The Mg$^{2+}$-containing Water Cluster of Mammalian Cytochrome c Oxidase Collects Four Pumping Proton Equivalents in Each Catalytic Cycle*

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Bovine heart cytochrome c oxidase (CcO) pumps four proton equivalents per catalytic cycle through the H-pathway, a proton-conducting pathway, which includes a hydrogen bond network and a water channel operating in tandem. Protons are transferred by H$_2$O$^+$ through the water channel from the N-side into the hydrogen bond network, where they are pumped to the P-side by electrostatic repulsion between protons and net positive charges created at heme a as a result of electron donation to O$_2$ bound to heme a$_3$. To block backward proton movement, the water channel remains closed after O$_2$ binding until the sequential four-proton pumping process is complete. Thus, the hydrogen bond network must collect four proton equivalents before O$_2$ binding. However, a region with the capacity to accept four proton equivalents was not discernable in the x-ray structures of the hydrogen bond network. The present x-ray structures of oxidized/reduced bovine CcO are improved from 1.8/1.9 to 1.5/1.6 Å resolution, increasing the structural information by 1.7/1.6 times and revealing that a large water cluster, which includes a Mg$^{2+}$ ion, is linked to the H-pathway. The cluster contains enough proton acceptor groups to retain four proton equivalents. The redox-coupled x-ray structural changes in Glu$^{198}$, which bridges the Mg$^{2+}$ and Cu$_{a_1}$ (the initial electron acceptor from cytochrome c) sites, suggest that the Cu$_{a_1}$-Glu$^{198}$-Mg$^{2+}$ system drives redox-coupled transfer of protons pooled in the water cluster to the H-pathway. Thus, these x-ray structures indicate that the Mg$^{2+}$-containing water cluster is the crucial structural element providing the effective proton pumping in bovine CcO.

Cytochrome c oxidase (CcO)$^3$ reduces molecular oxygen (O$_2$) in a reaction coupled with a proton pumping process. After binding of O$_2$ to the O$_2$ reduction site (which includes two redox-active metal sites, heme a$_3$ and Cu$_{b_2}$), four electron equivalents are sequentially donated from cytochrome c via two additional redox active metal sites, Cu$_{a_1}$ and heme a. Each of the four electron transfers is coupled to the pumping of a single proton equivalent (1, 2).

High resolution x-ray structural studies together with mutational analyses for bovine CcO show a possible proton pumping pathway, known as the H-pathway, which includes a hydrogen bond network and a water channel functioning in tandem (1, 2). The water channel provides access of water molecules (or H$_2$O$^+$ ions) inside the mitochondrial inner membrane (the N-side) to one end of the hydrogen bond network that extends to the outside of the mitochondrial inner membrane (the P-side). The hydrogen bond network interacts tightly with heme a by forming two hydrogen bonds between the formyl group of heme a and Arg$^{386}$ in the hydrogen bond network and between the A-ring propionate of heme a and a fixed water molecule in the hydrogen bond network (1, 2). The net positive charge increase in heme a, which occurs upon electron donation from heme a to the O$_2$ reduction site to be delocalized to the formyl and propionate groups, leads to proton pumping through the hydrogen bond network by electrostatic repulsion (3).

Because the proton pumping is driven by electrostatic repulsion, backward proton leakage from the hydrogen bond network must be prevented to provide the unidirectional proton transfer required for efficient proton pumping. X-ray structural analyses show that such leakage is blocked by closure of the water channel and that the water channel is open only when heme a$_3$ iron (Fe$_{a_3}$) and Cu$_{b_2}$ are in the reduced and ligand-free state (4). Therefore, in each catalytic cycle, four proton equivalents must be collected and pooled in the hydrogen bond network before binding of O$_2$ to the O$_2$ reduction site (4). How

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The atomic coordinates and structure factors (codes 5B1A and 5B1B) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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$^3$The abbreviations used are: CcO, cytochrome c oxidase; A type CcO, CcO that has two proton-conducting pathways, K and D, both connecting the N-side to the O$_2$ reduction site; B type CcO, CcO that has only a single proton-conducting pathway, analogous to the K pathway; MR, molecular replacement; MR/DM, molecular replacement followed by density modification.
ever, a region with sufficient capacity to accept four protons has not been clearly detectable in any of the x-ray structures of the hydrogen bond network in the H-pathway reported thus far (4, 5). In other words, one of the critical structures for the proton pumping function of the H-pathway has not been identified, although the proton-pumping function of the H-pathway has been well established by mutational and theoretical analyses of bovine heart cytochrome c oxidase (6, 7).

An alternative proton pumping mechanism has been proposed for some of the bacterial A-type CcOs based on mutational analyses that indicate that one of the possible proton-conducting pathways connecting the N-side space with the O2 reduction site, known as the D-pathway, transfers both pumping and water-forming protons (1, 8). The A-type CcOs have a putative proton-conducting pathway structure highly similar to the H-pathway of mammalian CcOs. However, the proton pump function was not found to be influenced by mutations of the critical amino acid residues (9). The discrepancy between the mutational effects on the proton pumping activity between bovine and bacterial CcOs indicates that the proton pumping system of CcO is not completely conserved. The lower proton pumping efficiency of B-type CcOs also suggests that there is some diversity in the function of CcOs (10).

Here, we report a reexamination of x-ray structures of bovine heart CcO with improved resolution and demonstrate that a water cluster that includes a Mg2+ ion has sufficient proton storage capacity to store four proton equivalents and that this site can donate protons to the H-pathway from the water cluster coupled with electron transfer from CuA to heme a iron (Fea).

Results

Purified bovine heart CcO as isolated under aerobic conditions has a peroxide ion bridged between Fea3 and CuB. Not involved in the catalytic cycle, the CcO species is designated as the resting oxidized CcO (1, 2). The structures of the resting oxidized CcO except for the ligand structure in the O2 reduction site is highly likely to be identical with those of the oxidized CcO under turnover conditions (1, 2). In this paper, “oxidized/reduced” denotes fully oxidized resting (or as-isolated)/fully reduced (with dithionite), respectively.

Improvement of the Resolution of X-ray Structures—The significantly improved conditions for soaking crystals in antifreeze reagent as described under “Experimental Procedures” were found to be critical for determination of the structures of the oxidized/reduced forms of bovine CcO at 1.5/1.6 Å resolution. The x-ray structures in both oxidation states are highly isomorphous with each other, as indicated by the lattice constants listed in the legend to Fig. 1, which allow direct structural comparisons between the oxidized and reduced forms of CcO. An \( (F_{\text{ox}} - F_{\text{red}}) \) electron density map is superimposed with the main chain structures in the oxidized state in Fig. 1. This shows that most of the redox-coupled conformational changes at 1.6 Å resolution, as given in the two close-up views, are restricted to subunit I, the largest subunit of CcO, which contains the three redox active metal sites. The restriction of conformational changes is expected to contribute to the highly efficient energy transduction and to effective prevention of spontaneous exposure of the transition metals to O2, which could produce various reactive oxygen species.

The Locations of the Water Clusters—X-ray structures at 1.5/1.6 Å resolution identify three large water clusters within the positive side half of the CcO molecule (Fig. 2A). The two clusters, located on opposite sides of the Mg2+–containing water cluster (hereafter referred to as the “Mg/H2O cluster”), have no direct pathways for proton exchange with the Mg/H2O cluster. One of the water clusters includes a long array of water molecules. The shortest distance between the water cluster and the Mg/H2O cluster is 4.7 Å, as represented by the O–O bond distance between the two water molecules located at the closest positions between the two water clusters (Fig. 2B). Protons are unlikely to be exchanged between the two water molecules within the physiologically relevant time scale. In addition, proton exchange through a long array of water molecules is seriously suppressed by the hydrogen bonds between the water molecules and the protein moiety (11). The present x-ray structure indicates that all of the water molecules in this array are hydrogen-bonded to the protein moiety. Thus, this water array is unlikely to function as a proton-conducting pathway within the physiologically relevant time scale. It is likely that the water cluster strengthens the tight specific interaction between subunits I and II by providing a hydrophilic array in the middle of the hydrophobic surfaces of both subunits. A similar arrangement has been observed in immune systems (12). Contact between the Mg/H2O cluster and the other water cluster, which
is also open to the P-side aqueous phase, is blocked by two peptide main chains (Fig. 2C). The water-accessible surfaces, calculated using a probe of 1.0 Å radius after manually eliminating water molecules, indicate that contact between these three water clusters is not possible.

Although a channel-like structure is detectable between the P-side surface and the water-accessible surface of the Mg/H₂O cluster, the 1.0 Å probe analysis shows that the Mg/H₂O cluster has no direct contact with the P-side bulk water phase (Fig. 2D). Furthermore, the channel-like space is surrounded by a proline-rich protein moiety including Pro₁³₀, Pro₁³₁, Pro₂²₂, and Pro₂²₈ of subunit I and Pro₁⁷⁶ of subunit II (Fig. 2D). These residues are likely to stiffen the protein moiety, thereby contributing to effective blockage of water exchange between the P-side phase and the Mg/H₂O cluster.

The water-accessible surface for the O₂ pathway provided by the space connecting the O₂ entrance at the transmembrane surface of subunit III with the O₂ reduction site and the water molecule exit pathway from the O₂ reduction site has no direct contact with the surface of the Mg/H₂O cluster (the O₂ transfer function of the O₂ pathway has been shown by dicyclohexylcarbodiimide binding experiments (13)). The O₂/H₂O pathway is located close to the Mg/H₂O cluster near the two propionate groups of heme a₃, as shown in Fig. 2E. Although both of the carboxyl groups of the propionate groups of heme a₃ are connected to the water-accessible space of the Mg/H₂O cluster, the -CH₂-CH₂- moiety of the propionates and the surrounding amino acid residues provide a clear hydrophobic barrier between the water cluster and the O₂ pathway. The imidazole of His²⁹¹, one of the three imidazole groups ligated to CuB, is hydrogen-bonded to one of the water molecules (water 10) in the Mg/H₂O cluster (Fig. 2E). However, this imidazole group is tightly fixed by a π-π stacking interaction with Trp²³₆, which is fixed in turn by the phenol group of Phe²³₅, as shown in Fig. 2E (inset). Thus, the proton of the imidazole group is unlikely to be transferred to the Cu₃-effec site in the O₂ pathway. The structure suggests that a mobile water molecule in the O₂ pathway would not exchange protons with the Mg/H₂O cluster (Fig. 2E). The other two water clusters, which are located closer to the P-side surface than the Mg/H₂O cluster (as shown in Fig. 2A), are also unlikely to exchange protons with the water molecules in the O₂ pathway.

A possible water molecule exit pathway visible in our improved x-ray structure connects the transmembrane surface with the O₂ reduction site, as shown in Fig. 2E by a red arrow. The location of the exit in the transmembrane surface, which is covered by the fatty acid tails of phospholipids of the mitochondrial inner membrane, is probably to facilitate effective prevention of backward leakage of protons from the P-side to the O₂ reduction site. The water molecule exit pathway is unlikely to...
be used for collection of O₂ because dicyclohexylcarbodiimide modification of the O₂ pathway in subunit III has been confirmed to completely block the O₂ reduction function (13).

The Mg/H₂O cluster is connected to the H-pathway via a short hydrogen bond network, which includes the guanidino group of Arg⁴³⁹ and the two propionate groups of heme a, bridged by a fixed water molecule, which is indicated in Fig. 3 (gray shadow). The A-ring propionate of heme a is hydrogen-bonded to a fixed water molecule of the hydrogen bond network of the H-pathway, which is marked by the red curve in Fig. 3. The Mg/H₂O cluster is indicated with the blue area in Fig. 3. The terminal amino group of Arg⁴³⁹ receives protons from Glu¹⁹⁸(II) for proton transfer to the propionate.

The Redox-coupled Conformational Changes in the Mg²⁺ Site—The main chain carbonyl group and the side chain carbonyl group of Glu¹⁹⁸(II) are coordinated to the Cuₐ and Mg²⁺ sites, respectively (Fig. 4A). The Mg²⁺ ion has a hexacoordinate structure with Glu¹⁹⁸(II), Asp⁶⁹⁰, and His⁴⁳⁶ and three water molecules acting as ligands. Reduction of the Cuₐ site requires charge neutralization at the Cuₐ site ligands. The coordination structural change also induces redox-coupled structural changes of hydrogen bonds, as shown in Fig. 4B. In the oxidized state, the carbonyl group of Glu¹⁹⁸(II) is connected to the guanidino group of Arg⁴³⁹ with a bridging water molecule (water 1). A U-shaped hydrogen bond network including three fixed water molecules (waters 21, 15, and 11) is drawn at the 3.5σ level.

4 Amino acid residues included in subunit II are indicated with (II) following the residue numbers. The other residues given in this paper belong to subunit I. No subunit mark is given in the figures for the sake of simplicity.

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FIGURE 3. The structure of the short hydrogen bond network (gray area) connecting the Mg/H₂O cluster (blue area) with the hydrogen bond network (red curve) of the H-pathway. To improve clarity, only the hydrogen bonds in the short hydrogen bond network and the hydrogen bond network of the H-pathway are shown with broken lines. The Arg⁴³⁸ guanidino group, salt-bridged to the D-ring propionate of heme a (on the left pyrrole of heme a), is not shown. The structure of the Mg²⁺ site is the structure adopted when the enzyme is in the oxidized state. The red and blue curves denote the approximate location of the hydrogen bond network and the water channel of the H-pathway, respectively. The fifth ligands of hemes a and a₃, His⁴⁷⁶ and His⁴⁷⁶, both of which are included in helix X, are also shown.

FIGURE 4. The redox-coupled conformational changes in the Cuₐ and Mg²⁺ sites. The purple and blue structures indicate those in the oxidized and reduced states, respectively. A, the redox-coupled conformational changes in the Mg²⁺ site and Cuₐ site. To preserve clarity, only the water molecules (orange spheres) that are associated with the conformational changes are shown. The broken and dotted lines indicate hydrogen and coordination bonds, respectively. The redox-coupled coordination structural change occurring at the Glu¹⁹⁸ carboxyl group is shown in the inset. B, the redox-coupled changes in the hydrogen bond network structure connecting Glu¹⁹⁸(II) and Arg⁴³⁹. The color code for the oxidation state is identical to that of A. C, MR/DM map of the oxidized state is shown by a stereoscopic pair. The electron density cages of the same range as that of A are drawn at the 3.5σ level.
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and 18) and the Thr127 OH group is detectable between Glu199(II) and Arg439. The network extends to the two water molecules water 16 and water 11. Upon reduction, the water 1 bridge is replaced by the water 18 bridge, whereas the hydrogen bond between the Thr127 OH and water 18 is broken. The structural changes occurring upon reduction suggest that there is a significant decrease in proton transfer efficiency between Glu199(II) and Arg439.

The molecular replacement MR/DM map of Fig. 4C clearly shows the structure of Glu198(II) bridging CuA and Mg2+, oxygen atoms of water molecules, and some other residues. Structures of this region of the oxidized and reduced states were clearly distinguished from each other by comparing the MR/DM maps of the present resolution.

The Water Structure of the Mg/H2O Cluster—Fig. 5 (A and B) shows the arrangement of all water molecules in the Mg/H2O cluster in the oxidized and reduced states, respectively. Twenty-four water molecule sites were assigned in the Mg/H2O cluster in the MR/DM map and the \((F_o - F_c)\) map of the oxidized form. Two of the 24 sites (waters 19a and 19b) are close to each other and therefore represent multiple sites that do not coexist. In the reduced form, 22 water molecule sites were identified in the corresponding region. The peak heights and positions in the maps of the oxidized and reduced forms suggest that 15 sites (waters 1–15) are at full occupancy and are visible in both states; their B-factors are similar within each form. Their averaged B-factors are 21.64 ± 0.95 and 20.27 ± 0.94 Å² for the oxidized and reduced forms, respectively. To estimate occupancies of the other water molecule sites of each form, several sets of structure refinements were conducted, with water occupancies decreasing by 5% at each step (i.e. four steps, from 90 to 75%). The best occupancy was chosen when the resultant B-factor was closest to the averaged B-factor of each form. The occupancies and B-factors of both states are listed in Table 1. The number of water molecules in the Mg/H2O cluster of the oxidized form was found to be 20.75, essentially identical to the 20.30 water molecules identified in the reduced form. These results strongly suggest that there is no exchange of these water molecules with water molecules located outside of the cluster, consistent with the water-accessible surface analysis for the Mg/H2O cluster, as described above, supporting the absence of water exchange of the Mg/H2O cluster with water molecules outside of the cluster. On the other hand, changes in the occupancy and location of some of the water molecules located inside the cluster, which occur with changes in oxidation state, indicate that these water molecules are mobile and are able to easily participate in proton exchange within the water cluster (Fig. 5C).

Candidate Proton-accepting Sites in the Mg/H2O Cluster—The Mg/H2O cluster includes many protonatable amino acid residues and heme a₃ propionates, hydrogen-bonded to the water molecules of the cluster, as shown in Fig. 5D (to preserve clarity, water molecules are not shown). The four protonatable groups, Tyr129, Asp173(II), Asp369, and the A-ring propionate of heme a₃ are not directly ligated to any metal site, as indicated in Fig. 5D. In addition to these groups, His291 ligated to the Mg²⁺ is likely to accept protons reversibly because Glu198(II) and Asp369 neutralize the positive charge of the Mg²⁺. His291 is also likely to accept protons because three histidine residues, including His291, are ligated to Cuₜ, Arg338, which forms a tight salt bridge with the D-ring propionate of heme a₃, shows clear redox-coupled conformational changes (see below), suggesting significant conformational flexibility in the salt bridge. Thus, both groups could accept protons reversibly. Arg439, although defined as one end of the short hydrogen bond network (Fig. 3), is directly hydrogen-bonded to water molecules in the Mg/H₂O cluster (Fig. 4B). Thus, the basic residue also increases the proton-accepting capacity of the cluster.

Two propionates in the short hydrogen bond network and Arg38 in the hydrogen bond network of the H-pathway (Fig. 3) are capable of storing protons and of exchanging them with the Mg/H₂O cluster. Although the structural characteristics and locations of these groups suggest that their primary roles are in proton relay during the catalytic cycle, these proton-accepting sites are likely to stabilize the protons stored in the cluster by increasing the proton-accepting capacity.

It is well known that the proton affinity of a deprotonatable group in solution is influenced strongly by the polarity of the solvent (15). In fact, a pKᵢ increase is attained by exchanging the medium from H₂O to methanol (15). In general, the protein interior provides a low dielectric environment, which could suppress reversible protonation with the protein exterior. Thus, the 20–21 water molecules, polar but non-charge residues (Ser162(II), Ser197(II), Thr124, Thr127, Thr291, and Glu332), and the peptide groups included in the cluster, which are tightly hydrogen-bonded with each other, as shown in Fig. 5, are likely to provide a dielectric environment similar to that of the N-side aqueous phase to facilitate the reversible proton-accepting capacity of the cluster. The above structures of the Mg/H₂O cluster clearly show that the cluster has sufficient capacity for storage of four equivalents of protons.

Structural Basis for Prevention of Back-leakage of Protons Used in the Proton Pumping Process—Timely closure of the water channel is critical for effective proton pumping. As described in our previous paper (4), binding of CO (and O₂) triggers conformational changes in helix X to close the water channel (blue curve in Fig. 3). The water channel extends to Arg38, which is hydrogen-bonded to the heme a-formyl group. The conformational changes in helix X occur upon complete oxidation of the reduced CcO as well as upon CO binding to the reduced CcO (3, 4). The structural bases of the water channel closure are obtained from the analysis of the redox-coupled conformational changes of CcO occurring in the heme and helix regions. At the present resolution, migration of the heme a₃ plane occurs without affecting the level of the porphyrin plane. This is clearly detectable upon oxidation of CcO and can be seen when Fig. 6A (the structure in the reduced state) is compared with Fig. 6B (the structure in the oxidized state). The most prominent migration is detectable in the position of the vinyl group of the C-ring, which occurs without significant movement of the propionate of the A-ring. The pairs of two non-polar carbon atoms with atomic distances shorter than 4.0 Å are marked by dotted lines, and the atomic distances are indicated in Å. The vinyl group movement induced upon oxidation induces a significant migration of the C₉-C₉ axis of Leu384 with
FIGURE 5. The structure of the Mg/H₂O cluster. A, location of water molecules and hydrogen bond network in the Mg/H₂O cluster of the oxidized state, illustrated by a stereo view. The dotted lines indicate hydrogen bonds. The color coding used is identical to that in Fig. 4. Polar amino acid residues included in the Mg/H₂O cluster are marked by the one-letter residue codes with numbers. Nonpolar amino acid residues interacting with the Mg/H₂O cluster with their main chain C=O or N-H groups are also shown. The beige sphere denotes the position of the Mg²⁺ ion. The purple spheres indicate the positions of water molecules. The numbering used for the water molecules is the same as in Table 1. B, the location of water molecules and the hydrogen bond network in the Mg/H₂O cluster of the reduced state are illustrated by a stereo view. The positions of water molecules are denoted by light blue spheres. C, superposed structures of the oxidized and reduced states showing the redox-coupled structural changes in the location of water molecules in the Mg/H₂O cluster, illustrated by a stereo view. For the sake of simplicity, hydrogen bonds have been deleted. D, location of proton-accepting functional groups included in the Mg/H₂O cluster. To preserve clarity, water molecules detectable in the cluster are not shown. The thin sticks indicate non-polar amino acid residues interacting with their main chain groups and polar groups that are unlikely to accept protons reversibly.
a turn of about 180° against the C₆₋C₇ axis to give the conformation of the fully oxidized state, as shown in Fig. 6B.

Concomitant with this conformational change, a bulge structural transition occurs upon oxidation as follows. In the reduced state, the main chain carbonyl group of Val380 is not included in the α-helix structure of helix X and instead forms a bulge conformation, which is marked by a circle in Fig. 6A. Upon oxidation, the conformational change in Leu381 induces two new bulge conformations in Ser382 and Met383 (two circles each in Fig. 6B), concomitantly with elimination of the bulge conformation at Val380, consistent with a damage-free x-ray structure at 1.9 Å resolution (16). Met383 is in a bulge/α-helix multiple conformation. The finding demonstrated in Fig. 6 provides direct structural basis for the water channel closure mechanism. Although the redox-coupled migration of the heme a₃ plane has been detected at 2.1 and 1.8 Å resolution, the coupling between heme a₃ and helix X via the two vinyl groups was not clearly identified.

As shown in Fig. 2E, His291 ligated to Cu₇ is hydrogen-bonded to water 10 located in the Mg²⁺-containing water cluster. The structure suggests that Cu₇, sensing the proton saturation via water 10 in the cluster and/or His291, increases the O₂-binding affinity of Fea₃²⁺ for facilitating the timely closure of the water channel.

**Structural Basis for the Selective Electron Transfer from Cu₄ to Heme a**—When the internal electron transfer starts from Cu₄ oxidation, electrons are selectively transferred to heme a, and not to heme a₃, despite the proximity of the two heme units, followed by quantitative reduction of heme a₃ by heme a. Direct electron transfer from Cu₄ to heme a₃ is not detected. It is
noteworthy that this selective electron transfer also provides a critical contribution to effective proton pumping, because heme a oxidation drives proton active transport through the hydrogen bond network in the H-pathway. This well-established experimental aspect suggests that the redox potential of heme a3 is significantly increased upon heme a reduction. Because electron transfer from CuA to heme a via heme a is likely to be coupled with proton uptake for water formation from the N-side, it has been proposed that protonation of heme a3 is the primary factor involved in increasing its redox potential (17). Thus, a system for controlling the timing of protonation is critical.

Fig. 7 shows the redox-coupled conformational changes that occur in the present x-ray structures located between the CuA and heme a3 sites. The possible electron transfer pathway from CuA to heme a, including the imidazole group of His204(II), the peptide bond between Arg438 and Arg439, and the main chain C=O group of Arg438, is detectable in the reduced state as shown by a narrow short hydrogen bond network in the Mg2+/H2O cluster. The O2-reduction site is represented by CuB1+ and Fea32+.

The redox-coupled conformational changes in the region intervening between the CuA and Mg2+/H2O sites and the two hemes. The color coding used is identical to that in Fig. 4. The green and beige balls indicate the CuA and Mg2+/H2O sites. A magnified view of the redox-coupled conformational changes occurring at Arg438 together with a MR/DM map of the reduced state is shown in the inset. The electron density cage is drawn at the 3.5σ level.

![Diagram of the redox-coupled conformational changes](image)

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change in Arg438 induced upon heme a reduction as well as the conformational change in His204(II) could trigger a small conformational change in heme a3 to increase the proton affinity and/or the redox potential of heme a3. This is the first possible structural basis for selective electron transfer.

The Crucial Involvement of the Mg/H2O Cluster in the Catalytic Cycle of Bovine Heart CcO Deduced from the Present X-ray Structural Findings—The contribution of the present finding to understanding of the mechanism of the proton pump could be shown schematically in Fig. 8. Each CcO molecule in the figure includes the H-pathway composed of the hydrogen bond network and the water channel in tandem. To the former, heme a is attached, as marked by Fea33+ or Fea2++. The Mg/H2O cluster containing the CuA-Mg2++ site is connected to the hydrogen bond network of the H-pathway with a short hydrogen bond network. The proton-accepting sites in the Mg/H2O cluster include the guanidino, carboxyl, and imidazole groups, as shown in Fig. 5D. Thus, in the scheme, the proton-accepting sites are shown by the four hollows in the wall of the Mg/H2O cluster. If the cluster includes two guanidino groups and two carboxyl groups as the proton-accepting sites, the total charges in the fully protonated and deprotonated states would be +2 and −2, respectively. The small total charges could be crucial for the proton storage capacity of the Mg/H2O cluster. The O2-reduction site is represented by CuB1+ and Fea32++.

Fig. 8A (a) shows the completely deprotonated state after completion of a single catalytic cycle, and the water channel is in the open state induced by the fully reduced ligand-free state of heme a3 (Fea3). The Mg/H2O cluster is fully protonated by protons transferred by hydronium ions from the N-side phase, as represented by A (b). The proton saturation is sensed by CuB1+ to increase the O2-binding affinity of Fea3 as shown by the dotted curves. The O2-bound heme a3 (Fea32++-O2) induces the water channel closure, as shown in A (c). The channel closure is triggered by the heme a3 plane migration, as shown in Fig. 6.

After the channel closure, as schematically shown in Fig. 8A (d), four electrons and four protons for making water molecules are transferred from cytochrome c and from the N-side phase through the two proton-conducting pathways (different from the H-pathway) to reduce the O2 molecule at Fea3. The electron transfers are sequential. Each electron transfer from CuA to heme a3 induces the channel open to provide the original state (Fig. 8A (a)).

The electron/proton-coupled transfer process is schematically shown by Fig. 8B. Upon electron transfer to CuA2++ from cytochrome c (Fig. 8B, a and b), CuA2++ induces proton collection from the peptide bond between Arg438 and Arg439 with the peptide bond between Arg438 and Arg439, and the main chain C=O group of Arg438, is detectable in the reduced state as shown by a narrow short hydrogen bond network in the Mg2+/H2O cluster. The O2-reduction site is represented by CuB1+ and Fea32++. The electron/proton-coupled transfer process is schematically shown by Fig. 8B. Upon electron transfer to CuA2++ from cytochrome c (Fig. 8B, a and b), CuA2++ induces proton collection from one of the proton-accepting sites to the Mg2++ site, due to the proton affinity increase in the Mg2++ site, as marked by the shape of the line connecting the two metals. As described above, reduction of CuA2++ suppresses the proton transfer through the short hydrogen bond network from the Mg2++ site, as represented by a narrow short hydrogen bond network in B (b). This proton collection function seems crucial especially for...
proton release from the protonated groups with high proton affinity in the cluster. Electron transfer from CuA to Fea induces the proton release from the Mg\(^{2+}\) site. The net negative charge increase in heme a (the Fea site) induces transfer of the released protons to the hydrogen bond network of the H-pathway. In turn, oxidation of Fea upon electron transfer to the O\(_2\) reduction site creates an electrostatic repulsion between the protons and the net positive charge of heme a (or Fea site) shown by a thick orange arrow. This electrostatic repulsion induces a unidirectional proton transfer to the P-side because the water channel is closed. An additional three cycles of this electron/proton-coupled transfer provide the fully deprotonated state, as shown in A (a).

Changes in the oxidation and ligand binding states of Cu\(_B\) and Fea\(_3\) in the intermediate states during the O\(_2\) reduction process corresponding to Fig. 8, A (d) and B (d), are not described in the figure for the sake of simplicity, although it has been well established that both Cu\(_B\) and Fea\(_3\) critically participate in the O\(_2\) reduction by direct electron donations to the bound O\(_2\) molecules (1, 2, 18).
Discussion

The observation of lower proton pumping efficiency (H^+ / e^- = 0.5) in CcOs that lack the Mg^{2+}-containing water cluster (B and C type CcOs) relative to the A type CcOs (H^+ / e^- = 1.0) also provides support for a crucial role of the Mg^{2+}-containing water cluster in the proton pumping process (1).

Based on a resonance Raman finding, Egawa et al. (19) proposed that the breakage of the hydrogen bond between Ser382 and the hydroxyfarnesylethyl group, which is detectable upon full reduction of CcO, has the effect of closing the proton transfer pathway from the N-side. However, the breakage of the hydrogen bond is accompanied by conformational changes of both Ser382 and the hydroxyfarnesylethyl group to introduce the large water cavity in which 2–3 mobile water molecules are trapped. The breakage of the hydrogen bond cannot block proton transfer between the two groups because the mobile water molecules inside the cavity rapidly transfer protons within the cavity.

As shown in Fig. 8A (d), four cycles of the proton/electron-coupled processes, each coupled with one equivalent of proton pumping, occur after the water channel closure. When the fully reduced CcO is treated with an excess amount of O_2, the initial two cycles of the proton/electron-coupled processes proceed, because the fully reduced CcO contains two electron equivalents in Cu_A and Fe_a, each of which could drive the single cycle of the proton/electron-coupled processes as shown in Fig. 8B. The kinetics of proton release and uptake during the initial two cycles of the electron/proton-coupled processes was analyzed using proteoliposomes of bacterial CcO by giving pH dye to the inside of the liposomes or to the outside to evaluate the proton uptake from the inside or the proton release to the outside, respectively (20). In each of the initial two cycles (as given in Fig. 8B), uptake of two protons coupled with one proton release has been reported to conclude that, in each cycle, one pumping proton and one chemical proton (protons for making waters) are taken up from the inside, assuming that each electron transfer is coupled with one chemical proton uptake. The results seem inconsistent to the present results. The pH dye, however, is able to count only the total number for proton uptake, not the number of the chemical or pumping protons independently. Therefore, an alternative interpretation is that two chemical protons are taken up in each of the initial two cycles of the electron/proton-coupled processes, which is consistent with the present structural findings. Furthermore, the results suggest that the number of the chemical proton uptake depends on the stage of the overall process. (The experimental condition for this analysis cannot be applied for the third and fourth cycles given in Fig. 8B; thus, at present, no information is available for the number of the chemical proton uptake in these cycles.) For the sake of simplicity, one chemical proton uptake (the average number) is given in Fig. 8B.

The mechanism shown in Fig. 8 shows complete separation of the O_2 reduction system from the proton pumping system to block any proton exchange between the two systems, which is necessary for effective energy coupling (21). The separation of the two system represents a major point of distinction with respect to a recently proposed mechanism (22).

Conclusion

The present work shows that the Mg/H_2O cluster, linked to the hydrogen bond network of the H-pathway, has sufficient capacity to accept four protons from the N-side, driven by the acid-base equilibrium between the cluster and the aqueous phase of the N-side. The four protons are pumped to the P-side, coupled with sequential transfer of four electron equivalents from cytochrome c, whereas the water channel remains closed after O_2 binding until the fully reduced O_2 reduction site ready to accept O_2 for the next catalytic cycle is regenerated without opening of the water channel, thereby blocking backward proton leakage with a minimal free energy requirement for the process. Two important findings obtained in the presently improved x-ray structure are (i) the involvement of the two vinyl groups of the hemes in the changes in helix X during opening and closure of the water channel and (ii) the redox-coupled conformational changes occurring at Arg_438, which is coupled with selective electron transfer from Cu_A to heme a. These findings have also improved significantly our understanding of the proton-pumping mechanism.

Experimental Procedures

Preparation of Oxidized and Reduced Crystals of Bovine Heart CcO—CcO in the fully oxidized state was purified from bovine heart mitochondria and crystallized with a batch-wise method as described previously (23). The oxidized crystal was soaked at 4 °C in 40 mM sodium phosphate buffer, pH 5.7, containing 0.2% (w/v) n-decyl-β-D-maltoside, 5% (w/v) polyethylene glycol 4000 (Merck), and 45% (w/v) ethylene glycol, which was attained by a multistep increase in the concentration from 0 to 45% with manual exchange of the soaking medium. To reduce the crystals, 5 mM sodium dithionite was added to the solution, which was supplemented with a system for complete elimination of contaminating O_2, including 5 mM glucose, 1 μM glucose oxidase, and 0.5 μM catalase. Full reduction of each crystal was spectroscopically confirmed as described previously (3, 4, 24). The crystals were frozen in a cryo-nitrogen stream and preserved in liquid nitrogen.

X-ray Diffraction Experiments of Oxidized and Reduced Crystals of Bovine Heart CcO—All x-ray experiments providing the results shown in the figures and tables were carried out at beamline BL44XU/SPring-8. The beamline was equipped with an MX225HE CCD detector. The x-ray beam cross-section for x-ray diffraction experiments was 50 × 50 μm or 50 × 30 μm at the crystal, and the wavelength was 0.9 Å. For low resolution data collection, photon flux was reduced using an aluminum attenuator. The photon number at the sample position was 3.0 × 10^{11} photons/s. For data acquisition at 50 K, the crystals were frozen in a cryo-helium stream. Each frame was taken with a 10-s exposure. Each crystal was translated by 100 μm after each round of 10 shots to reduce radiation damage of the crystal. A total of 16 oxidized and 8 reduced crystals were used for acquisition of the full data sets. Data processing and scaling were carried out using DENZO and SCALEPACK (25). A total of 1,723 images of the oxidized form and 1,120 images of the reduced form were successfully processed and scaled. The structure factor amplitude (|F|) was calculated using the CCP4.
X-ray diffraction data for fully oxidized and fully reduced CcOs

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Oxidized form</th>
<th>Reduced form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beam line</td>
<td>BL44XU at the SPring-8</td>
<td></td>
</tr>
<tr>
<td>Wavelength (Å)</td>
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<td></td>
</tr>
<tr>
<td>Beam size</td>
<td>50 µm × 50 µm, 50 µm × 30 µm</td>
<td></td>
</tr>
<tr>
<td>Detector</td>
<td>MX225HE</td>
<td></td>
</tr>
<tr>
<td>Number of crystals used</td>
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<td>7</td>
</tr>
<tr>
<td>High-resolution data</td>
<td>140</td>
<td>116</td>
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<tr>
<td>Low-resolution data</td>
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<td>320</td>
</tr>
<tr>
<td>Oscillation angle (°)</td>
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<tr>
<td>High-resolution data</td>
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<tr>
<td>Low-resolution data**</td>
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<td>10.0</td>
</tr>
<tr>
<td>Temperature (K)</td>
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<td>50</td>
</tr>
</tbody>
</table>

X-ray Structural Analyses of the Oxidized and Reduced CcOs—Both structure determinations were performed according to the same procedure. Initial phase angles of structure factors up to 4.0 Å resolution were obtained by the MR method (28) using the fully oxidized structure, previously determined at 1.8 Å resolution (Protein Data Bank entry 2D7YR) (13). The phases were extended to 1.5 and 1.6 Å resolutions for the oxidized and reduced forms, respectively, by density modification (29) coupled with noncrystallographic symmetry averaging (30, 31) using the CCP4 program DM (32). The resultant phase angles (ΩMR/DM) were used to calculate the electron density map (MR/DM map) with Fourier coefficients |Fo| exp(iΩMR/DM), where |Fo| is the observed structure factor amplitude. Inspection of the electron density maps around Asp51 of subunit I, where the oxidized and the reduced CcOs have different conformations, confirmed that the phase extension procedure removed the model bias from the map. During the structure refinement of the oxidized crystal, an O–O distance of peroxide was determined by damage-free XFEL crystallography (16). Furthermore, no electron density suggesting the existence of the reduced CcO is detectable in the present electron density map of the oxidized CcO. No significant x-ray effects on the absorption spectra and x-ray structures are detectable for the reduced CcO crystals.

**Protein atoms**

- Oxidized form: 30.9
- Reduced form: 36.0

**Heavy metals**

- Oxidized form: 23.1
- Reduced form: 24.8

**Lipids and detergents**

- Oxidized form: 73.5
- Reduced form: 71.5

**Waters**

- Oxidized form: 53.3
- Reduced form: 52.4

**All atoms**

- Oxidized form: 38.0
- Reduced form: 36.7

**Number of amino-acid residues**

- Oxidized form: 3,614
- Reduced form: 3,614

**Determined**

- Oxidized form: 3,558
- Reduced form: 3,558

**Multiple conformation**

- Oxidized form: 86
- Reduced form: 73

**Notes**

1. A and B indicate two independent enzyme molecules in an asymmetric unit.

2. † Redundancy is the number of observed reflections for each independent reflection.

3. ‡ Rmerge is the conventional crystallographic R-factor, R = Σihkl |Fo| − |Fc| / Σihkl |Fo|, where |Fo| is the measured structure factor amplitude.

4. †† Rp value is the free R-factor for the 5% of the reflections that were excluded from the refinement.

5. ** Root mean square deviation.

6. *** Numbers in parentheses are given for the highest resolution shells.
per, and zinc atoms were imposed on the calculated structure factors. Because two crystallographically independent monomers were found to pack differently (35) in the crystal, each monomer was assigned to a single TLS group in the REFMAC refinement. The crystal structure was refined under non-crystallographic symmetry restraints between two monomers. Because one monomer was converged to lower averaged B-factor than the other monomer by 4–5 Å$^2$ (Table 3) and any significant structural difference between two monomers was not observed for each state, structural details were described for the monomer with a lower B-factor. The quality of the structural refinement was characterized by the R and R$_{free}$ values. The ($F_o - F_e$) maps were calculated with the Fourier coefficients \([|F_o| - |F_e|] \exp(\alpha_i)\), where \(|F_o|\) and \(\alpha_i\) are the calculated structure factor amplitude and phase, respectively, obtained in the structural refinement. The refinement statistics are listed in Table 3. Of 3,614 amino acid residues, 56 residues could not be located in the electron density maps of the oxidized and the reduced enzyme. A total of 86 and 73 residues of the oxidized and reduced enzymes, respectively, have multiple conformations.


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**References**


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