Review

Proton pumping mechanism of bovine heart cytochrome c oxidase

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Abstract

X-ray structures of bovine heart cytochrome c oxidase at 1.8/1.9 Å resolution in the oxidized/reduced states exhibit a redox coupled conformational change of an aspartate located near the intermembrane surface of the enzyme. The alteration of the microenvironment of the carboxyl group of this aspartate residue indicates the occurrence of deprotonation upon reduction of the enzyme. The residue is connected with the matrix surface of the enzyme by a hydrogen-bond network that includes heme a via its propionate and formyl groups. These X-ray structures provide evidence that proton pumping occurs through the hydrogen bond network and is driven by the low spin heme. The function of the aspartate is confirmed by mutation of the aspartate to asparagine. Although the amino acid residues of the hydrogen bond network and the structures of the low spin heme peripheral groups are not completely conserved amongst members of the heme-copper terminal oxidase superfamily, the existence of low spin heme and the hydrogen bond network suggests that the low spin heme provides the driving element of the proton-pumping process.

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1. Introduction

Cytochrome c oxidase is the terminal oxidase of the cell respiration which reduces molecular oxygen (O2) to water, in a reaction coupled with a proton pumping process. Elucidation of the reaction mechanism of this enzyme is one of the most intriguing subjects in the field of bioenergetics.

Redox-driven unidirectional proton active transport (i.e., the redox-driven proton pump) could be facilitated by conformational changes which alter the accessibility of a proton-accepting site between two different aqueous phases, if the conformational changes are coupled with a pKa change of the proton-accepting site. It has also been proposed that protons can be pumped as a result of electrostatic interactions between the protons used for pumping and protons used for water formation protons without the occurrence of significant conformational changes [1]. For effective coupling between the proton pumping process and O2 reduction, having a proton-pumping site located in close proximity to the O2 reduction site may be advantageous. On the other hand, effective insulation to prevent spontaneous leakage of the pumping protons to the O2 reduction site is indispensable [2].

In any case, a high resolution X-ray structure of the enzyme is prerequisite for elucidation of the proton pumping mechanism. In fact, various functional investigations have been stimulated by the X-ray structural determinations of bovine and bacterial cytochrome c oxidases in 1995 [3,4]. Vibrational spectroscopy is also a very important tool for elucidation of the proton pumping mechanism, since the chemical reactivity of a functional group may be greatly influenced by a structural change that is too small to detect by even the highest possible X-ray resolution.

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X-ray structures of bovine heart enzyme have been improved up to 1.8 Å resolution since 1995 when the first 2.8 Å X-ray structures were reported. These X-ray structures provide various new insights into the mechanism of proton pumping. In this article, the proton pumping mechanism will be discussed based on these X-ray structures and site-directed mutants of cytochrome c oxidase.

2. X-ray structure of the possible proton active-transport pathway from the matrix surface to the intermembrane surface

Asp51 is located within subunit I, near the molecular surface facing the intermembrane space. Upon reduction, this residue becomes exposed to the bulk water phase as shown in Fig. 1 [5]. No other X-ray structural change at 1.8/1.9 Å resolution in the oxidized/reduced states is detectable for other amino acid residues near Asp51. In the oxidized state, the carboxyl group of Asp51 is hydrogen bonded with two serine OH groups and two peptide NH groups. For the carboxyl group, these interactions provide a dielectric microenvironment similar to that of methanol. Upon reduction, the conformational change of the carboxyl group is roughly equivalent to exchange of the medium from methanol to water. This suggests that the \( \text{pK}_a \) of the carboxyl group should decrease by about 5 pH units [6]. The ionization of Asp51 upon reduction is confirmed by an FTIR analysis [5]. The IR spectral change assignable to deprotonation of Asp51 is detectable upon reduction of the mixed-valence CO derivative, which indicates that the \( \text{O}_2 \) reduction site does not control the conformation of Asp51 [5]. A reductive titration of the cyanide-bound oxidized enzyme shows that a single electron equivalent ionizes a single Asp51 equivalent [5]. The redox site that controls the conformation (and the protonation state) of Asp51 is more likely to be heme \( \text{a} \) rather than CuA, since proton ejection is detectable upon \( \text{O}_2 \) oxidation of the fully reduced enzyme reconstituted in proteoliposomes [7,8]. CuA reduction is not included in the oxidation process of the fully reduced enzyme, while heme \( \text{a} \) transfers one electron equivalent from CuA to the \( \text{O}_2 \) reduction site during the \( \text{O}_2 \) reduction process.

The net positive charge of heme \( \text{a} \) could lower the activation energy for migration of the dissociated carboxyl group into the interior of the protein upon oxidation of heme \( \text{a} \). Similarly, the positive charge of CuA also contributes to decrease in the activation energy. However, the electrostatic interactions from these metal sites are eliminated upon protonation of the carboxyl group. Thus, the four hydrogen bonds to Asp51 in the oxidized state appear to be the major stabilizing factor of the conformation of Asp51 in the oxidized state.

Asp51 is located at one end of a hydrogen bond network. The other end is connected with the matrix surface by a water channel through which bulk water molecules are accessible, as shown schematically in Fig. 2 [5]. The hydrogen bond network interacts with heme \( \text{a} \) via two hydrogen bonds, between the formyl group and Arg38 and between the propionate and a

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Fig. 1. Schematic representation of the hydrogen-bonding structure of Asp51 in the oxidized and reduced states. The smooth thick curve represents the molecular surface accessible to water molecules in the intermembrane space. The balls represent the fixed water molecules. The dotted lines denote hydrogen bonds. The double-headed dotted arrows indicate possible movement of the water molecule from Arg38 to Tyr371.
water molecule in the network (Fig. 2). The network is called the H-pathway. Heme a is in the six-coordinated low spin state. In its reduced state, the two positive charge equivalents of the iron (Fe^{2+}) are neutralized by the two negative charge equivalents of the porphyrin. Upon oxidation of heme a, one equivalent of positive net charge is created, since the heme, located in the protein interior, cannot obtain a counter ion. The positive charge of the heme a iron (Fe^{2+}) is delocalized to the porphyrin peripheral groups. Evidence for this delocalization is clearly revealed by a 40 cm\(^{-1}\) redox-coupled shift in the C\(=\)O stretch band of the heme a formyl group\[9,10\]. The decrease in electron density must decrease the proton affinity of the guanidino group of Arg38 which is hydrogen-bonded to the formyl group at the end of the hydrogen bond network extending to Asp51. Thus, the electrostatic interaction could be a driving force for proton active transport through the hydrogen bond network. In the reduced state, the guanidino group extracts protons from the water channel. Although the pK\(_a\) of the guanidino group is significantly lower in the protein interior, relative to the pK\(_a\) in the aqueous solution, the proton affinity could still be high enough to enable the guanidino group to extract protons from the water channel.

Upon oxidation of the heme iron, the net positive charge could cause a decrease in the electron density of the propionate carboxyl groups although they are not directly conjugated with the porphyrin \(\pi\)-electron system. One of the propionates is hydrogen-bonded to one of the fixed water molecules in the hydrogen bond network. This interaction could accelerate the process of proton transfer through the hydrogen bond network. Thus, the X-ray structures suggest that the two hydrogen bonds that connect heme a to the hydrogen bond network could be crucial for coupling the proton and electron transfer processes of cytochrome c oxidase.

The hydrogen-bond network includes a peptide bond (Fig. 2) whose N–H group is hydrogen-bonded to Asp51 when the enzyme is in the oxidized state. Protons can be transferred...
through the peptide bond which is capable of forming an imidic acid intermediate, \((-\text{COH})\text{==N}^+\text{H}^-\) [11]. After extraction of the proton from the imidic acid to produce the enol form of the peptide \((-\text{COH})\text{==N}^-\), proton transfer for tautomerization to the keto form \((-\text{CO}^-\text{NH}^-\)\) is thermodynamically favorable. This intrinsic property of the peptide bond provides unidirectionality in the proton transfer process through the hydrogen bond network. In the process of tautomerization, the OH hydrogen migrates to the nitrogen atom of the enol form \((-\text{N}^-\). In aqueous solution, the transition readily proceeds by proton transfer mediated by solvent water. In the protein, some conformational flexibility of the peptide bond is required for the transition at a physiologically-relevant rate [12].

A large increase in the capacity of the water channel is induced by conformational changes in the hydroxyfarnesylethyl group and movement of a helix, as schematically shown in Fig. 2 [5]. This conformational change would promote effective exchange of water molecules in the water channel with the bulk water in the matrix phase. The ovals in Fig. 2 show the probable locations of water molecules. The gray rectangles denote water pathways through which water molecules can migrate depending on the dynamic nature of the amino acid residues surrounding the pathways. The upper end of the pathway extending to the formyl/Arg38 site is fairly narrow. The structure suggests that the interaction between Arg38 and the water molecule transferred from the matrix space through the channel is tightly controlled by the conformational change at the upper end. This conformational change could effectively block the reverse proton transfer process. The intrinsic proton affinity of the guanidino group suggests that Arg38 can extract protons from the water molecules to leave OH\(^-\) ions. The redox-coupled capacity change in the water channel would provide the means to effectively drain these OH\(^-\) ions out to the matrix space.

3. Mutational analyses of H-pathway

The X-ray structures of bovine heart cytochrome c oxidase in the oxidized and reduced states indicate the proposed proton pumping system driven by heme \(a\), as given in the previous section. However, in general, X-ray structures do not provide direct evidence for the function of the protein. In fact, it has been suggested that the H-pathway might act as a system for discharging the membrane potential to retain the potential below the allowable limit. Therefore, direct examination of the function of the system is necessary for evaluation of the proposal described above. Site-directed mutagenesis provides the means to probe the function of specific amino acid residues.

The H-pathway is embedded in the largest subunit (subunit I) of the enzyme, which is encoded by a mitochondrial gene [5,13]. In order to develop a site-directed mutagenesis system, a stable gene expression system for subunit I of bovine heart cytochrome c oxidase was constructed in HeLa cells [5]. A signal peptide of subunit IV which is a nuclear-coded subunit of this enzyme is attached on the N-terminal side of the subunit I. Thus, the subunit I peptide synthesized by cytosolic ribosomes in the transfected HeLa cells is guided by the signal peptide and transferred to the mitochondrial inner membrane. The bovine subunit I peptide is assembled with another 12 subunits of human cytochrome c oxidase. The yield of the hybrid enzyme evaluated by Western blot analyses is about 80% [5]. The absorption spectrum of the mitochondrial suspension clearly shows the \(\alpha\)-band of cytochrome c oxidase at 604 nm in the reduced state, suggesting that the hybrid enzyme adopts a native conformation. The hybrid enzyme activities (proton pumping and \(O_2\) reduction) are identical to those of the non-hybrid human enzyme [5]. These results indicate that the subunit assembly process and the conformation of the hybrid enzyme are identical to those of the native human enzyme. It is quite remarkable that the subunit I peptide which is composed of 514 amino acids including 12 transmembrane helix regions, is transferred from the opposite side of the mitochondrial inner membrane to attain its proper association with the other 12 subunits to form the native enzyme.

Proton pumping activity is abolished in a site-directed mutant of the hybrid enzyme in which Asp51 has been replaced with asparagines [5]. However, the \(O_2\) reduction activity of this mutant determined by oxidation of ferrocytochrome c under aerobic conditions is about 1.5 times higher than that of the hybrid enzyme. These results confirm the proposal that Asp51 functions as the proton pumping site. In the oxidized state of the enzyme, the Asn51 residue participates in the hydrogen bond network and provides the same hydrogen bonding functionality to the acid-amide group as the carbonyl group of the Asp51. This indicates that the capability of deprotonated Asp51 to extract protons within hydrogen bonding distance of the peptide bond is critical for the proton pumping process. The driving force provided by heme \(a\) for proton active transport through the hydrogen bond network from Arg38 to the carbonyl group of the peptide bond is not itself strong enough to release protons to the molecular surface via the peptide bond and the acid amide group of Asn51.

The mutation results argue against previous suggestions that Asp51 has a role in discharging the membrane potential [14,15]. The discharge system would not function in the presence of valinomycin.

X-ray structures of cytochrome c oxidase from two different bacteria indicate the existence of a potential proton transfer system which is homologous to the H-pathway. One notable difference is observable at the upper end of the proton channel where a glycine residue and a water molecule are found at the location occupied by Asp51 bovine cytochrome c oxidase [16,17]. The H-pathway of the two bacterial enzymes has been extensively examined by site directed mutagenesis analyses. No significant effects on electron transfer activity or proton pumping activity were observed except for the case of mutation of the residue equivalent to Arg38 in bovine cytochrome c oxidase [16,17]. Replacement of Arg38 with methionine decreases the electron transfer activity to a small percentage of that of wild type enzyme. Proton pumping activity is also significantly affected to give the pumping proton / electron ratio of unity [18]. Based on these analyses, it has been concluded that at least in these bacterial enzymes, the H-pathway is not involved in the proton pumping process. However, it should be
noted that the methionine thioether can form two hydrogen bonds as the hydrogen acceptor to provide a link within a proton transfer pathway. In fact, methionine residues participate in the hydrogen bond networks for transfer of water-forming protons (within the K and D pathways as described below). However, the methionine sulfur is not able to form a hydrogen bond with the formyl group. On the other hand, the removal of the hydrogen bond to the formyl group by the mutation could have the effect of increasing the redox potential which would decrease the rate of electron transfer from heme \( a \) to the \( O_2 \) reduction site. The slower electron transfer process could then couple with proton transfer at the propionate / water hydrogen bonding site. Thus, an alternative interpretation for the electron/pumping proton ratio of unity observed for the Arg38Met mutant is that this result provides support for our proposal that proton pumping occurs through the H-pathway.

Many amino acid residues in the water channel may be replaced via site-directed mutagenesis with less bulky residues with no significant inhibitory effects [16,17]. This does not argue against the role of the water channel because less bulky residues would not be expected to block the process of migration of water molecules through the channel. In recent report, a double mutant was created that has selected residues in the water channel replaced by bulkier residues without causing any significant inhibitory effects [19]. However, the X-ray structure suggests that the bulkier residues significantly influence the conformation of the water channel to provide an extended space large enough to promote water migration instead of blockage of the water channel. Furthermore, Tyr371 (bovine) which is located within the hydrogen-bond network has been replaced by phenylalanine without significant inhibitory effects [16,17]. If the phenylalanine occupies the same site as the tyrosine it replaced, a space remains which is large enough for a water molecule to occupy. This water molecule would function as a link within the proton transfer pathway in a manner similar to the native tyrosine. Although these mutation results do not provide direct support of the proposal that the H-pathway plays a major role in the proton-pumping system, they also do not provide conclusive proof against this proposed role of the H-pathway.

4. Evolutional aspects of the proton pumping system

The proposed proton-pumping site, Asp51, is not highly conserved. Cytochrome \( c \) oxidases of plants and bacteria do not have a homologous aspartate at the site corresponding to bovine Asp51. These findings do not provide conclusive evidence against the proposal that the hydrogen bond network plays a major role in proton pumping because different amino acid residues may have similar functionalities. Two bacterial \( aa_3 \) type cytochrome \( c \) oxidases have proton transfer pathways which are essentially homologous to that of the bovine pathway. The pathways are composed of a hydrogen bond network and a water channel. However, Asp51 is replaced by a glycine residue and a cavity of sufficient size to accommodate one or two water molecules. The results of site-directed mutagenesis hybrid enzyme suggest that the water molecules are not able to extract protons from the hydrogen bond network through the peptide bond as effectively as Asp51. However some differences in the other residues of the pathway are detectable between the bovine enzyme and the bacterial enzymes. In the bovine enzyme, the propionate group of heme \( a \), which interacts with the hydrogen-bond network via a water molecule which is further hydrogen-bonded to the peptide carbonyl group is also hydrogen-bonded to a tyrosine. On the other hand, instead of tyrosine, the bacterial enzymes have tryptophan which does not form a hydrogen bond to the propionate. The lack of this hydrogen bond to the propionate is expected to strengthen the electrostatic interaction for accelerating the proton transfer across the peptide bond. In this proton pumping system, protons are released upon oxidation of heme \( a \) in contrast to the proton release upon heme \( a \) reduction in the case of the bovine enzyme. The difference in the timing of the proton release is consistent with a recent report [20].

Some of the bacterial terminal oxidases which reduce \( O_2 \) in a reaction coupled with proton pumping have heme \( b \) instead of heme \( a \) [21]. In these enzymes, the propionate group may be the main site for interaction between the low spin heme and the hydrogen bond network. Furthermore, a bacterial \( ba_3 \) type cytochrome \( c \) oxidase has a hydrogen bond network across the enzyme (known as the Q-pathway) which is not homologous to that of the bovine enzyme [22]. The bacterial enzyme structure suggests a proton pumping mechanism which is somewhat different from that of the bovine enzyme, although driven by the low spin heme. On the other hand, the structure of the \( O_2 \) reduction site, which is composed of a high spin heme and a copper ion with three histidine imidazoles in a trigonal planar coordination geometry, is well conserved amongst cytochrome \( c \) oxidases from different species with the exception of the peripheral heme substituents. Some bacterial enzymes contain heme \( o \) or heme \( b \) instead of heme \( a_3 \) [21]. Reduction of \( O_2 \) to water without release of active oxygen species requires a complex chemical process. Perhaps, no other system is more effective for the process than the heme-copper system of cytochrome \( c \) oxidase. The \( O_2 \) reduction site is well sequestered from the \( H \) and \( Q \) pathways. The structural aspect is highly conserved among different species. This evolutionary conservatism argues strongly for a requirement for complete insulation against the spontaneous leakage of protons participating in the process of pumping protons to the \( O_2 \) reduction site [2]. In general, examination of evolutionary conservation is a powerful means for identification of amino acids involved in the functional site of a protein. However it should be noted that the empirical approach cannot be conclusive. The wide varieties of structures of potential proton transfer systems interacting with the low spin heme cannot provide conclusive evidence against these systems functioning in the proton-pumping process.

5. The proton pumping mechanism including the proton transfer pathway for water-forming protons

Cytochrome \( c \) oxidase has two possible proton transfer pathways detectable in the X-ray structures and mutational analyses of bacterial enzymes which are known as the D and K-pathways. The pathways extend from the molecular surface of
the matrix side to the O\textsubscript{2} reduction site. Glu242 (bovine) is located at the upper end of D-pathway. A Glu242Gln mutation abolishes both proton and electron transfer activities. Similar mutation results have been reported for Asp91 which is located within the D-pathway near the molecular surface of the matrix side [23]. These results indicate that proton transfer is tightly coupled with electron transfer, although direct structural interactions between the electron transfer pathway and proton transfer pathway are not observable. Furthermore, the K-pathway which also functions as a pathway for water forming protons, does not transfer protons in these D-pathway mutant enzymes, suggesting that tight coupling exists between the D and K pathways. The proton transfer channel blocked by the mutations must be employed for protons used for water formation since electron transfer to the O\textsubscript{2} reduction site (the site of water formation) is inhibited. A D-pathway mutation, Asn98Asp, abolishes the proton pumping process without affecting the electron transfer activity or with increasing the activity [23,24]. It has been proposed that the two types of mutations in D-pathway, the Glu242Gln type which blocks proton transfer for water formation and the Asn98Asp type which blocks only transfer of protons used in the proton pumping process, indicate that the D-pathway is used for transfer of protons used for pumping and protons used for water formation.

However, it should be noted that these observations do not provide conclusive proof that transfer of protons used for proton pumping occurs through the D-pathway. Asn98 cannot act as the proton pumping site because it is located near the entrance of the D-pathway. Replacement of Asn98 with Asp would not be expected to block proton transfer through the pathway. In fact, proton transfer for water formation is not inhibited by the mutation. Although mutations at sites within the D-pathway inhibit the proton pumping process, they do not provide conclusive evidence that the D-pathway acts as the proton pumping pathway. The mutation result rather suggests that the D-pathway is not involved in the proton pumping process. If the D-pathway transfers protons used for both proton pumping and water-forming protons, mutation of residues within the pathway should influence both proton transfer activities to an equal extent.

An interpretation of these observations is that a long range interaction exists between Asp98 and Glu242 [25]. Glu242, which is located at the upper end of the D-pathway has been proposed to be the site where switching of the direction of the proton transfer from the O\textsubscript{2} reduction site to the putative site for accepting pumping protons at the propionate of D-ring of heme a\textsubscript{3} [26]. For this switching function, the pK\textsubscript{a} of Glu242 must be lower than the pK\textsubscript{a} of the pumping-proton accepting site and the pK\textsubscript{a} of the O\textsubscript{2} reduction site. The observation that the mutation abolishes the proton-pumping activity without decreasing the O\textsubscript{2} reduction activity could be the result of increasing the pK\textsubscript{a} of Glu242 to the higher level than the pK\textsubscript{a} of the proton accepting site, but lower than that of the O\textsubscript{2} reduction site [26]. It should be noted that the mutation results themselves do not identify the long range interaction. An alternative interaction may equally exist between Asp98 and Arg38 which would increase the pK\textsubscript{a} of Arg38 and cause it to lose its function as a proton donor to the hydrogen bond network extending to Asp51.

Direct experimental evidence that transfer of protons used in the proton pumping process occurs through the D-pathway is not yet available. However, this point is not well recognized and experimental proof is highly desirable. With regard to the H-pathway, real time vibrational spectroscopic analyses of the enzyme reaction are expected to aid in the evaluation of the H-pathway proposal.

References


