Biophysics 490M Project:

Exploring the Structure and Function of Cytochrome bo3 Ubiquinol Oxidase from *Escherichia coli*

Lai Lai Yap

Department of Biochemistry
The respiratory systems of bacterial are branched in that many distinct terminal oxidases are present, unlike most eukaryotic mitochondrial systems in which only cytochrome c oxidase is present. These oxidases have different substrates (ubiquinol versus cytochrome c), oxygen affinities, and heme types and metal compositions. Together, they form a closely-related superfamily called the heme-copper oxidase superfamily, which also includes the eukaryotic mitochondrial oxidases. The study of these bacterial oxidases in the prokaryotic systems offers experimental advantages in the exploration of the structures and the functional mechanisms common to the members of the superfamily.

Cytochrome bo₃ ubiquinol oxidase is a terminal oxidase in the aerobic respiratory chain of *Escherichia coli* and is also a member of the heme-copper oxidase superfamily. It consists of four subunits and catalyzes the two-electron oxidation of ubiquinol-8 (Q₈H₂) at the periplasmic side of the cytoplasmic membrane and the four-electron reduction of oxygen to water at the cytoplasmic side (Fig. 1). In addition to the protolytic scalar reactions, cytochrome bo₃ ubiquinol oxidase also functions as a proton pump by translocating protons across the cytoplasmic membrane to establish an electrochemical proton gradient. The transmembrane proton and voltage gradient thus generated is then converted to more useful energy forms through energy conserving systems such as the ATP synthase.

![Fig. 1 Electron and proton transfer in cytochrome bo₃ ubiquinol oxidase.](image-url)
Three redox metal centers are located in subunit I (the largest subunit), namely, low-spin heme \( b \), high-spin heme \( o \) and \( \text{Cu}_b \) (Fig. 1 and 5). Together, heme \( o \) and \( \text{Cu}_b \) form the binuclear center where oxygen binds and is reduced to water (Fig. 1 and 5). Subunits I, II and III are homologous to the counterparts of cytochrome c oxidase, another member of the heme-copper oxidase superfamily. However, in contrast to cytochrome c oxidase, which uses a water-soluble cytochrome c as the electron donor, cytochrome bo\(_3\) ubiquinol oxidase uses a membrane-soluble ubiquinol-8 (consisting of eight isoprenoid units in the hydrophobic tail) as the electron donor. Furthermore, cytochrome c oxidase contains an additional metal center, \( \text{Cu}_A \), in subunit II, which accepts electrons from cytochrome c; this is absent in cytochrome bo\(_3\) ubiquinol oxidase since it uses a different type of electron donor. Instead, two ubiquinol-8 binding sites with two distinct functional roles have been proposed for cytochrome bo\(_3\) ubiquinol oxidase. In one possible mechanism, ubiquinone bound at the high affinity site (\( Q_{H} \)) acts as a cofactor and mediates electron transfer from the ubiquinol substrate, which binds at the low-affinity site (\( Q_{L} \)), to the low-spin heme \( b \). The reduced heme \( b \) then provides electrons to the binuclear center for the reduction of oxygen to water to occur (Fig. 1).

The crystal structure of cytochrome bo\(_3\) ubiquinol oxidase from \textit{E. coli} has been solved at 3.5Å. The electron density for most of the transmembrane helices was clear enough to allow for sequence alignment; however, this could not be done for many of the loop regions due to lack of resolution. In all, 967 residues out of 1291 residues in cytochrome bo\(_3\) ubiquinol oxidase could be assigned. The overall structure (in ribbon representation and spherical representation) of cytochrome bo\(_3\) ubiquinol oxidase is shown in Fig. 2A and B, and it is very similar to that of cytochrome c oxidase from \textit{Paracoccus denitrificans} (Fig. 3). Both structures can also be superimposed on each other (Fig. 4).

![Fig. 2A](image)

Overall structure of cytochrome bo\(_3\) ubiquinol oxidase from \textit{E. coli} parallel to the membrane in ribbon representation. The dotted circle represents the location of the electron donating substrate substrate ubiquinol at the posterior of the protein within the membrane.
Fig. 2B  Overall structure of cytochrome bo₃ ubiquinol oxidase from *E. coli* parallel to the membrane in spherical representation.

Fig. 3  Overall structure of cytochrome c oxidase from *P. denitrificans* and cytochrome bo₃ ubiquinol oxidase from *E. coli*, showing the cytochrome c binding site and ubiquinol binding site respectively.
All three redox metal centers in cytochrome bo$_3$ ubiquinol oxidase are associated with subunit I, and therefore, subunit I functions as the reaction center of the oxidase complex. It has been shown by magnetic circular dichroism, EPR (electron paramagnetic resonance) and X-ray absorption fine structure studies that histidines are the axial ligands of the metal centers (Fig. 5). His$^{106}$ and His$^{421}$ are axial ligands of low-spin heme b, and His$^{284}$, His$^{333}$ and His$^{334}$ are ligands of the Cu$_B$ center. Last but not least, His$^{419}$ is the proximal ligand of high-spin heme $o$. The distance from His$^{106}$ and His$^{421}$ to the heme $b$ Fe is 2.189 Å and 2.212 Å respectively, while that of His$^{284}$, His$^{333}$ and His$^{334}$ to Cu$_B$ is 2.162 Å, 2.145 Å and 2.218 Å respectively. His$^{419}$ is 2.247 Å away from heme $o_3$ Fe.

Fig. 4 Superposition of cytochrome c oxidase from *P. denitrificans* and cytochrome bo$_3$ ubiquinol oxidase from *E. coli*.

Fig. 5 The redox metal centers in cytochrome bo$_3$ ubiquinol oxidase with the coordinating ligands.
Two proton transfer pathways have been identified in subunit I in the crystal structure of cytochrome c oxidase from *P. denitrificans*. These two proton transfer pathways, called the D- and K-channels, are also observed in subunit I of cytochrome bo₃ ubiquinol oxidase (Fig. 6A and B). Both these channels contain amino acid residues that are highly conserved in cytochrome c oxidases and ubiquinol oxidases, thus suggesting that they have similar functions. The D-channel is so called because it begins with a highly conserved Asp residue (Asp₁³⁵ of cytochrome bo₃ ubiquinol oxidase in *E. coli*), while the K-channel contains a conserved Lys residue (Lys³⁶² of cytochrome bo₃ ubiquinol oxidase in *E. coli*) in the middle of the pathway. Both these channels form polar cavities that originate on the cytoplasmic side, leading to the binuclear center for proton pumping and water formation (Fig. 6A). The D- and K-channels are also lined with water molecules that form a network of hydrogen bonds for proton transfer to occur; however, the water molecules are excluded from the figure as they are not found in the PDB file.

The D-channel begins with Asp₁³⁵ and proceeds through Asn¹²⁴, Thr²¹¹, Asn¹⁴², Asn¹²⁴, Tyr⁶¹, Thr²⁰⁴, Ser¹⁴⁵, Thr²⁰¹, Thr¹⁴⁹ and ends at Glu²⁸⁶ (Fig. 6A and B). Both Asp₁³⁵ and Glu²⁸⁶ are conserved in the proton pumping oxidases: the D₁³⁵N mutant lost proton pumping activity but retained half of the enzyme activity and wild-type properties of the redox metal centers, while the E₂₈₆Q and E₂₈₆A mutants have severely reduced enzyme activity and a perturbed binuclear center. Hence, Asp₁³⁵ appears to be important for vectorial proton translocation whereas Glu²⁸⁶ seems to serve as the immediate proton donor to the binuclear center. On the other hand, the K-channel starts with Ser³¹⁵ and continues up to the binuclear center through Ser²⁹⁹, Lys³⁶², Thr³⁵⁹, the OH group of the hydroxyethylfarnesyl tail of heme *o*₂ and Tyr²⁸⁸ (Fig. 6A and B). The Y₂₈₈F mutant has severely reduced enzyme activity and Cu₉ binding in addition to perturbed high-spin heme environment. This suggests that Tyr²⁸⁸ is crucial for binding both high-spin heme *o* and Cu₉. Studies have shown that the D-channel is involved in the uptake of both chemical and pumped protons, while the K-channel is used for loading the enzyme with protons at some earlier catalytic steps.

A major difference between ubiquinol oxidase and cytochrome c oxidase is that the former uses a membrane soluble electron donor (ubiquinol) for reduction of oxygen to water, whereas the latter uses a water-soluble electron donor (cytochrome c). When the ubiquinol oxidase is solubilized with the detergent *n*-dodecyl-β-D-maltoside (DM), the high affinity ubiquinol binding site (Q₉) is able to maintain a tightly bound ubiquinone molecule. However, the enzyme was crystallized using the detergent *n*-octyl-β-D-glucopyranoside (OG), and under these conditions, crystals were obtained without bound ubiquinone. Hence, the ubiquinol binding site was identified by surveying the structure and mapping potential binding sites based on biochemical data, and using known structural motifs in membrane proteins that bind ubiquinone.

Previously, it was proposed that the ubiquinol binding site was located in subunit II of the enzyme (at its interface with subunit I), as the structure of this domain is similar to the corresponding domain in cytochrome c oxidase (except that Cu₉ is absent in the ubiquinol oxidase). It has also been proposed that the Cu₉ center in cytochrome c oxidase may be replaced by a ubiquinol binding site in ubiquinol oxidase. Despite this, the sequence motif search for a ubiquinol binding site in subunit II of cytochrome bo₃ ubiquinol oxidase proved to be futile.
The crystal structure of cytochrome bo$_3$ ubiquinol oxidase, however, suggests a potential ubiquinol binding site in the membrane domain of subunit I (Fig. 7). This membrane domain consists predominantly of hydrophobic $\alpha$-helices (Fig. 2A), and contains a patch of conserved polar and charged residues. The fact that such a hydrophilic patch is localized within the membrane seems to contradict the paradigm of membrane protein structure, and it suggests that there must be an underlying reason for maintaining such an energetically unfavorable structure in this environment. This polar cluster (including Arg$_{71}$, Asp$_{75}$, His$_{98}$ and Gln$_{101}$) is located near the Cu$_A$ center in cytochrome c oxidase. In order to investigate the functional relevance of this polar cluster, site-directed mutagenesis was performed. The enzyme activity of the R71L, R71Q, D75N and H98N mutants were all blocked, while the conservative Q101N mutant had only 25% activity compared to wild-type enzyme. The loss of fast electron transfer from bound ubiquinol was also observed in the Q101N mutant by electrochemical studies. The spectrum of heme $b$ was slightly shifted in the mutants, indicating that the loss of bound ubiquinol affects the spectrum of heme $b$. Therefore, this also implies that the ubiquinol binding site is relatively close to heme $b$ and also that mutations in this site cause a change or loss of ubiquinol binding. Moreover, the steady state level of reduction of heme $b$ was decreased during turnover in all the mutants, suggesting that impaired electron transfer from ubiquinol to the heme group resulted in the inhibition of enzyme activity.

Fig. 6 The possible D- and K-channel proton pathways in subunit I of cytochrome bo$_3$ ubiquinol oxidase.
A. The helices lining both D- and K-channels are shown in light blue.
B. The polar side chains, heme $b$ and the binuclear center in the D- and K-channels.
Based on both biochemical and structural evidence, a ubiquinone molecule is modeled into the proposed ubiquinol binding site (the modeled ubiquinone was not included in the PDB file), which is exposed to the membrane bilayer (Fig. 8). The ubiquinone molecule is liganded to Arg\textsuperscript{71}, Asp\textsuperscript{75}, His\textsuperscript{98} and Gln\textsuperscript{101} with a distance of 3.939 Å, 3.222 Å, 2.579 Å and 2.500 Å respectively. This proposed ubiquinol binding site is similar to the ubiquinol binding sites of other membrane proteins, for instance, it adopts a similar binding conformation to the ubiquinone in the Q\textsubscript{i} and Q\textsubscript{o} binding sites of cytochrome bc\textsubscript{1} complex. A possible electron path from the ubiquinone to the binuclear center (consisting of heme o\textsubscript{3} and Cu\textsubscript{B}) via heme b is shown in Fig. 9.

EPR studies have shown that the bound ubiquinone can be stabilized as a semiquinone anion radical which displays an X-band EPR spectrum with characteristic hyperfine structure, and this has been used to study and identify the residues involved in ubiquinone binding proposed by the crystal structure. The results show that H98 and R71 are required for ubiquinone binding and stabilization of the semiquinone radical formed during catalytic turnover. These two mutants, H98F and R71H, have less than 1% wild-type activity and complete loss of activity respectively, and the semiquinone radical signal could not be detected in both mutants. The ubiquinol binding site of the wild-type enzyme as well as that of both mutants are shown in Fig. 10. The residual activity of the H98F mutant suggested that some electron transfer may still occur with ubiquinone being stabilized as a radical. Since the D75H mutant still retains the radi-

Fig. 7  Location of modeled ubiquinone (shown in spherical representation) in subunit I of cytochrome b\textsubscript{0}3 ubiquinol oxidase (ribbon structure).
Fig. 8  A possible ubiquinol binding site in subunit I of cytochrome bo$_3$ ubiquinol oxidase with modeled ubiquinone (in green).

Fig. 9  View of subunit I of cytochrome bo$_3$ ubiquinol oxidase along membrane normal from the periplasmic side with modeled ubiquinone (in green), showing possible electron path (dotted line).
cal signal, D75 is not directly involved in radical stabilization, but it is possible that it is interacting with the radical directly (probably by hydrogen bond formation).

Another low-affinity Q$_L$ binding site has been proposed previously next to the high-affinity Q$_H$ site, but the crystal structure reveals no other potential binding site near the one described. However, there is a possibility that the second site is created only after the first ubiquinone molecule is bound, so that electrons can then be delivered by a new ubiquinone molecule via the one already bound. Yet, it is also possible that the second binding site does not exist at all, and the ubiquinol binding site described here is sufficient for the oxidation of ubiquinol by the oxidase.

Fig. 10  The ubiquinol binding site of (A) wild-type enzyme, (B) H98F mutant and (C) R71H mutant.
All crystal structures were generated using Chimera (http://www.cgl.ucsf.edu/chimera). The superpositioned structures were generated in Chimera using the match command, and the in silico mutageneses were performed using the swapaa command.

References


