordinated to hydroxyl (–OH) group. One or two ethylic acids are modeled near the proximal histidine to reproduce the effect of H-bonding. No restrictions were applied in the models. Charge population analysis based on Mulliken approach was performed for the binuclear models, and the results are shown on selected atoms in Fig. 4. Population analysis allowed us to check the validity of the optimized structures for the intermediates under study. The calculated spins for iron and copper for all models (A1–A3) demonstrated that the heme iron was indeed in the +4 oxidation state and that copper was in the +2 oxidation state. These results were consistent with spin population analysis for ferryl-oxo species (35).

Structures A1, A2, and A3 differed in the distance of \( \text{Cu}_{\mu} \) from \( \text{Fe}_{\alpha3} \) (\(-4 \) Å) at a maximum of \( \pm 0.098 \) Å as \( \text{Cu}_{\mu} \) approached Fe(IV)\( =O \) and as the H-bonding interaction of proximal His changed from null to two. The TURBOMOLE software was applied for the calculation of \( \Delta \nu \) and as shown in Table 1, these values represented real shifts that do not suffer...
FIGURE 4. Geometry-optimized structures of ferryl conformations in the heme αβ-Cuβ binuclear center with different hydrogen bonding to proximal histidine. Spin (with charge in parentheses) population analysis based on the Mulliken approach is also shown for selected atoms. Numbering of amino acids is the same as in Fig. 1.

TABLE 1
Calculated frequencies (in cm⁻¹) in TURBOMOLE riBLYP/TZVP level are shown without and with (× 1.022) scaling factor. Scaling factor was chosen to derive the experimental 804 cm⁻¹ ν(Fe-O) vibrational frequency.

| Fe(IV) models | ν(Fe=O)² | ν(Fe=O) | ν(Fe=O) × 1,022 | ν(Fe=O) × 1,022 |
|---------------|----------|----------|-----------------|-----------------|
| Ferryl + 0× H-bond | 787 | 755 (Δν = 32) | 804 | 772 (Δν = 32) |
| Ferryl + 1× H-bond | 779 | 750 (Δν = 29) | 796 | 766 (Δν = 30) |
| Ferryl + 2× H-bond | 763 | 728 (Δν = 35) | 780 | 744 (Δν = 36) |
| Ferryl π-cation radical (triplet) | 774 | — | 791 | — |
| Ferryl π-cation radical (quintet) | 774 | — | 791 | — |

² No scaling.

TABLE 2
Calculated high frequency Raman frequencies in TURBOMOLE riBLYP/ TZVP level

| Fe(IV) (¹⁸O) models | ν₁₅⁰² | ν₂₅⁰² | ν₃₅⁰² | ν₄⁰² |
|---------------------|-------|-------|-------|-------|
| Ferryl + 0× H-bond | 1558  | 1508  | 1422  | 1373 (1173) |
| Ferryl π-cation radical (triplet) | 1561  | 1512  | 1411  | 1360 (1179) |
| Ferryl π-cation radical (quintet) | 1549  | 1509  | 1413  | 1329 (1176) |

² No scaling.

from accuracy restrictions of the method used because the peaks involved have large intensities. For example, the modes at 787 (A1) and 763 (A3) cm⁻¹ have intensities of 89.28 and 106.98 km/mol, respectively (significantly higher than adjacent normal modes). Calculated frequencies are shown with and without scaling in Table 1. The Fe-Cuβ distance calculated from the unconstrained group of models did not differ significantly from that observed in the crystal structures of CcO (7–9). Based on the crystal structures, the hydrogen bonds are asymmetric, and O–N(H) distances vary from 2.78 to 3.52 Å. In our models, the O–H(N) distances varied from 1.94 to 2.51 Å, representing the hydrogen-bonding network in vivo. Cleavage of the O–O bond was linked to an Fe(V) = O moiety. However, iron in +5 oxidation state was not stabilized and was converted to a more stable Fe(IV) π-cation radical iso-electronic configuration. Thus, in addition to oxo-ferryl intermediates (doublet species) with different proximal hydrogen-bonding strength presented above, we have investigated the possibility that either a porphyrin π-cation radical or an Fe(V) species appeared in the CcO catalytic cycle. Although π-cation radical models (triplet and quintet with total charge of +2) containing Fe(IV) and Cu(II) metal sites were optimized in geometry (riBLYP/TZVP), the DFT calculations gave no Fe(V) species in a scan of different multiplicities and geometries. These results are in agreement with those reported by Dey and Ghosh (36). They have reported that Fe(V) porphyrin complexes are rare and less stable by 3.7–5.8 kcal/mol as compared with π-cation radicals (A₂u, A₁u). A triplet iron-oxo π-cation radical gave a spin population 1.27 on iron and 0.42 on Cuβ, suggesting, based on Mulliken population analysis, Fe(IV) and Cuβ(II) oxidation states. A spin population of 0.77 was found on the porphyrin ring. For the quintet iron-oxo π-cation radical, spin populations were calculated to be 1.24, 0.43, and 0.80 on iron, copper, and the porphyrin ring, respectively.

A close inspection of the data presented in Table 1 shows that the ν₁₅⁰(Fe-O) is mainly affected by the interaction of His-411 with the protein environment and by the formation of a π-cation radical. In the latter case, significant differences were calculated for the ν₂ and ν₄ porphyrin modes as shown in Table 2. Moreover, the ν(Fe(IV) = O) = 774 cm⁻¹ remained unaffected in the case of the two calculated π-cation radicals. In the case of a ferryl species, the calculated ν₁₅₀, ν₂, ν₄, and ν₄ modes shown in Table 2 are in agreement with those reported in the RR experiments (12, 19, 25). The His proximal to heme α₂ exerted similar distances to residues such as Gly, Thr, or His found in the proximal area on the crystal structures of different cytochrome oxidases (7–10). Although these H-bonding interactions appear rigid, we have observed their dependence on the protonation state of the heme α₁ propionate A/Asp-399 pair (37). In detail, based on these earlier simulations, we probed the His-411-Gly-386 and His-411-Thr-389 distances for the α₁, α₂ from the P. denitrificans model system. Molecular dynamics simulation trajectories exerted variable distances of H-bonding interac-
FIGURE 5. Correlation diagrams for selected interactions. Left panel: the combined mean value of His-411-Gly-386 distance and mean value for His-411-Thr-389 distance. Right panel: the effect of the variation in Fe(IV)=O (ferryl) and Fe-N (His-411) bond lengths on the ν(Fe-O) mode. The abbreviations used are: C, fully deprotonated propionate-A/Asp-399 pair; A, fully protonated propionate-A/Asp-399 pair; D, only the propionate-A is protonated; B, only Asp-399 is protonated; GH, Glu-278 is protonated.

In summary, the following points emerge from the present study. First, the detection of two ferryl-oxo intermediates at the same oxidation level (P) establishes crucial links between their structures. Second, it clearly demonstrates that the frequencies detected for the ferryl-oxo intermediates are independent of the formation of the P or F intermediate but rather depend on the equilibrium between the H- and the non-H-bonded O=Fe-His-411, yielding either the 790 cm⁻¹ or the 804 cm⁻¹ species. This idea finds support in the experiments on oxygenated MV intermediates. The present data indicate that the chemical energy produced by the cleavage of the O–O bond is stored in the chemical bonds of the oxygen reduction products in the heme \(a_3\)-Cu₅ center.

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Ferryl-Oxo Intermediates of Cytochrome c Oxidase

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