Proton-Pumping Mechanism of Cytochrome c Oxidase

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Abstract
Cytochrome c oxidase (CcO), as the terminal oxidase of cellular respiration, coupled with a proton-pumping process, reduces molecular oxygen (O2) to water. This intriguing and highly organized chemical process represents one of the most critical aspects of cellular respiration. It employs transition metals (Fe and Cu) at the O2 reduction site and has been considered one of the most challenging research subjects in life science. Extensive X-ray structural and mutational analyses have provided two different proposals with regard to the mechanism of proton pumping. One mechanism is based on bovine CcO and includes an independent pathway for the pumped protons. The second mechanistic proposal includes a common pathway for the pumped and chemical protons and is based upon bacterial CcO. Here, recent progress in experimental evaluations of these proposals is reviewed and strategies for improving our understanding of the mechanism of this physiologically important process are discussed.
INTRODUCTION

Cytochrome c oxidase (CcO) reduces molecular oxygen (O\textsubscript{2}) coupled to the pumping of protons across the mitochondrial or bacterial membrane. This enzyme is one of the most important elements of cellular respiration. Extensive efforts have focused on elucidating the atomic mechanism of the reaction, since the reports 15 years ago on the X-ray structures of CcO from bovine and bacterial origins at 2.8 Å resolution (16, 43).

CcO contains four redox active metal sites, Cu\textsubscript{A}, heme \( a \), heme \( a_3 \), and Cu\textsubscript{B}, each of which reversibly receives one electron equivalent. The dinuclear copper site, Cu\textsubscript{A}, receives electrons from cytochrome c in the P-phase (the intermembrane, or periplasmic, phase) and subsequently donates these electrons to heme \( a \). The O\textsubscript{2} reduction site of CcO includes heme \( a_3 \) and Cu\textsubscript{B}. The two hemes are fixed perpendicularly to the membrane plane by coordination to side chains in the transmembrane \( \alpha \)-helices of subunit I, the largest of the 13 different distinct subunits of bovine CcO (44). Each of the four propionate groups of hemes is directed toward the P-phase. The two heme planes are at the same level relative to the membrane plane and are located sufficiently close to each other [at a minimal edge-to-edge distance of 4.7 Å giving an interplane angle of about 100° (44)] to induce electron tunneling between the peripheral groups as shown in Figure 1. In fact, the intrinsic electron transfer occurs at the nanosecond level (31).

CcO has two aqueous-exposed surfaces, each facing the P-phase or the N-phase (the matrix or cytoplasmic phase, respectively) (Figure 1). The O\textsubscript{2} reduction site is connected to the hydrophilic surface of CcO facing the N-phase by two hydrogen-bond networks, designated the K- and D-pathways. The two pathways are expected to transfer protons (the chemical protons) necessary for forming water at the O\textsubscript{2} reduction site (16, 44). A third possible proton transfer pathway of bovine CcO, the H-pathway, forming a connection between the two hydrophilic surfaces, is for pumping protons (the pumped protons) associated with the formation of a transmembrane proton gradient (51) (Figure 1). The H-pathway is located near heme \( a \). The D-pathway is a pathway for both pumped and chemical protons, based on the mutational analyses of bacterial CcOs (20). All
Figure 1
X-ray structure of bovine heart cytochrome c oxidase. Structures and locations of redox active sites and possible pathways for protons, $O_2$, water, and electrons. Inset: Location of the redox active sites in the protein moiety.
P-phase: the space outside the mitochondrial inner membrane or in the periplasmic side of the bacterial cell membrane

N-phase: the region located on the opposite side of the membrane from the P-phase; it has a negative membrane potential relative to the P-phase

Chemical protons: protons required to generate water during the reduction of O₂

Pumped protons: protons translocated across the membrane to store the energy released during the transfer of electrons to O₂ through CcO and by reducing O₂ to water

H-pathway: a proton transfer pathway connecting the N-phase with the P-phase

these networks and metal sites are located in subunits I and II, which are the largest and third largest of the constituent subunits, respectively. The structures and locations of these metal sites and possible pathways for protons and electrons are fairly well conserved in bacterial and eukaryotic aa₃-type CcOs. It is impossible to estimate the scale of the conformational changes sufficient to enable the proton-pumping function. Thus, major efforts have been undertaken to improve the resolution of X-ray structures of CcO. In this review, the basic functions and structures of the above redox active metal sites reported thus far summarized the proton-pumping mechanism is discussed.

STRUCTURES AND FUNCTIONS OF THE O₂ REDUCTION SITE

The oxidation and ligand-binding states of intermediate species during CcO reaction discussed in this article are summarized in Table 1.

In order to identify the chemical structure of the intermediate species of the O₂ reduction reaction by CcO, the reaction of fully reduced CcO with excess O₂ was followed by resonance Raman spectroscopy using a flow-flash method. The initial intermediate (A) has a band at 571 cm⁻¹. The isotopic shift effects using ¹⁸O₂ and ¹⁶O=¹⁸O indicate that this band corresponds to a Fe–O₂ stretch band similar to those of oxyhemoglobins and oxymyoglobin. The second and third intermediates (P and F) exhibit bands at 804 cm⁻¹ and 785 cm⁻¹, respectively (19). The isotope shift effects indicate that both of these bands originate from oxide-bound iron (Fe⁴⁺=O²⁻). The oxide structure of the intermediate P indicates that the O-O double bond has been cleaved at this stage. Thus, a possible structure of the O₂ reduction site in this intermediate is [Fe⁴⁺=O²⁻, CuB₂⁺-OH⁻, tyrosine radical]. The tyrosine radical is likely to be that of Y244 located near the O₂ reduction site. The fourth intermediate (O) is characterized by a band at 450 cm⁻¹ that is assignable to the Fe-O stretching mode of Fe³⁺-OH⁻ (19). The absorption spectra of the A, P, and F intermediates have been characterized as having peaks at 590 (11), 607, and 580 nm (19), respectively. When the fully reduced (or four-electron-reduced) CcO reacts with O₂, the intermediate P is formed significantly faster compared with the reaction between O₂ and the two-electron-reduced enzyme in which Cu₆ and heme α are in the oxidized state. The oxidation state of heme α

Table 1 Intermediate species in the cytochrome c oxidase reaction

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>States</th>
<th>Oxidation and ligand binding states</th>
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| Oₓ             | resting fully oxidized | Fe₄⁺⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓–→<br>"Yoshikawa • Muramoto • Shinzawa-Itoh"
influences the rate of intermediate P formation (19). Furthermore, when the O₂ reduction reaction is initiated from fully reducedCcO, intermediate P formation is coupled to heme a oxidation. Thus, this intermediate, P₁, is in an overall oxidation state of the O₂ reduction site (including the Y244-OH group) one equivalent lower than that of the intermediate P₃m, which is produced from two-electron-reducedCcO. Upon formation of P₃m and P₁ from A, the O=O double bond is broken to provide O₂⁻⁻ and OH⁻. This is a nonsequential four-electron reduction process. By this strategy, this enzyme reduces O₂ to water without formation of reactive oxygen species. Extensive X-ray structural analyses of various O₂ analog derivatives of bovine heartCcOs show that the O₂ reduction site induces a conformational change to facilitate the nonsequential donation of three electrons to O₂⁻⁻ bound at Fe₃₅₅⁺, which is formed upon O₂ binding to Fe₃₅₅⁺ (26). These results indicate that, in normal enzymatic turnover, when both metals in the O₂ reduction site are in the reduced state (R), CcO binds O₂ to form Fe₃₅₅⁺ − O₂ (A). The bound O₂ takes up four electron equivalents to form P₃m. Then, P₃m sequentially receives four electron equivalents (one at a time) from ferrocychrome c, generating intermediates F, O, and E (a one-electron-reduced form of O) to regenerate R (Table 1).

Fully oxidized bovine heart CcO, as isolated under aerobic conditions (the resting, fully oxidized CcO, O₅), requires six electron equivalents for complete reduction (24). During this reductive titration, the slope of the titration curve monitoring absorption spectral changes during the addition of the initial two electron equivalents is shallow relative to the slope of the curve observed for another four electron equivalents yielding maximal absorption changes. This observation suggests that a majority of the two initial reducing equivalents is consumed by the reduction of an electron acceptor other than the four redox active metal sites of CcO. On the other hand, fully reduced CcO is completely oxidized by four oxidation equivalents donated by O₂. After the oxidative titration, when the oxidized CcO solution is exposed to excess O₂ for 30 min, the CcO sample, which requires six electron equivalents for complete reduction, is regenerated. These results suggest that the O₅ state has a peroxide bound to the O₂ reduction site (24).

The proposed structure of the O₂ reduction site of the O₅ state is consistent with its X-ray structure. The F₀-F₄ map, obtained from X-ray diffraction experiments carefully conducted to minimize the effect of hydrated electrons from a third-generation synchrotron radiation facility such as SPring-8, shows that a peroxide is bridged between Fe₃₅₅⁺ and CuB ions. The O-O bond distance is 1.7 Å, which is longer than the O-O bond distance of typical peroxide model compounds (1.55 Å), suggesting that the peroxide is in an activated state (22). However, the resting oxidized CcO can be kept at 4°C in the crystalline state for several months without any significant changes to its absorbance spectrum (22). Although the X-ray structural results have been confirmed by a recent resonance Raman investigation showing a band at 755 cm⁻¹ assignable to the bridging peroxide (37), it remains desirable to improve the resolution of the X-ray structure to identify the structural origins for the stability of this bridging peroxide.

Fully oxidized CcO generated immediately after complete oxidation, corresponding to the fully oxidized CcO under turnover conditions, the O state, pumps protons upon the addition of one or two electron equivalents, whereas the O₅ state does not (47). On the basis of these results, it was proposed that the free energy obtained by O₂ reduction is stored in intermediate P, with half of it released in the oxidative phase. However, the rest of this energy is lost as heat unless the reductive phase starts immediately after complete oxidation of CcO (47). However, the proton pump is detectable upon one-electron reduction of CcO in the E state, prepared by the two-electron reduction of F by CO (35). This E intermediate is surely not prepared from the oxidized form produced immediately after the complete oxidation of CcO. Thus, the proton pump activity of the E intermediate strongly suggests that there are some chemical structural differences between

Oxygenated form (species) (A): O₂-bound intermediate species

F: the third intermediate species next to P₃m under turnover conditions

P₁: the second intermediate species next to the O₂-bound form in the reaction between the four-electron-reduced CcO with O₂

P₃m: the second intermediate species next to the O₂-bound form in the reaction between the two-electron-reduced CcO with O₂

Resting, fully oxidized CcO (O₅ state): fully oxidized CcO as isolated under aerobic conditions

Turnover, fully oxidized CcO (O state): fully oxidized CcO generated immediately after the reduction of O₂ to water, which corresponds to the fully oxidized CcO produced under turnover conditions

E: one-electron-reduced intermediate species of CcO
O₂ and O. The structural differences in the O₂ reduction site between O and Or \{[Fe₄³⁺-OH⁻] (19) versus [Fe₄³⁺-O⁻-O⁻-Cu₆++] (3)\} are likely to cause the difference in the proton-pumping activity.

The O₃ state was observed also in a ba₃-type CcO from *Thermus thermophilus*. Thus, the formation of O₃ is not induced by damage during the course of purification as had been suggested (40). The physiological significance of O₃ is expected to prevent the formation of active oxygen species under low levels of electron flow in the respiratory chain, in which the fully oxidized CcO is likely to be accumulated. The structure of O [Fe₄³⁺-OH⁻, Cu₆²⁺-OH⁻, TyrOH] is expected to react with O₂ spontaneously under aerobic conditions to generate various active oxygen species.

**COUPLING BETWEEN THE PROTON-PUMPING AND O₂ REDUCTION CYCLES**

A quantitative evaluation of the protons ejected by the reduction of CcO was performed using the O₃ state reconstituted into liposomes (6). After quantitative anaerobic addition of electron equivalents by ruthenium (III) hexamine, O₂ was added to initiate the reaction. Significantly, CcO that received two electron equivalents or fewer showed no proton ejection. The number of ejected proton equivalents was essentially proportional to the number of electron equivalents above two equivalents. At four electron equivalents above two equivalents (six electron equivalents in total), the maximal number of protons (four proton equivalents) was ejected. The titration curve (figure 2 in Reference 6) is similar to that of the reductive titration curve of the O₃ state under strictly anaerobic conditions (24). The initial lag in the titration curve suggests that the initial two electron equivalents are used for reducing the bridging peroxide in the O₂ reduction site as described above.

In order to confirm that the ratio of protons to electrons is unity in the reductive phase, the electric potential generated during this process was measured following donation of a single electron via a laser flash system to the O state. Quantitative analyses of the charge separation using the potential generation assignable to the electron transfer from Cu₆ to heme a as the internal standard indicate the ejection of one proton equivalent per electron equivalent injected (6). These experimental results indicate that each electron transfer from ferrocytochrome c to CcO under turnover conditions is coupled with pumping of one proton equivalent.

**ANALYSES OF THE ELECTRON AND PROTON TRANSFER PROCESSES DURING THE ENZYME REACTION**

**Flow-Flash Analyses**

The CcO reaction has been analyzed extensively by a flow-flash method in which the reaction of fully reduced CcO with an excess amount of O₂ is initiated by a flash-photolysis technique to release CO from the CO-bound, fully reduced CcO in the presence of O₂. Essentially, the same absorbance spectral changes were observed for CcOs isolated from different sources. The time course of this reaction was resolved into at least four phases with approximate time constants (half-lives) of 7, 24, 72, and 800 μs, respectively. These phases are the R→A, A→Pr, Pr→F, and F→O transitions, respectively (1). As described above, oxidation of heme a is coupled to the formation of Pr. Thus, the Pr→F transition does not include electron transfer. The Pr→F and F→O transitions are coupled to both the pumped and the chemical proton transfers. The release and uptake of the protons during these phases have been carefully analyzed using a pH-sensitive dye to measure the change of pH outside and inside proteoliposomes reconstituted with CcO from *Rhodobacter sphaeroides*. One proton equivalent is released to the outside and two proton equivalents are consumed inside the lipid bilayer.
between the two hemes during transitions such as \( \text{A} \rightarrow \text{P} \), is unlikely to be electrogenic. However, time-resolved electron and proton transfer (charge translocation) measurements have shown that at neutral pH, the \( \text{A} \rightarrow \text{P} \) transition is superimposable with the initial phase of the \( \text{P} \rightarrow \text{F} \) transition (5). However, at pH 10.5, at which the \( \text{P} \rightarrow \text{F} \) transition is negligible (27), \( \text{P} \) formation followed by absorbance at 595 nm was coupled to a simultaneous charge separation. The mechanistic implication of the charge separation is discussed below (5).

**Single-Electron Injection Analyses**

A single-electron-reduction technique using ruthenium bispyridyl was applied to the O state from *Paracoccus denitrificans* to analyze the internal electron and proton transfer reaction (4). After the initial reduction of \( \text{CuA} \), a rapid electron transfer to heme \( \text{a} \) with a half-life of about 10 \( \mu \)s occurs concomitantly with membrane potential formation. This 10-\( \mu \)s phase is insensitive to pH and \( \text{H}_2\text{O}/\text{D}_2\text{O} \) exchange. Therefore, this electron transfer step is not coupled to proton transfer. When the electron movement is finished, 30% and 70% of electron equivalents are distributed between \( \text{CuA} \) and heme \( \text{a} \), respectively (31). However, no electron equivalent is distributed in the \( \text{O}_2 \) reduction site. As described above, hemes \( \text{a} \) and \( \text{a}_3 \) are located sufficiently close to each other so that the intrinsic electron transfer rate could be on the nanosecond scale. Thus, the absence of electron transfer from heme \( \text{a} \) to heme \( \text{a}_3 \) indicates that the redox potential \( (E_{\text{ref}}) \) of heme \( \text{a}_3 \) is much lower than that of heme \( \text{a} \) \((\Delta E_{\text{ref}} > 100 \text{ mV})\). The amplitude of membrane potential formation is 12% of the total potential formation. The reduction of heme \( \text{a} \) increases the redox potential of heme \( \text{a}_3 \) significantly to induce the electron transfer to heme \( \text{a}_3 \) in the next 150-\( \mu \)s phase. At the end of this phase, 40% and 60% of the electron equivalents are distributed between heme \( \text{a} \) and the \( \text{O}_2 \) reduction site (heme \( \text{a}_3/\text{CuB} \), respectively. Namely, \( \text{CuA} \) is completely oxidized during this phase by significant increment of \( E_{\text{in}} \) of heme \( \text{a} \), induced by reduction of heme \( \text{a}_3 \). The electron transfer from heme \( \text{a} \) to heme \( \text{a}_3 \), although parallel to the membrane surface, contributes to the formation of a large membrane potential (about 42% of the total) due to the proton translocation from the N-phase. The absorbance spectrum at the end of the next phase (800 \( \mu \)s) shows an absorption peak near 640 nm, suggesting that \( \text{CuB} \) is fully reduced and all the other metal sites are in the fully oxidized state (4).

It has been proposed that the absorption peak at 640 nm is assignable to a band due to \( \text{CuB}^{3+} \) because this is blue-shifted from the charge transfer band of the \( \text{CuB}^{2+} \) site at 665 nm (4). However, independent experimental confirmations are needed to validate this proposal. If the final destination of the single electron equivalent is the \( \text{CuB} \) site, the \( E_{\text{in}} \) of \( \text{CuB} \) must be much higher than that of all the other metal sites. This 800-\( \mu \)s phase also includes a large membrane potential formation (30% of the total), again suggesting that proton translocation occurs. The charge separation takes place in 800 \( \mu \)s, is induced by the electron transfer parallel to the membrane surface, and is due to proton movement from the N-phase. As the final phase, membrane potential formation with a half-life of 2.6 ms is detectable without any corresponding absorption spectral change. The amplitude is approximately 16% of the total (4). The time constant, electron distribution, and membrane potential generation of each phase in the above single-electron injection process are summarized in Table 2.

The incremental increase in \( E_{\text{in}} \) of the \( \text{O}_2 \) reduction site upon protonation of the \( \text{O}_2 \) reduction site must be higher than that upon protonation of the loading site of the pumped proton, because the loading site must be separated from the \( \text{O}_2 \) reduction site. Therefore, the 150- and 800-\( \mu \)s phases are assignable to the transfer of pumped protons and chemical protons, respectively. The final 2.6-ms phase.
Table 2  Process of single-electron injection to the turnover fully oxidized intermediate

<table>
<thead>
<tr>
<th>Phases</th>
<th>Electron distribution</th>
<th>Membrane potential generation</th>
<th>Proton transfer</th>
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<tbody>
<tr>
<td></td>
<td>CuA</td>
<td>Heme a</td>
<td>Heme a3</td>
</tr>
<tr>
<td>&lt;0.5 μs</td>
<td>100%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 μs</td>
<td>30%</td>
<td>70%</td>
<td>0</td>
</tr>
<tr>
<td>150 μs</td>
<td>0</td>
<td>40%</td>
<td>60%</td>
</tr>
<tr>
<td>800 μs</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.6 ms</td>
<td>0</td>
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*Electron injection process by a laser pulse. The quantum yield is 10%–20%. The experimental details are given in Reference 4.

is for proton release from the proton-loading site, which is driven by conformational changes near the pumped-proton-loading site (4).

The single-electron injection technique has been applied to the transitions F→O and Pₘ→F using CcOs isolated from bovine and bacteria and for monitoring the formation of membrane potential (20, 40, 46, 52). Essentially, the same results obtained for the O→E transition described above were observed, including a rapid phase followed by a slower phase with at least two components.

The fact that the transitions Pₘ→F, F→O, and O→E cause similar electron and proton movements strongly suggests that the proton-pumping mechanism is independent of the oxidation and ligand-binding state of the O₂ reduction site. The low-potential site (CuA/heme a) transfers each of the four electron equivalents in the catalytic cycle through the same pathway to the high-potential site (heme a³/CuB). Conversely, four types of chemical reactions proceed at the high-potential site upon receipt of each electron equivalent, depending on the overall oxidation state of the high-potential site. Thus, it is likely that the low-potential site drives the proton pump.

The O₂ has also been analyzed by the single-electron injection technique. Using carefully prepared reconstituted CcO proteoliposomes to minimize spontaneous reduction, Verkhovsky et al. (48) showed that only the initial membrane potential formation, which is assignable to the electron transfer from Cu₄ to heme a, was detectable. As described above, the resting oxidized CcO has a bridging peroxide in the O₂ reduction site (3). The absence of the second phase in which heme a is oxidized suggests that the increase in Eₘ of the O₂ reduction site is prohibited by the negative charge of the peroxide. The second flash induces a slower phase (135 μs). However, the slower phase is not sufficiently intense to drive the proton pump (36, 48). These results are consistent with the observation that the O₂ state does not have the ability to pump protons upon the addition of one or two electron equivalents as described above (47). A major part of the two electron equivalents available upon the second flash would be used for reduction of the peroxide to yield two oxides or hydroxides (O₂⁻ or OH⁻). Both heme a₁ and CuB are likely to still be essential in the ferric and cupric states, respectively, even after the second flash. Electron donation to the metal sites in the O₂ reduction site seems to be a prerequisite for proton uptake to trigger proton pumping.

**ROLES OF PROTON TRANSFER PATHWAYS OF BACTERIAL ENZYMES**

**Mutational Analyses of the D-Pathway**

The site-directed mutagenesis technique has been applied extensively to bacterial aa₃-type CcOs from *P. denitrificans* and *R. sphaeroides* in order to identify the pathways for the chemical and pumped protons. The critical amino acid residues in the D- and K-pathways discussed in this review are conserved in the two bacterial and bovine CcOs. Thus, for the sake of
simplicity, the residue numbers of bovine CcOs are given in this review. Key residues in the D-pathway are E242 near the O2 reduction site and D91 near the entrance facing the N-phase. The E242Q mutant CcO of *R. sphaeroides* shows no electron transfer or proton-pumping activities under the steady-state turnover conditions. Upon single-electron injection to F state, slower membrane potential generation after rapid potential generation is abolished in the E242Q mutant, suggesting that the F→O transition is blocked by stopping proton transfer through the D-pathway (20). This proton transfer is tightly coupled to electron transfer from heme *a* to the O2 reduction site in the F state. The slower potential generation is composed of at least two components that are assignable to the proton translocations of the chemical and pumped protons. Thus, these results suggest that the D-pathway transfers both chemical and pumped protons (20).

A flow-flash analysis of the fully reduced E242Q mutant enzyme from *R. sphaeroides* shows that electron transfer to P* r* is impaired without significant oxidation of Cu A (2). Further, no net proton uptake is detectable. Thus, E242 donates protons to the O2 reduction site in the P* r*→F transition, inducing Cu A oxidation. The D91N mutant of CcO (which lacks both proton-pumping and O2 reduction activities) provides protons for the transition of P* r* up to the F state without any net proton uptake. In wild-type CcO, the P→F transition is coupled to electron transfer from Cu A to heme *a* as described above. However, the P→F transition in the D91N mutant of CcO does not induce electron transfer, indicative of tight coupling between the D91 and Cu A sites (2).

A mutation of the *R. sphaeroides* enzyme, N98D, located in the middle of the D-pathway, shows a peculiar phenotype with abolished proton-pumping activity and stimulated O2 reduction activity (150%–300%). The flow-flash analysis shows that the intermediate species are not perturbed by the mutation. More specifically, the O2 reduction mechanism has not been modified (29). The N98D mutant CcO generates a membrane potential corresponding to chemical proton uptake at the O2 reduction site, consistent with a lack of proton-pumping activity (41). A similar phenotype is reported for N98D and N163D in *P. denitrificans* CcO (30). The X-ray structure of the N98D mutant CcO of *P. denitrificans* at 2.32 Å resolution shows no significant structural change relative to wild-type CcO except for an alternative conformation of the E242 side chain (13).

**Mutational Analyses of the K-Pathway**

The K319M and T316A mutants in the K-pathway of the CcO of *R. sphaeroides* showed no influence on potential generation due to F→O transitions. These mutant enzymes have neither proton-pumping nor O2 reduction activities under turnover conditions. Furthermore, reduction of heme *a*3 in the mutant CcO is extremely slow. These results suggest that the K-pathway transfers chemical protons coupled to electron transfer to the oxidized O2 reduction site (Fe* a*3+/CuB2+) (20, 49). In fact, blocking the K-pathway impairs the electron-coupled transfers of both the chemical and pumped protons, triggered by electron transfer from Cu A to heme *a* upon injection of a single electron into the O state (6). The results indicate that the transfer of chemical protons through the K-pathway is coupled to the transfer of pumped protons. An alternative interpretation is that the K-pathway transfers both the chemical and pumped protons.

As a result of these extensive mutagenesis analyses of the K- and D-pathways, the original proposal (that the K- and D-pathways are used for chemical and pumped protons, respectively) (16) has been revised to the present proposal that the K- and D-pathways are used for chemical and chemical/pumped protons, respectively. Most of the experimental results have been interpreted according to the refined proposal.

**Mechanism of Proton-Pumping Driven by the D-Pathway**

As summarized above, all the phenotypes of the D-pathway mutant CcOs seem consistent
with the proposal that the D-pathway transfers both pumped and chemical protons. The proton-pumping site must be completely separated from the O₂ reduction site, because otherwise the pumped protons would be utilized for water formation, thereby generating a short circuit. Thus, the D-pathway must have at least one branching point for separating the pumped protons from the chemical protons. A candidate for the branching point amino acid residue is E242. The X-ray structure of the E242Q mutant of CcO shows that the conformation of Q242 is different from that of E242 in wild-type CcO, suggesting that disruption of a hydrogen bond between the carboxyl group of E242 and the peptide carbonyl group of M107 has occurred (42). Furthermore, the conformational change upon the E/Q exchange induces a translational shift of the indole ring of W126. The indole ring is hydrogen-bonded to the propionate group of the D-ring of heme A₁, which is salt-bridged to R438. The movement of W126 upon E242Q mutation influences the conformation of the propionate to break the salt bridge with R438 through the introduction of a water molecule between the guanidino group and the carboxyl group (42). Based on this structural change and assuming that Q is analogous to deprotonated E, it has been proposed that deprotonation of E242 induces the conformational change of the propionate group to increase its pKa value (9). (It is not clear why Q could be analogous to E in the negatively charged state.)

These structural changes are consistent with the following proton pump mechanism driven by the D-pathway (9). When E242 is in the protonated state, the propionate group salt-bridged to R438 has a low pKa value and is accessible only to the P-phase. Upon one-electron reduction of the O₂ reduction site, a proton is transferred from E242 to the O₂ reduction site for charge compensation. The deprotonation of E242 induces a conformational change in R438 and the D-ring propionate (the pumped proton acceptor site A) to increase its pKa value. At this stage, the proton accessibility of site A switches from the P-phase to the N-phase. Then, the pumped protons are transferred to site A through E242, followed rapidly by the chemical proton transfer to E242. The protonation of E242 induces a conformational change in site A to decrease its pKa value and to change its accessibility to the P-phase. Thus, the pumped proton on site A is released to the P-phase. See figure 7 in Reference 9 for a more detailed description of this proton pump proposal.

Sufficient X-ray structural basis for this proposal has not been obtained. For example, additional convincing evidence is required for concluding that the conformational change induced by E242Q mutation is analogous to the conformational change induced by deprotonation of E242, as described above. Furthermore, the structural change of the D-ring propionate of heme A₁ by the E/Q exchange does not clearly show the accessibility switching of site A from the P-phase to the N-phase.

Under high pH conditions, the P → F transition rate becomes slower, with a pKa of 9.4. The pH dependency of the amplitude of F formation also shows a pKa of 9.4. This pKa value has been ascribed to the ionization of E242 at the branching point of the D-pathway (27). On the other hand, the N98D mutant of R. sphaeroides CcO, which has full O₂ reduction activity but no proton-pumping activity, has a pKa of 11 for the pH dependency of the P → F transition rate. These results are consistent with the following proposed function of E242. The pKa value of site A of the wild-type enzyme is significantly higher than that of E242 but lower than that of the O₂ reduction site. Thus, the pKa for E242 in the N98D mutant of CcO is 11, higher than that of site A. This prevents E242 from transferring protons to site A, whereas proton donation to the O₂ reduction site with much higher pKa would be possible (28).

The above proposal that the pKa value determined by the P → F transition is critical for determining proton pump efficiency was confirmed by introducing a second mutation (D91N) into the N98D variant of CcO, which restored both the original pKa (9.5) and proton pumping during steady-state turnover (8).
Direct structural identification of the site used to control pH dependency for the \( P_r \rightarrow F \) transition is desirable, because CcO has protonatable functional groups other than E242, such as R38, K319, and the propionate groups of hemes \( a \) and \( a_3 \).

As described above, the \( A \rightarrow P_r \) transition is electrogenic (5). The charge separation during this transition is not blocked by the D91N mutation, which blocks proton uptake to the D-pathway. However, the E242Q mutation does block charge separation. On the basis of these results, it has been proposed that the \( A \rightarrow P_r \) transition is coupled to proton transfer from E242 to the pumped-proton-loading site. Thus, the \( P_r \rightarrow F \) transition is preceded by protonation of site \( A \) driven by electron transfer from heme \( a \) to heme \( a_1 \) (for \( P_r \) formation). The pumped protons at the loading site are released by chemical proton uptake through E242 near the \( O_2 \) reduction site. In other words, the proton pump during the \( P_r \rightarrow F \) transition, which does not include electron transfer as described above, also is driven by electron transfer to the \( O_2 \) reduction site. On the other hand, charge separation is not detectable in K319M mutant CcO, suggesting that the charge separation detectable in the \( A \rightarrow P_r \) transition is due to proton movement in the K-pathway (22). It is impossible to identify the charge separation site from only these mutational analyses.

E242 must transfer protons both to the proton acceptor site \( A \) and to the \( O_2 \) reduction site if it has the proposed branching function in the D-pathway. However, the X-ray structures of CcO reported thus far show no detectable proton transfer pathway to these two sites. E242 is located on the wall of the \( O_2 \) transfer channel from the molecular surface of the transmembrane region to the \( O_2 \) reduction site. The protons must be transferred to site \( A \) across the \( O_2 \) transfer channel. No chemical structure that could function as the proton pathway has been identified in the X-ray structure in the region between E242, the site \( A \), and the \( O_2 \) reduction site. [Although an extended space near site \( A \) is present as a branch of the \( O_2 \) path, the vacuum space cannot function as a controlled proton transfer pathway (45)]. Thus, possible locations of water molecules in this space were calculated by an energy minimization procedure (50), showing that four water molecules could be located in an L-shaped alignment in the region connecting E242 with the \( O_2 \) reduction site and site \( A \). These water molecules, placed in a narrow tube with a highly hydrophobic inner surface, are expected to form an oriented single array that facilitates unidirectional proton transfer. The direction of transfer depends on the charge distribution of these metal and proton-accepting sites to appropriately direct protons either to the \( O_2 \) reduction or to site \( A \) (50). Furthermore, the function of E242 as the valve to prevent reverse proton transfer has been proposed theoretically (17).

However, the following facts are not fully consistent with the presence of the ordered water molecules in this space. In the fully reduced state, water molecules are unlikely to be located in this space, because an \( O_2 \) molecule must be transferred though the narrow space. Thus, the four water molecules indicated in the theoretical studies must clear out before introduction of \( O_2 \). In fact, no water molecule is detectable in the X-ray structure of the fully reduced CcO, even at 1.8 Å resolution (26).

In X-ray diffraction experiments conducted using the strong X-ray beam of SPring-8, concomitantly with the decrease in peroxide electron density in the \( O_2 \) reduction site with increasing X-ray irradiation time, an increase in electron density assigned to a water molecule is detected at Y244OH. Y244 is covalently linked to one of the histidine imidazole groups ligated to CuB (3). These results suggest that Y244 functions as a scavenger of water in the \( O_2 \) reduction site including the space between E242 and the D-ring propionate. In other words, the absence of water at Y244 in the X-ray structures of bovine heart CcO provides strong evidence for the absence of water molecules in the space. Thus, CcOs in various oxidation and ligand-binding states reported thus far, except for the X-ray-irradiated resting oxidized form and the fully reduced \( CN^- \) bound form (26),
are unlikely to contain any water molecules in the space because they show no water at Y244.

Possible conformational changes in E242 and the G239 peptide carbonyl group would provide a hydrogen-bond network extending to the OH\(^-\) ion on Ca\(_B\) from the E242 COOH group, without introducing any water molecules into the O\(_2\) path (S. Yoshikawa, unpublished observations). Thus, the X-ray structure suggests that the role of E242 is to transfer only chemical protons to the O\(_2\) reduction site in the absence of any water molecules in the hydrophobic space.

Although the finding of one water molecule in the hydrophobic space above the top of the D-pathway in the X-ray structure of the fully oxidized CcO from \textit{R. sphaeroides} may represent positive evidence for the presence of these ordered water arrays (34), it is strongly desirable to establish more convincingly the presence of these water molecules experimentally. Randomly oriented water molecules in the space would not be detectable in the high-resolution X-ray structure. Randomly oriented water molecules would also be unable to be involved in controlling the directionality of proton transfer to site A or to the O\(_2\) reduction site.

In order to examine the possible role of the D-ring propionate of heme \(a_1\) and the arginine pair system as the site for the pumped proton acceptor site A, extensive mutational analyses on the arginine pair (R438 and R439) have been performed for bacterial CcO and the homologous \textit{Escherichia coli} quinol oxidase. However, the mutation results are not straightforward to interpret. Mutations of R439 (K, Q, N, and L) and R438 (K and Q) do not impair proton-pumping activity, whereas the R438Q/R439Q double mutant and the R439P and R438 (N and L) mutants do not have the proton-pumping activity (7, 32, 33). The fairly significant mutational changes in this study (R\(\rightarrow\)Q, N, L) reveal an unexpectedly weak influence on proton-pump activity.

The upper end of the branch of the O\(_2\) path is composed of the R438 and R439 pair and two D-ring propionates of the two hemes. The two guanidino groups of R438 and R439 are hydrogen-bonded to the propionates of heme \(a_1\) and heme \(a\), respectively. This Arg-propionate system (including the salt bridge between R438 and heme \(a_1\) propionate) has been proposed to be the pumped proton loading site, as described above. The X-ray structure indicates that the P-phase side of the arginine pair is protonically equilibrated with P-phase through hydrogen-bond networks inside the protein and that the arginine pair effectively blocks access of the branch of the O\(_2\) path to the P-phase (45). Therefore, the structure of the arginine-propionate system strongly suggests that it functions in reverse proton transfer to maintain mitochondrial membrane potential within an appropriate range, as previously proposed (23).

Thus, it is possible that the abolishment of pumping activity by these arginine mutations is due to an increased extent of proton leaking back through the branch to the O\(_2\) reduction site.

In spite of the extensive mutational analyses described thus far, the elements for the proton-pumping function driven by the D-pathway have not been identified experimentally. Even the branching function of E242 for the chemical and pumped protons has not been established. Perhaps the strongest experimental evidence for the proposal that the D-pathway transfers both chemical and pumped protons is provided by the results for the E242Q and D91N mutations of the bacterial D-pathway, which abolish membrane potential generations driving both proton-pumping and O\(_2\) reduction functions (20). However, these results do not identify the location of the pumping pathway but simply indicate that proton transfer through the D-pathway, electron transfer to heme \(a_1\), and proton pumping are all strongly coupled. In fact, the K-pathway mutation (K319M), which abolishes both pumped and chemical proton transfers (6), is also consistent with the proposition that the K-pathway transfers both chemical and pumped protons. Thus, experimental confirmation is indispensable for any proposal based on mutational analyses.
PROTON-PUMPING FUNCTION OF THE H-PATHWAY

The Proton-Pumping Site and Driving Element of the H-Pathway of Bovine Heart CcO

The H-pathway is composed of a hydrogen-bond network connected to the P-phase side and a water channel opening to the N-phase side (Figure 1 and Figure 2). The water molecules in the N-phase are accessible to R38 at the bottom end of the hydrogen-bond network through the water channel. As a result, R38 is protonically equilibrated with the N-phase. D51 of bovine CcO is located at the upper (P-phase) side of the hydrogen-bond network (26). A peptide bond between Y440 and S441 located next to D51 is proposed to facilitate the unidirectional proton transfer by the relative stability of the keto form of the peptide bond compared to the enol form. A theoretical analysis suggests that proton transfer through the keto form of the peptide bond occurs in the physiological timescale in bovine heart CcO because the enzyme has an energetically favorable proton path from the enol OH to the peptide nitrogen, which facilitates an effective enol-keto transition (-(COH) = N- → -(CO)-NH-) (18). The hydrogen-bond network forms two hydrogen bonds with heme a from the propionate and formyl groups of heme a to a fixed water molecule and R38 in the hydrogen-bond network, respectively. The water channel in the fully reduced CcO has five cavities, each of which is large enough to accommodate at least one water molecule (26, 45).

Figure 3 illustrates that D51, exposed to the bulk water phase of the P-side, becomes buried inside the protein interior upon oxidation of CcO (45). This conformational change of D51 strongly suggests that the protonation state of D51 is dependent on the redox state of CcO. Furthermore, upon oxidation, the accessibility of D51 to the P-phase is essentially eliminated and D51 becomes connected to the N-phase via the H-pathway. Although D51 is expected to function as the proton-pumping site, this residue is not conserved in bacterial and plant ccOs. The evolutionary fact is discussed below.

The positive charge created upon oxidation of heme a after donating an electron equivalent to the O2 reduction site is delocalized to the propionate and formyl groups, each of which forms a hydrogen bond to the hydrogen-bond network of the H-pathway. Thus, protons in the hydrogen-bond network are expected to be actively shuttled through the network, driven by the electrostatic repulsion. These X-ray structures strongly suggest that heme a is the driving element of proton pumping.

The Function of D51

The X-ray structures in Figure 3 suggest that protons are released to the P-phase upon reduction of heme a. However, several proton acceptor sites on the protein surface near D51

Figure 2
Schematic representation of the redox-coupled conformational changes in the H-pathway. The water channel is depicted by the gray, dark blue, and red areas. The light blue circles represent the positions of the fixed water molecules. The redox-dependent structural changes are highlighted in red and dark blue.
could trap the pumped protons released from D51 upon reduction. The pumped protons are expected to be distributed over these sites and stabilized electrostatically by the negative charge on D51 (38). The protons are released to the bulk water phase upon oxidation of CcO when the D51COO\(^-\) is buried inside the protein prior to being protonated by the peptide bond imidic acid, which is a protonated form of the peptide bond (-C(OH)==N\(^+\)H\(-\)). Protons are transferred through a peptide bond by forming the imidic acid form as an intermediate state (45). Proton release upon oxidation of heme \(\text{a}\) is consistent with the direct measurement showing the release of the pumped protons upon oxidation of the heme \(\text{a}/\text{Ca}_\text{a}\) site (10).

The Gating Mechanism for Proton Pumping Through the H-Pathway

Reverse proton transfer from the P-phase is effectively blocked by the peptide bond near the upper end of the hydrogen-bond network. However, the peptide bond is not able to block reverse proton transfer through the hydrogen-bond network below the peptide bond. Upon oxidation of CcO, one water cavity in the water channel is eliminated by a conformational change of helix X (Figure 2) (26, 45). This change narrows the water channel significantly to essentially block water's accessibility to the hydrogen-bond network from the N-phase. Thus, the hydrogen-bond network is not able to equilibrate with the N-phase (the closed state, hereafter) in the oxidized state, at least within the physiological timescale. The same conformational change in helix X occurs also upon binding of strong heme ligands, such as CO or NO, to the fully reduced CcO. Thus, binding of O\(_2\) (a strong heme ligand) is expected to trigger the elimination of the water cavity. Furthermore, preliminary X-ray structural analyses suggest that the P and F intermediates also show the water channel in the closed state. Thus, the water molecules in the N-phase are effectively accessible to the hydrogen-bond

Figure 3
Redox-coupled conformational changes in D51. (a) Stereoscopic drawing of the hydrogen-bond network in the fully oxidized and reduced states at 1.8 and 1.9 Å resolution, respectively, viewed from the P-phase side. (b) The hydrogen-bonding structure of D51 in the oxidized and reduced states. The smooth, thick curves denote the molecular surface to which the water molecules in P-phase are accessible. The conformational changes upon reduction of CcO are shown by the blue structures on the right. Reprinted from Reference 45.
network through the water channel (the open state), only when the O₂ reduction site is in the fully reduced state. Thus, the effective blockage of the proton back leakage through the H-pathway is facilitated by the peptide bond near the upper end of the hydrogen-bond network and the water channel located in the N-phase side of the hydrogen-bond network.

Mutational Analyses for the H-Pathway

A stable expression system for bovine CcO genes has been constructed in HeLa cells. Thus far, mutants of three critical sites in the H-pathway, D51N, S441P (the peptide bond in H-pathway), and V386L/M390W (the water channel), have been reported (38, 45). The proline mutation is designed to block formation of the normal protonated imidic acid intermediate (∂(C-OH) = N⁺H⁻). The torsion angles of the peptide between Y440 and S441 suggest that the replacement of S441 with proline would have minimal influence on the conformation near the site. The double mutation, V386L/M390W, was generated to block water from accessing the hydrogen-bond network by exchanging the residues on the inside surface of the water channel for the bulkier residues. All three mutant CcOs show the identical phenotype, complete elimination of proton-pumping activity without any significant influence on the function of O₂ reduction. These observations indicate that the functions of these residues are consistent with those proposed by X-ray structural analyses. Furthermore, the X-ray structures of the H-pathway and heme α suggest that the coupling between electron transfer and active transport of protons through the H-pathway is not tight. In other words, heme α could be oxidized without driving the active transport of protons by electrostatic repulsion, because both the delocalization of the net positive charge at Fe₃ and the location of the protons in the hydrogen-bond network are expected to be fairly flexible.

DIVERSITY OF THE PROTON TRANSFER PATHWAYS OF CcO

Bacterial (R. sphaeroides and P. denitrificans) aa₃-type CcOs have a possible proton transfer pathway analogous to the H-pathway but lack D51 (21). Extensive mutational analyses have not shown any positive evidence for involvement of the bacterial H-pathway in proton pumping (21). These mutational studies, as well as the absence of D51 from bacterial and plant CcOs, may be the strongest evidence against proton pumping driven by the H-pathway. A notable report has indicated that the ba₃-type CcO from a thermophilic bacterium has only one possible proton transfer pathway corresponding to the K-pathway (12, 15). Mutations of the amino acids only in the pathway impair both the oxidative and reductive phases of the catalytic cycle, indicating that no other proton pathway exists in this CcO. In other words, both the H-pathway and D-pathway are not conserved. The common elements across all families of the heme-copper oxygen reductases include the heme α₃/CuB dinuclear site, the four histidine imidazole groups that coordinate the metals, and the covalently linked His-Tyr moiety (one of the three imidazoles coordinated to CuB is covalently linked to a tyrosine phenol group). Thus, it has been suggested that none of the proton channels plays a central role in the pumping and gating mechanism; that is, roles of these proton channels are only for proton delivery (12).

However, an alternative interpretation of the diversity of proton-pumping systems in all families of the heme-copper oxygen reductases is possible. Reduction of O₂ without releasing active oxygen species is not a simple chemical reaction. No alternative system with comparable or higher efficiency than the Fe₃/CuB system has been obtained in the evolution of aerobic organisms. In contrast, proton pumping is a chemically simple reaction, and various amino acid residues can facilitate proton pumping in various ways. Thus, although D51 in bovine CcO is not conserved in bacterial and plant CcOs, this cannot be considered
conclusive evidence against the possibility that D51 plays a critical role in the proton pump of animal CcOs. Extensive efforts are required for the structural and functional analyses for each CcO. In this context, mutational analyses for the K- and D-pathways of bovine heart CcO are critical.

CONCLUSIONS

Although these structural comparisons concomitantly with site-directed mutagenesis analyses provide many important insights into understanding the mechanism of proton pumping, these empirical findings must be confirmed by nonempirical experimental results. For example, direct proton movement in CcO during the course of proton pumping must be determined directly, for example, by ultra-high-resolution X-ray crystallographic analyses coupled with time-resolved highly sensitive infrared measurements for elucidation of the proton-pumping mechanism of CcO.

SUMMARY POINTS

1. CcO must transfer protons in the interior of the protein moiety for proton pumping for energy conservation and for making water from molecular oxygen. Three potential proton transfer pathways, D, K, and H, have been identified by X-ray structural analyses of bovine and bacterial CcO. The roles of these pathways have been extensively examined by site-directed mutagenesis analysis.

2. Most of the structural and functional analyses of bacterial CcOs have been interpreted to assume that the D-pathway transfers both pumped and chemical protons. However, the structural elements indispensable for the D-pathway proton-pumping function have not been well characterized, specifically structures relevant for the loading of pumped protons, for switching accessibility between the N- and P-phases, for redox-coupled pKa changes, and for the branching function for the proton transfer direction either to the O₂ reduction site or to the pumped-proton-loading site.

3. Proton pumping through the H-pathway has been proposed based on the X-ray structural analyses of bovine CcO. The structural elements for proton pumping through the H-pathway, including the proton-pumping (loading) site, the structural changes for switching accessibility, and the driving unit, have been demonstrated by X-ray structural analyses at fairly high resolutions.

4. Mutational analyses using the bovine gene expression system have confirmed the functions of some critical H-pathway residues.

FUTURE ISSUES

1. Structural validations for the proposed functions of residues in the D-pathway for evaluations of the D-pathway proposal are needed.

2. Extensive mutagenesis analyses for the D- and K-pathways of bovine CcO would provide important insights into the proton-pumping mechanism.

3. Protons have never been directly observed during the proton-pumping process. In this context, X-ray structural analyses at resolutions sufficient to identify hydrogen and the time-resolved infrared analyses using site-directed stable isotope-labeling techniques are needed for further elucidation of the proton-pumping mechanism of CcO.
DISCLOSURE STATEMENT
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