THE INTERACTIONS OF CYTOCHROME BO3 FROM ESCHERICHIA COLI WITH ITS SUBSTRATES - UBIQUINONE AND OXYGEN

BY

SYLVIA KYEONG-MIN CHOI

DISSERTATION

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Doctoral Committee:
Professor Robert B. Gennis, Chair
Professor James A. Imlay
Professor Yi Lu
Professor Satish K. Nair
ABSTRACT

Heme-copper respiratory oxygen reductases reduce O$_2$ to water and use the redox free energy to generate the proton motive force. Among the heme-copper oxygen reductases are the quinol oxidases, which catalyze the 2-electron oxidation of ubiquinol or menaquinol instead of cytochrome c. *Escherichia coli* (*E. coli*) cytochrome $b_03$ is the best characterized quinol oxidase. Depending on the detergent used to solubilize the enzyme, cyt $b_03$, preparations of this enzyme contain between 0 to 2 equivalents of ubiquinone-8. Studies using ubiquinol-1, a soluble quinol substrate, indicate one high affinity quinone binding site, which acts as a non-exchanging co-factor, and a second, low-affinity site which is the substrate site. The residues interacting with the quinone at the high affinity $Q_H$ site are well defined through numerous mutagenesis and spectroscopic studies, but the identification of the low affinity $Q_L$ site has remained difficult.

To clarify whether the low affinity $Q_L$ exists, we used structural models to guide us. First is based on the new structure of cytochrome $b_03$ we have obtained by collaboration with the Stowell group. It shows two ubiquinone molecules bound to the enzyme. The benzoquinone headgroup of one bound quinone is located at the expected $Q_H$ site. The headgroup of the second bound quinone is, however, located on the opposite side of the molecule. Site-directed mutagenesis of residues located at the putative second site do not indicate that this second site is functionally important, leaving the question of the existence of the $Q_L$ site open. The second model is based on bioinformatics. Recent
publication from Bossis group used bioinformatics methods to identify a possible Q_L site. We have tested this model using site-directed mutagenesis but have found no evidence for disruption of a substrate binding site. Along with these two models, we have revisited the possibility of a Q_L site in subunit II with site-directed mutagenesis but did not find the Q_L site.

A strong argument in favor of the existence of the Q_L site is the inability of the quinone bound at the Q_H site to exchange during enzyme turnover. These experiments were performed using the soluble quinone analogue, ubiquinone-1. This experiment has been repeated with long chain quinol substrates to test the possibility that these might behave differently. If these long chain quinones exchange into the Q_H site during turnover, there would be no need to postulate the existence of the second Q_L site.

All of the previous attempts to exchange the quinone at the Q_H were done in the purified micelles-form of enzyme with short chain quinone analogue, UQ1 or inhibitors. In my work, I will demonstrate that, in the lipid bilayer this “tightly bound” quinone can not only dissociate, but also can exchange with an exogenous quinone. Cyt bo_3 embedded liposomes allowed us to reconstitute a respiratory chain using with a monotopic membrane protein, NDH-2, and have a good turnover with hydrophobic long chain UQ10. Moreover, we show that the “tightly-bound” quinone can readily exchange with UQ2 even in micelles depending on incubation time and concentration of the substrate. Therefore, proving that this “cofactor-like” quinone can actually dissociate from the enzyme and exchange with exogenous quinone, allows us to argue that there is only one quinone binding site.
To Dad, Mom, Diana Jaemin, Jhinkyu, Grandma,

& Snowball
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모두 사랑하고 감사합니다.

#THISWASAGOODRUN

#I_DID_IT

#GENNISLAB4EVA
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I. INTRODUCTION

1. Overview of the heme copper oxygen reductases

Heme-copper oxygen reductases (HCO) are terminal oxidases from aerobic respiratory chain (Fig. 1.1), membrane proteins catalyzing reduction of oxygen to water. The redox energy is coupled to proton translocation, which contributes to the electrochemical proton gradient that drives energy-requiring processes such as ATP-synthesis. The protons are taken from the negative (N) side of the membrane and are released to the positive (P) side membrane through proton channels [1-8].

\[
8H^{+}_{in} + O_{2} + 4e^{-} \rightarrow 4H^{+}_{out} + 2H_{2}O
\]

Among HCOs, subfamilies A, B and C are distinguished based on the phylogeny, genomic sequence, and structural information. Each of these subfamilies pumps protons coupled to reduction of oxygen. However, the proton pathways are different among the subfamilies. The sequence homology of the main subunits (I and II) among HCOs in the same subfamily show significant sequence similarity (>35% identity) while HCOs outside of the subfamily show much less sequence homology (<20% identity). This shows the large divergence among the subfamilies [6, 8, 9].

HCOs are also differentiated on the basis of the electron donor. If the electron donor is quinol, the oxidase is categorized as a quinol oxidase (e.g., cytochrome bo₃ from Escherichia coli). If the of electron donor is a cytochrome c, the oxidase is categorized as a cytochrome c oxidase (CcO). The difference of electron donor correlates with structural differences. CcOs have a di-copper site (Cuₐ) in subunit II, whereas the quinol oxidases have a quinol binding site instead within subunit I and do not have a Cuₐ site [1, 8, 10].
HCOs are found in all three domains of life [9]. The bovine heart mitochondrial cytochrome oxidase is among the most studied HCO, as is the bacterial CcO from *Rhodobacter sphaeroides*. Cytochrome (cyt) *aa*₃ from *R. sphaeroides* is an A-family enzyme, which consists of four subunits. Subunits I, II and III are homologues of the equivalent subunits in the mitochondrial enzyme. Subunits I and II contain all that is required for catalytic function, including proton pumping. Subunit I is the largest (15 transmembrane helices) and most conserved subunit. The redox active metals in subunit I are embedded within the membrane: the low spin heme *a*, the high spin heme *a*₃ and CuB. Heme *a*₃ and CuB form the binuclear center, which is considered to be the catalytic site. The copper is ligated to three conserved histidines. One of the histidine ligands is crosslinked to a tyrosine, which forms a novel His-Tyr crosslinked cofactor [4, 7, 10].

2. The respiratory chain and quinones, in *E. coli*

Prokaryotes usually have a simplified respiratory chain. In the case of *E. coli*, the respiratory chain has a two NADH dehydrogenase (complex I and NDH₂), succinate dehydrogenase, and three terminal oxidases (cyt *bo*₃, *bdI* and *bdII*). Different organisms contain different quinone species as electron carriers. In case of *E. coli*, there are three types of quinones: ubiquinone (UQ₈) is the main quinone for aerobic respiration while menaquinone (MK₈) and demethylmenaquinone (DMK₈) are the main quinones present during anoxic growth conditions. Once reduced, both UQ₈ and MK₈ quinones are ultimately oxidized by the respiratory quinol oxidase which reduces oxygen to water. Under conditions of high aeration, cyt *bo*₃ is highly expressed and uses ubiquinol (UQ₈)
as an electron donor while cyt bdI or bdII are expressed in other situations. Both cyt bd enzymes have a high oxygen binding affinity and utilize menaquinol (MK₈) as a substrate. Demethylmenaquinone is the main electron source under high nitrate respiration [6, 11-13].

The midpoint potentials the quinones are +0.11 mV, -0.08 mV, and +0.04 mV corresponding each to UQ₈, MK₈, and DMK₈. The values are lower than oxygen (+0.82 mV) and all these quinones are energetically suitable to serve as an electron donor for the terminal O₂ reductases or for the NO₃ reductase. (+0.42 mV for nitrate reductase) E. coli has evolved a complex network of parallel electron transfer pathways, allowing the cells to adapt under different environments and also broadening the range of conditions for cells to live [11-14]. Not all of the dehydrogenases can readily couple to the terminal reductases and such combinations are restrictive [14]. Under different conditions in E. coli, where different types of quinones are deficient or not able to be expressed, other quinones are limited by their function and there is an overall decrease in efficiency [15].

3. The cytochrome bo₃ ubiquinol oxidase from E. coli

Cytochrome bo₃ is the HCO in E. coli. Cyt bo₃ has two hemes, low spin heme b and high spin heme o₃, with a copper-binding site, CuB. The binuclear site is composed of heme o₃ and CuB where the dioxygen binds and reduced to water. Cytochrome bo₃ is a well-known A-family oxidase, which has two proton pathways, the D-channel and K-channel, that lead up to the binuclear center. (Fig 1.2) The D-pathway starts from residue
D135 and progresses via N124, T211, N142, Y61, T204, S145, T201, and T149 and ends at E286. The K-pathway contains S315, S299, K362, T359, and Y288 [7, 8].

Structurally A-family HCOs are very similar but cytochrome $bo_3$ is a quinol oxidase. It is missing the dinuclear Cu$_A$ copper site in subunit II and also does not have a cytochrome $c$ binding site. Since there is no cytochrome $c$ in the aerobically grown enzyme and it utilizes ubiquinol as an electron donor, the ubiquinone binding site was first thought to be in subunit II which is where the cytochrome $c$ binds in the A-family enzymes that utilize cyt $c$ as the electron donor [7, 8]. In 2000, Abramson et. al., obtained the crystal structure of cytochrome $bo_3$. Using sequence homology and site-directed mutagenesis, they identified a likely ubiquinone binding site [8]. The ubiquinone is bound to the enzyme in subunit I, 13 Å far from low spin heme $b$. Residues R71, D75, H98, and Q101 are indicated as the high affinity site ($Q_H$). It is not clear whether two binding sites exist or just one, but in this study there was no second binding site ($Q_L$) to be indicated.

Four protons per O$_2$ are taken up for oxygen chemistry and four additional protons are pumped across the membrane. This causes generation of an electrochemical gradient which drives ATP synthesis. Both structural analysis and site-directed mutagenesis have identified two conserved proton entry channels in the A-family HCOs: the D-channel and K-channel. These channels convey protons from the cytoplasm both for proton pumping and for chemistry at the active site (formation of H$_2$O). The residues in each channel are conserved within the family. Proton transfer occurs through a series of internal water molecules, which provides a continuous sequence of hydrogen bonds. Mutagenesis studies have shown that the D-channel is used for the input of all pumped
protons (4 per O₂) and provides two of the four protons required for chemistry of O₂ reduction at the active site. Mutating the conserved residues at the beginning and end of the D-channel (D132 and E286 in the R. sphaeroides cyt aa₃) eliminates catalytic function [16-19]. In contrast, mutating N139 to aspartate eliminates proton pumping without altering the catalytic rate. The K-channel in the A family HCOs, on the other hand, does not convey pumped protons, but delivers two chemical protons for the O₂ reduction [3, 20-23]. The redox chemistry catalyzed by the enzyme is coupled to proton pumping that generates proton motif force. Unlike protons which use specific pathways, water diffuses randomly out of the enzyme without any specific pathways [24].

4. Kinetics of electron and proton transfer

As mentioned above, the novel electron donor for cyt bo₃ is UQ8. During the catalytic cycle, electrons are transferred to the active site in consecutive steps: Qₙ → heme b → heme o₃ [2]. The electron transfer rate \( k_{eT} \), as described by the semiclassical Marcus theory, is the reaction between the electron donor and acceptor at a fixed distance.

\[
k_{eT} = \frac{2\pi}{\hbar} |H_{AB}|^2 \frac{1}{\sqrt{4\pi \lambda kT}} \exp \left( -\frac{(\Delta G + \lambda^2)}{4\lambda kT} \right)
\]

where \( \Delta G \) is the driving force of the reaction, \( H_{AB} \) is the electronic coupling between the donor and acceptor, and \( \lambda \) is the reorganization energy [25-27]. Intramolecular electron transfer rate between heme o₃ and heme b rate (9 Å) is \( 1.1 \times 10^5 \text{s}^{-1} \) at 25 °C which is similar to cyt aa₃ from R. sphaeroides (1.2 \( \times \) 10⁵ s⁻¹) [26]. During the process of electron transfer from heme b to heme o₃ the reaction is coupled to proton movement [28].
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Fig. 1.1 Schematic representation of the *E. coli* respiratory chain and ATP synthase.
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6. References


17. Zhu, J., et al., *Decoupling mutations in the D-channel of the aa(3)-type cytochrome c oxidase from Rhodobacter sphaeroides suggest that a continuous*


II. CHARACTERIZING THE DIFFUSION OF O$_2$ IN TWO A-FAMILY OXYGEN REDUCTASES: CYTOCHROME BO$_3$ FROM E. COLI AND CYTOCHROME AA$_3$ FROM R. SPHAEROIDES

1. Introduction

It is critical for the substrate O$_2$ to reach the active site for the enzyme to function. At room temperature, the oxygen solubility in water is about 250 uM. O$_2$ has been shown to be concentrated 4~5 times higher in the biological membranes than in water [1]. This is presumably why the hydrophobic channel in the HCOs starts from the lipid bilayer and leads to the active site [2]. The oxygen pathway to reach the active site will be functionally important only under circumstances when the rate of O$_2$ diffusing to the active site becomes rate limiting. Under steady state conditions, this will only occur when $[O_2] << K_M$. Under these conditions, the enzyme reaction rate will depend on the product of the oxygen concentration, the enzyme concentration and the second order rate constant for the reaction.

\[
\text{rate} = k_i [O_2][E] \quad \text{or} \quad \frac{\text{rate}}{[E]} = k_i [O_2]
\]

The rate constant can be expressed in terms of the Michaelis-Menten parameters.

\[
k_i = \frac{k_{cat}}{K_M} \quad \text{units:} \quad \frac{s^{-1}}{M} = s^{-1}M^{-1}
\]
Using this simple approach, we can ask under what conditions the second order rate constant, $k_1$, will be rate limiting. This condition implies that

$$k_1[O_2] < k_{cat} \quad \text{or} \quad [O_2] < \frac{k_{cat}}{k_1}$$

where $k_{cat}$ is determined by the rate of the slowest step in the chemistry that follows the binding of $O_2$. For cyt $bo_3$ and cyt $aa_3$ this is about 1000 s$^{-1}$.

We can also write this as

$$[O_2] < \frac{k_{cat}}{k_1} = \frac{1000}{k_1}$$

This means that when the $O_2$ concentration is lower than $\frac{1000}{k_1}$ second order rate constant will limit the rate of catalysis. If it is important for the enzyme to react as fast as possible, even at low $[O_2]$, then one would expect the enzyme to have evolved to have the value of the second order rate constant $k_1$ to be as large as possible [1]. For example, if $k_1 = 10^9$M$^{-1}$s$^{-1}$, then at concentrations of oxygen below 1 µ the rate of oxygen reaching the enzyme active site by diffusion will be rate limiting.

The question addressed in the research project is whether the hydrophobic channel in subunit I of the *E. coli* cyt $bo_3$ or *R. sphaeroides* cyt $aa_3$ is a critical structure to facilitate rapid diffusion of $[O_2]$ to the enzyme active site. These enzymes are expressed under high oxygen condition; therefore the need for a special channel to facilitate diffusion of oxygen could be questionable. It has been suggested, but not
demonstrated, that the rate of O$_2$ diffusion through the protein would be much slower in the absence of the specific pathway “designed” for this purpose [3, 4].

2. Structural information on the hydrophobic channels

Employing gas pressurization and freeze-trap kinetics, Fee’s laboratory successfully solved X-ray structures of cyt $ba_3$ (B-family) from *Thermus thermophilus* showing several binding sites of xenon [5] and krypton [6] along a hydrophobic channel that appears to be accessible to the catalytic site from the membrane [7, 8]. The binding of Xe and Kr has suggested the potential function of this particular channel as the putative O$_2$ channel. Xe and Kr are good analogues of O$_2$ because they are gases, which are soluble in non-polar environments and are also similar in size. The van der Waals diameters of O$_2$ are ~4.2 Å (O-O bond), Xe (~4.3 Å) and Kr (~4.0 Å).

Other enzymes have been shown to favor O$_2$ pathway. Hydrophobic pathways have been identified in the following proteins: cholesterol oxidase, copper amine oxidase, hemoglobin, and more [9-15]. Copper amine oxidase (CuAO) from *E.coli* structure used Xe to probe the putative oxygen-entry pathway. It is common to use Xe for mapping the pathway from crystallography and use site-directed mutagenesis method to confirm this oxygen pathway [7, 8, 11].

Xe binding sites in cyt $ba_3$ structures form a bifurcated Y shaped channel with two entry point, converges directly to the catalytic site. This hydrophobic channel is also found in X-ray structures of A-family CcOs from *R. sphaeroides*, *P. dentrificans*, and Bovine heart. As opposed to B-family from *T. thermophilus* cyt $ba_3$, A-family CcOs
exhibit only one entrance into the channel [7, 8]. Superimposing the hydrophobic channels on top, one can obtain a secondary structure-based sequence alignment. This well conserved feature, allows us to measure the O₂ binding rate. Cyt ba₃, which has the second order O₂ binding rate of $1 \times 10^9$ M⁻¹s⁻¹, is 10 fold faster than bovine aa₃ or R. sphaeroides cyt aa₃ [16, 17]. With site directed mutagenesis, we can increase the hydrophobic area in the cyt bo₃/aa₃ to see if it can increase the binding rate to that of bring it up as cyt ba₃.

3. Cytochrome bo₃ from E. coli [18]

1. Methods

Site-directed mutagenesis

pET cyo is a pET-17b derived plasmid, with T7 promoter and encodes the operon cyoABCDE including histidine tag at the C-terminus of subunit II and has Ampicillin antibiotic resistance (constructed by previous group member, Lailai Yap). All oligonucleotides were ordered from IDT (Integrated DNA technologies). (Table 2.1) PCR was used to perform site-directed mutagenesis using Agilent (Santa Clara, CA) kit. PfuUltra High-Fidelity DNA Polymerase was used to lower the error rate. DpnI was used to digest the template for at least 4 hours of incubation at 37 °C. After using PCR purification, using GeneJET™ Kit (Thermo Scientific, Bannockburn, IL), the plasmids were transformed into XL10-Gold ultracompetent cells (Agilent). This was later spread onto Ampicillin plate and incubated at 37 °C overnight.

Colonies that were picked and grown in LB media were later spun down and the plasmids were extracted using GeneJET™ Plasmid Miniprep Kit (Thermo Scientific,
Bannockburn, IL). The sequence verification of mutagenesis products was performed at the University of Illinois Urbana-Champaign (UIUC) Biotechnology Center. All chemicals of the purest grade available were used.

*E. coli* C43 (DE3) (Avidis, France) strains were used for expression of membrane proteins [19]. CLY strain is a derivative of C43 (DE3) strain, that has *bo*3 knockout replaced by a Kanamycin resistance factor cassette, was used for this purpose. After transforming the mutated plasmids into CLY competent cells and grown in 5 ml of LB, the cells were centrifuged and went through miniprep for another sequence verification at the same facility. A 500 µl aliquot of the cells was used to make a frozen glycerol stock (final concentration of glycerol 25%, stored in -80 °C freezer).
<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>W170Ffor</td>
<td>CGC AGA CCG GCT TTC TGG CCT ATC C</td>
</tr>
<tr>
<td>W170Frev</td>
<td>GGA TAG GCC AGA AAG CCG GTC TGC G</td>
</tr>
<tr>
<td>W170Yfor</td>
<td>GCA GAC CGG CTA TCT GGC CTA TC</td>
</tr>
<tr>
<td>W170Yrev</td>
<td>GAT AGG CCA GAT AGC CCG TCT GC</td>
</tr>
<tr>
<td>V108Ffor</td>
<td>CCG CGC ACG GCG CGA TTA TGA TCT TC</td>
</tr>
<tr>
<td>V108Frev</td>
<td>GAA GAT CAT AAT CGC GCC GTG CGC GG</td>
</tr>
<tr>
<td>L246Ffor</td>
<td>CCT TCC CAA TTT TCA CGG TTA CCG TC</td>
</tr>
<tr>
<td>L246Frev</td>
<td>GAC GGT AAC CGT GAA AAT TGG GAA GG</td>
</tr>
<tr>
<td>W282Tfor</td>
<td>GAT TTG GGC CAC GGG CCA CCC GGA AG</td>
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<td>W282Trev</td>
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</tr>
<tr>
<td>F112Ffor</td>
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<tr>
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<tr>
<td>I111Mfor</td>
<td>CAC GGC GTG ATT ATG ATG TTC TTC GTA GC</td>
</tr>
<tr>
<td>I111Mrev</td>
<td>GCT ACG AAG AAC ATC ATA ATC ACG CCG TG</td>
</tr>
</tbody>
</table>

**Table 2.1** List of primers used in the Site-directed mutagenesis.

**Cell growth and purification**

Cells were inoculated from the glycerol stock, with antibiotics (Ampicillin 100 μg/mL, Kanamycin 50 μg/mL) and grown in LB media at 37 °C, 210 rpm which later was transformed into a pre-inoculum of minimal media (M63) with Thiamine (10 μg/mL), CuSO₄ (1 μM), MgSO₄ (1 μM), and glucose (2 g/L). Minimal medium was autoclaved prior to adding additional vitamins. This pre-inoculum was grown overnight, and was used for a larger growth the next day. After reaching OD₆₀₀ of 0.6, the cells were induced with IPTG (0.5 mM) and left to grow for 4~5 hours more. The cells were
harvested by centrifugation at 8,000 rpm at 4 °C for 10 minutes per run in a Sorvall GS3 rotor and the supernatant was discarded and the pellets were collected. The cell pellets were resuspended in 50 mM KPi at pH 8.3 and MgSO4 was added along with Protease Inhibitor Cocktail (Sigma, Saint Louis, MO), and Deoxyribonuclease I from bovine pancreas (Sigma). This was gently shaken it to mix it well. The cells were broken by a microfluidizer (Microfluidics Corp. Waltham, MA), filled with ice around the device to keep the temperature down. The cell lysates were centrifuged at 8,000 rpm for 15 min at 4 °C to remove the cell debris. The membranes were isolated from the supernatant after 4 hours centrifugation at 40,000 rpm, 4 °C in a Beckman Ti45 rotor. At this point the membranes could be stored at -80 °C freezer for later use. The isolated membranes were resuspended in 50 mM Kpi, pH 8.3 buffer with final concentration of 1 % dodecyl maltoside (DDM) detergent (Anatrace, Maumee, OH) by stirring at 4 °C. The unsolubilized material was removed by centrifugation at 42,000 rpm for 45 min at 4 °C temperature using the Beckman Ti45 rotor. The supernatant that contained the solubilized protein was incubated with nickel-nitrilotriacetic acid (Ni-NTA) resin with 5 mM imidazole for ~1 hour with gentle stirring in the cold room (4 °C). Everything from this point was done in the cold room. After an hour, the Ni-NTA resin with the protein bound was loaded onto the column and washed with 50 mM KPi, pH 8.3, 0.05% DDM, 10 mM imidazole buffer with 10 column-volumes of Ni-NTA resin. After that, the column was washed with 5 volumes of 50 mM KPi, pH 8.3, 0.05% DDM, 15 mM imidazole buffer. Finally the enzyme was eluted with 50 mM KPi, pH 8.3, 0.05% DDM, ~150 mM imidazole buffer. The eluted protein was concentrated using a 100-kDa cutoff Amicon (Millipore), followed by buffer exchange into 50 mM KPi, pH 8.3, 0.05% DDM buffer, a
total of three times. The protein with 5% glycerol was flash-frozen in liquid nitrogen and stored at -80 °C for future use.

**Traditional CO flow-flash**

The traditional way to do the photolysis experiment with HCOs is to first bind the reduced HCO to carbon monoxide [20-23]. In a stopped flow, the CO-enzyme adduct is mixed with a solution with about 1 mM O₂. Within a few seconds, a laser flash ejects the CO from the enzyme active site and allows O₂ to bind [8, 11, 21, 23, 24]. In the cases of cyt bo₃ and cyt aa₃ the Einarsdottir group has shown that caged O₂ and caged NO show the same O₂ rate of binding (data not shown). Therefore, the caged substrate method was not used.

The reduced enzymes (used ascorbic acid and ruthenium hexamine) were incubated with CO to make sure it was fully reduced and CO-bound (incubation for 30 - 60 min). The fully reduced, CO-bound enzyme was mixed 1:1 volume ratio with O₂ or NO- saturated buffer, followed by photolysis to release CO from the heme o₃ using a Q-switched DCR-11 Nd:Yag laser (532 nm) [16, 17].

The data obtained were analyzed using single value decomposition (SVD) and global exponential fitting using programs from Matlab.

**CO Flash-Photolysis**

The experiment is similar to the flow-flash method, but instead of mixing, the photolysis solution remains in an anaerobic quartz-cuvette. The sample was reduced with
ascorbate and ruthenium hexamine or dithionite. After reducing the enzyme, the samples were exposed to CO for 15 minutes to form reduced, CO-bound enzyme complex. The CO recombination rate was measured by photolysis of the reduced, CO-bound enzyme using the time-resolved optical absorption spectroscopy [25]. The photolysis used the same laser as for the flow-flash experiment. All spectra were analyzed using SVD and global exponential fitting using Matlab.

2. Results

The O₂ binding rates to different HCOs have been previously measured [8, 11, 16, 17, 21, 24, 26, 27]. However, in the case of cyt bo₃, the enzyme preparations contained a substantial impurity with heme o replacing heme b in the low spin site, yielding cyt oo₃ [28-30]. With the improvement of expression strains optimized for membrane protein expression and using a system using the T7 promoter, the improved cytochrome bo₃ has the expected 1:1 ratio of heme b : o which is essential to get accurate measurements.

The flow-flash experiment reveals the rate constant for the initial O₂ binding as well as the subsequent electron transfer and proton uptake rates. This is all monitored by following the changes in the heme absorption. Whereas the cytochrome c oxidases use a 1-electron donor, cyt bo₃ uses a 2-electron donor, ubiquinol. Cyt bo₃ has a ubiquinone bound to its Q₁ site after purification using DDM. The flow-flash experiment follows one turnover of the fully reduced enzyme as it interacts with O₂. Starting from fully reduced, CO-bound enzyme, following photolysis, the full optical absorption spectra were recorded as a function of time (Fig 2.1). In addition to the studies using O₂, NO was also used as a substrate analogue which does not have subsequent redox chemistry following
the initial binding to the active-site heme. This provides an alternate measure of the initial rate of ligand binding. The first spectrum is obtained after a $1 \, \mu s$ delay and the last at $20 \, ms$ following photolysis.

**Fig 2.1** The full optical absorbance difference spectra of WT cyt $bo_3$ recorded at different times after photolysis $500 \, ns – 20 \, ms$.

The previous analysis of the rates obtained with the $aa_3$-type bovine cytochrome $c$ oxidase, was used as point of comparison. However, the initial fully reduced cytochrome $c$ oxidase enzyme contains 4 reducing equivalents, just enough to reduce $O_2$ to $2 \, H_2O$, whereas the fully reduce quinol oxidase contains 5 reducing equivalents since the 1-electron reduced $Cu_A$ is replaced by the 2-electron reduced ubiquinol. Hence, there is an “extra” electron left over after one turnover.
After obtaining the spectra and doing the SVD analysis and global exponential fitting, the time-resolved spectra were analyzed assuming a kinetic model with five sequential steps with characteristic times of 42 μs, 22 μs, 30 μs, 470 μs and 2.0 ms. The spectroscopic changes with each step were analyzed by comparison to the independently obtained spectra of different oxygenated forms of the enzyme. Our results differ from the conventional sequential mechanism of O₂ reduction. The conventional mechanism is a linear series of sequential steps with progressively slower rate constants from R → A → P → F → O. In our case, comparing the spectra of each step, we observed that the first step R → A takes 42 μs but that the following step rapidly converts A to a combination state of P and F with a characteristic time of 22 μs. Hence this is called the “slow-fast” mechanism. The short lifetime of the A intermediate state would result from a fast proton transfer leading to a mixture of P and F. In 30 μs the complete F intermediate state is formed by partial reduction of heme b by the bound quinol (forming a semiquinone). The oxoferryl heme o₃ intermediate in the F state is reduced to the ferric hydroxyl form by an electron from the quinol/semiquinone in 470 μs without further oxidation of the ferrous heme b. This state corresponds to the O state found in cytochrome c oxidase, but differs due to the presence of one “extra” electron (shared between heme b and the bound ubiquinone) and is therefore called the O₁ state. The final 2 ms transition is not well defined and may involve reaction with a second ubiquinol molecule, where the reduced heme b becomes oxidized and this electron reduces the heme o₃.

By this kinetic analysis, the second order binding constant of O₂ to cyt bo₃ is 3.8 × 10⁷ M⁻¹ s⁻¹. This is 25-fold slower than T. thermophilus cyt ba₃ and 2.5-fold slower than bovine cyt aa₃ or R. sphaeroides cyt aa₃ [16, 17]. Site-directed mutagenesis was
performed on residues that appear to form a constriction in cyt \( bo_3 \) within the putative \( O_2 \) channel (in comparison to cyt \( ba_3 \), where the rate constant is 25-fold greater) in an effort to increase the diffusion of \( O_2 \) to the active site of cyt \( bo_3 \) (Table 2.2, bold, italic) [31].

<table>
<thead>
<tr>
<th>Enzyme species</th>
<th>Constriction point</th>
<th>Hydrophobic branch$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R.\ sphaeroides\ aa_3 )</td>
<td>( F282 )</td>
<td>( W172 )</td>
</tr>
<tr>
<td>( E.\ coli\ bo_3 )</td>
<td>( W282 )</td>
<td>( W170 )</td>
</tr>
</tbody>
</table>

**Table 2.2** Structure and sequence homology. Four cytochrome c oxidases were selected for comparison [7]. There are several hydrophobic branches but here only one was selected. The hydrophobic branches converge to the constriction point where a single pathway leads to the binuclear center.

Five different mutants were examined by flow-flash and the results are summarized in Table 2.3, with the time-resolved difference spectra shown in Figures 2.2, 2.3 and 2.4. The results are in each case similar to those of the wild type, indicating no substantial change in the rate constant for \( O_2 \) binding to the active site due to these mutations.
Figure 2.2 $b$-spectra (A, C) for a two-exponential fit of the TROA difference spectra recorded for the reaction of the photo-produced O$_2$ with the fully reduced I$^{I11}M$ and F$^{I12}I$ *E. coli* bo$_3$ mutants, respectively. The $b_0$ is the difference between time zero and the last time point recorded, and represents the difference spectra extrapolated to infinite time.

Figure 2.3 $b$-spectra (A, C) for a two-exponential fit of the TROA difference spectra recorded for the reaction of the photoproduced O$_2$ with the fully reduced W$^{282}T$ and W$^{170}Y$ *E. coli* bo$_3$ mutants, respectively.
Figure 2.4 (A) TROA difference spectra (post-minus-pre-photolysis) of the reaction of the fully reduced V108F E. coli bo3 enzyme with photo-produced O2 in the absence of CO. The Soret and visible spectra were recorded together at logarithmically spaced time delays of 2 µs - 50 ms. (B) b-spectra resulting from a two-exponential fit.

Table 2.3 The apparent lifetimes and second-order rate constants for O2 binding in cyt bo3 constriction point and hydrophobic pocket mutants. The factor increase is versus wild-type (WT) enzyme.
3. Discussion and conclusions

As mentioned earlier, cytochrome \( bo_3 \) has not been studied thoroughly due to previous issues with the heme content of recombinant enzyme. With the new strain provided by previous lab member, and using minimal medium (M63) instead of LB, it is possible to get the heme \( b:o \) ratio to 1:1, which was not possible before. Once that was possible, using the flow-flash measurement, we were able to measure the second order \( O_2 \) binding rate and moreover, were able to see the mechanism of \( O_2 \) reduction in cyt \( bo_3 \). [18]

The \( O_2 \) binding rate is slower than other A-family oxidases by about 3-fold and slower than the B-family oxidase cyt \( ba_3 \) by 25-fold. The mutants that we made to increase the binding rate resulted in at most an increased up to two-folds. It is likely that cyt \( bo_3 \) contains more than one pathway for \( O_2 \) to reach the active site and therefore, a single mutation does not have much of an impact. It has previously been shown that the mutation of residues close to the active site can have a very large effect on the rate of reaction with \( O_2 \) [32-34].

To reduce \( O_2 \) to \( H_2O \), four electrons are required. In cytochrome \( c \) oxidases, cyt \( c \) is the electron donor for the chemical reaction. Cyt \( bo_3 \), which is an ubiquinol oxidase, uses ubiquinol (UQ8) as an electron donor. While cyt \( c \) carries only one electron, ubiquinol carries two electrons. The reduced form ubiquinol (UQH\(_2\)) donates electrons sequentially one at a time, turning into ubisemiquinone, than to ubiquinone (UQ). Because of this extra (fifth) electron there are changes in the sequence of intermediates formed during the single turnover of \( O_2 \) with the fully reduced enzyme. Previous measurements were not able to detect the initial complex of heme \( o_3 \) bound to \( O_2 \), the A
intermediate state [26]. In our experiments this state is observed and the second order rate constant for its formation is $3.8 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, which is 25-fold slower than *T. thermophilus* cyt *ba*$_3$ and 2.5-fold slower than bovine cyt *aa*$_3$ or *R. sphaeroides* cyt *aa*$_3$ [16, 17].

The first state observed following A in the flow-flash experiment is a mixture containing both states P and F, and is mostly F. These states are distinguished by a protonation of a group at the active site. The P state in cyt *bo*$_3$ can be observed by reacting the oxidized enzyme with H$_2$O$_2$ [35], but is very unstable as an intermediate in the reaction of O$_2$ with the reduced enzyme, decaying to F very rapidly. The very short lifetime of P state must be due to very fast proton transfer, leading into an instant conversion of P to F state, so the pure P state is not observed.

The other main difference between the flow-flash results with cyt *bo*$_3$ and previous results with cyt c oxidases is the complications due to the bound 2-electron reduced quinone in the cyt *bo*$_3$ as well as the presence of some additional reduced quinone in the preparation. This results in formation of an O$_1$ state that has an extra reducing equivalent present, shared between the quinone and heme *b*, and a slow reaction that appears to be due to subsequent reduction by some contaminating ubiquinol.

Site-directed mutagenesis was performed to increase the O$_2$ second order binding rate. However, as the flow-flash results show, the binding rate constant increased by at most two-fold. The very rapid rate constant for the B-family cyt *ba*$_3$ is likely the result of evolution under the stress of growth conditions with very low oxygen concentration. It is also likely that cyt *bo*$_3$ contains more than one pathway for O$_2$ to reach the active site and therefore, a single mutation does not have much of an impact. It has previously been
shown that the mutation of residues close to the active site can have a very large effect on the rate of reaction with O₂ [32-34].

4. Cytochrome aa₃ from *R. sphaeroides*

1. Methods

Site-directed mutagenesis

The Quik-Change mutagenesis kit was used to introduce mutations. Plasmid pUC19 (Ampicillin 100 µg/ml) was used as the template for mutations (only encodes subunit I) and the sequence verification of mutagenesis products was performed at the University of Illinois Urbana-Champaign (UIUC) Biotechnology Center. (Table 2.4) All chemicals of the purest grade available were used. After verification of the mutations, the cyt aa₃ gene was digested and ligated into pRK415 using Quick Ligation™ Kit (New England BioLabs, Ipswich, MA). After ligation, the expression plasmid pRK-415 (tetracyclin, 15 µg/ml) was introduced into *R. sphaeroides* JS100 strain by biparental conjugation using *E. coli* S-17-1 as a donor. (JS100 has a subunit I replacement in the genome with a streptomycin/spectinomycin resistance cassette.) All the *E. coli* cells were grown at 37 °C. All of the *R. sphaeroides* cells were grown at 30 °C, at 210 rpm using 2L flasks, each with only 800 ml of Sistrom’s medium to maintain high aeration.) After each step of transformations the sequence of the plasmids were verified.
<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequences</th>
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</thead>
<tbody>
<tr>
<td>W172Yfor</td>
<td>GGC ATC GGC TAT GTG CTC TAT CCG</td>
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<tr>
<td>W172Yrev</td>
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</tr>
<tr>
<td>F282Tfor</td>
<td>GCA CAT CCT GTG GTT CAC CGG CCA CCC</td>
</tr>
<tr>
<td>F282Trev</td>
<td>GGG TGG CCG GTG AAC CAC AGG ATG TGC</td>
</tr>
</tbody>
</table>

Table 2.4 List of primers used in the site-directed mutagenesis.

2. Results

As mentioned before, the cyt $aa_3$ has an $O_2$ binding rate constant of $1 \times 10^8 \text{M}^{-1}\text{s}^{-1}$. This is 10-fold slower than $T.\ thermophilus\ ba_3$. From the Stout group’s structural comparison, the hydrophobic cavity volume is smaller than cyt $ba_3$ [7]. Comparing cyt $aa_3$ to cyt $ba_3$ hydrophobic pathways, cyt $ba_3$ has two separate pathways converging to the binuclear center from the membrane, while $aa_3$ has only one pathway. We attempted to expand the pathway by site-directed mutagenesis to increase the binding rate constant. Designing the pathway to become larger, the main target we focused on was to change the residues that appear to form a constriction point or a bottleneck to diffusion of oxygen. This was determined by comparison with the pathway in the $R.\ sphaeroides\ cyt\ aa_3$ with that in cyt $ba_3$. The putative constriction point in the $R.\ sphaeroides\ cyt\ aa_3$ corresponds to where the two pathways converge in cyt $ba_3$. The residues at this location are W172 and F282 (Fig 2.5).
Fig 2.5 Cyt $aa_3$ active site with W282 and F172 residues that form the constriction point. The dark area indicated by the arrow is the hydrophobic pocket.

Fig 2.6 UV/Vis spectra of WY/FT double mutant. The lowest intensity (black) is the oxidized spectrum with peak at 415 nm, the highest intensity (blue) is the dithionite reduced CO-bound enzyme with peak at 426 nm and the spectrum on the far right (red) is the dithionite reduced spectrum with peak at 435 nm.
Residue W172 was mutated to tyrosine and F282 was mutated to threonine. The double mutant has a perturbed spectrum (Fig 2.6), and the expression yield of the enzyme, compared to the WT was low. Due to the low amount of available mutant enzyme, we could not perform the flow-flash to obtain the O₂ second order-binding rate. We did measure the CO recombination of the mutant and these data were compared to those from the WT enzyme.

The enzymes were maintained under anaerobic conditions to assure full reduction by either ascorbic acid/ruthenium hexamine or dithionite. After fully reducing the enzyme, CO was added for 15 minutes to form the reduced CO-bound enzyme complexes. The CO ligand was photolyzed by 532 nm laser flash and the spectroscopic changes were determined on a 200 ns - 500 ms time scales. The difference spectra were analyzed using SVD and global exponential fitting. (Fig 2.7)

The peak and the trough of the double mutants (W172Y/F282T) have both shifted and became broader compared to the WT. The time-resolved spectral changes were analyzed using a four exponential fit for the WT with characteristic times of 0.9 μs, 54 μs, 500 μs, and 23 ms. (Fig 2.7) In the meantime, the W172YY/F282T spectral analysis was satisfied by a three exponential fit with characteristic times of 0.6 μs, 44 μs, and 300 μs. Fig 2.8 shows the comparison of b-spectra of the first three lifetimes. The b-spectra of W172YY/F282T have been blue shifted ~5 nm to match those of the WT. The process corresponding to the highest amplitude b-spectrum is the CO recombination to heme a₅. This recombination time for the WT is 23 ms but for the W172YY/F282T mutant, the recombination time is 44 μs, 500 times faster.
Fig 2.7 Time-resolved optical absorption difference spectra (post minus pre-photolysis) recorded following flash-photolysis of (a) the fully reduced CO-bound the wild-type fully reduced CO-bound cyt $aa_3$ and (b) the W172Y/F282T R. sphaeroides cyt $aa_3$. 
Fig. 2.8 Comparison of the first three $b$-spectra of the wild-type $R. sphaeroides$ $aa_3$ (blue curves, 4-exponential fit) and W172Y/F282T (green curves, 3-exponential fit).
3. Discussion and conclusions

From previous measurements we know cyt aa₃ has an O₂ binding rate constant of $1 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$. A double mutant, W172Y/F282T, was made in an attempt to reduce a bottleneck in the hydrophobic channel leading to the active site. Due to the poor yield of the protein, only CO-recombination was studied to observe the influence of these mutations compared to the WT.

The double mutant could not be completely reduced using ascorbate and ruthenium hexamine. The mutations clearly altered the structure of the cyt aa₃ (Fig 2.6) as determined by the altered absorption spectrum. One of the double mutant residue, W172 is very close to the low spin heme a so it is plausible that the W172Y mutation might result in perturbations leading to different conformers as well as a change in the reduction potential [36]. The main effect is a 500-fold faster rate of CO recombination. This is most likely to result from the mutations preventing the exit of CO from the enzyme following photolysis. A similar “caged” active site has been observed previously by a mutation that is much closer to the active site of the heme [37]. Further studies are needed to clarify this situation.
5. References


III. BIOPHYSICAL STUDY TO ELUCIDATE THE LOW AFFINITY UBIQUINONE BINDING SITE (QL)

1. Introduction

Heme-copper oxidases (HCO) reduce O₂ to water and use the redox free energy to generate proton motive force. The HCOs are categorized depending on the electron donor as to cytochrome c oxidases or quinol oxidases. Cyt bo₃ from E. coli is the most studied ubiquinol oxidase along with menaquinol oxidase aa₃-600 from Bacillus subtilis. In 2000 the Wikström group published the structure of the cyt bo₃ with four subunits, two low spin hemes and a Cu₈ site (1FFT.pdb) [1]. However, this structure is missing a substantial part of the protein as well as the natural substrate ubiquinol-8 (UQ₈). The study was used to successfully locate the Q₉ site based on sequence analysis and site-directed mutagenesis, but has not identified the Q₉₈ site.

There has been a question as to whether cyt bo₃ has two ubiquinone binding sites or one binding site. A recent study from our group has concluded that there are two ubiquinone binding sites using inhibition (HQNO, AC-10) measurements (The inhibitor could not replace the bound ubiquinone.) [2-4]. However, the exact location of the Q₉₈ site could not be identified. In this chapter, we show attempts to locate the Q₉₈ site using site-directed mutagenesis based on the new structure provided by Dr. Michael Stowell (Univ. of Colorado, Boulder) (unpublished). The mutagenesis was guided by suggestions from the analysis of the new structure, from knowledge of conserved residues that might conceivably participate in hydrogen bonding to quinone and from a bioinformatics study predicting the location of the Q₉₈ site from the work of Bossis et al. [5]. The expectation is
that mutations that perturb the \( Q_L \) site will either lower the activity (or \( V_{max} \)) or alter the \( K_M \) for ubiquinol, so these parameters were measured [5].

2. The new structure, and the search for the low affinity ubiquinone binding site

The cyt \( bo_3 \) structure previously published [1] is incomplete and the resolution is poor (3.5 Å). This structure includes two low spin hemes and a Cu\(_B\) site but is missing many residues and substrate UQ\(_8\) [1]. The study located the \( Q_H \) site based on the location of a set of conserved residues that were thought likely binding partners to ubiquinone, however the \( Q_L \) site was not identified. Due to the missing residues and the substrate UQ\(_8\) in the published structure of cyt \( bo_3 \) many questions remain unanswered. The new structure provided by Dr. Stowell is an improvement and contains what appear to be two bound quinone molecules. In this chapter we will show a new structure and describe experiments to test the proposed \( Q_L \) site by directed mutagenesis.

3. Methods

Site-directed mutagenesis

The same methods were used as Chapter 2. All oligonucleotides were ordered from IDT (Integrated DNA technologies). (Table 3.1) PCR was used for site-directed mutagenesis using Agilent (Santa Clara, CA) kit. The sequence verification of
mutagenesis products was performed at University of Illinois Urbana-Champaign (UIUC) Biotechnology Center. All chemicals of the purist grade available were used.

**Steady state enzymatic activity assay**

The steady state oxygen reduction activity of cyt bo₃ was measured at 25 °C using YSI model 53 oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) that has an oxygen electrode chamber of 1.8 mL. The temperature is achieved using a water bath hooked up to the chamber. The reaction mixture was made using 50 mM Kpi, pH 7.0, 0.05% DDM with 2 mM dithiotherol and ~300 uM ubiquinone-1 (Q1, Sigma, Saint Louis, MO) with constant stirring. The oxygen saturated buffer at 25 °C is estimated to have an oxygen concentration around 250 𝜇𝑀. With all substrates added, the reaction is initiated as soon as ~4 𝑝𝑀 amount of enzyme is added into the mix. Turnover number (𝑒⁻¹/sec/enzyme) of the enzyme is calculated based on the consumption of oxygen slope and subtracting the background slope prior to the addition of the enzyme. The 𝑘_{cat} and 𝐾_{M} values were determined by measuring the turnover number as a function of the ubiquinol-1 concentration over a range 1 𝑢𝑀 to 330 𝑢𝑀, and fitting the data to the Michaelis-Menten equation (below).

\[ V_0 = \frac{V_{max}[S]}{K_M + [S]} \]
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<th>Name</th>
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Table 3.1 List of primers used in the Site-directed mutagenesis.
4. Results

Native cytochrome \( bo_3 \) from \( E. \ coli \) was purified and crystallized in nonyl-glucoside and the structure determined using X-ray crystallography by the collaborator’s lab. Phase improvement was achieved through multiple rounds of model building, density modification, and refinement. The new structure resolved some of the issues, adding most of the missing residues and in addition has two ubiquinones bound to the enzyme. The final model contains 659 out of 663 residues of subunit 1, 306 out of 315 residues of Subunit 2, 203 out of 203 residues of Subunit 3, 109 out of 109 residues of Subunit 4. The \( R_{\text{cryst}} \) and \( R_{\text{free}} \) are 0.374 and 0.446 at 3.8 Å with good stereochemistry and MOLPRO statistics, Fig. 3.1.

The complete four-subunit enzyme adopts a dimeric quaternary structure, facilitated by a large dimer interface, and a long amphipathic helix, which wraps around the adjacent monomer. Two bound ubiquinones per monomer are observed on opposite sides of the putative membrane boundary. The position of the first ubiquinone (Q\(_H\)) is similar to that previously modeled and confirmed by mutagenesis and is located near the periplasm as predicted [1, 2, 6-8]. The second ubiquinone (head group especially) is located near the cytoplasm at a water channel. (Fig. 3.1) The second bound quinone should define the Q\(_L\) site but, as shown below, this proved not to be the case.

To determine if the observed Q\(_L\) binding site is functional we performed site directed mutagenesis of potentially important residues based upon the new structure. (Fig. 3.2) Table 3.2 lists the mutated residues and the relative activity of each compared to that of wild type (WT). The data show that each mutant exhibits near the same activity as the
WT. For each mutant, the $K_M$ of ubiquinol-1 was determined to see if any mutant perturbed this parameter, which might be expected if the off-rate constant of the substrate were increased. (Table 3.3) The mutants show no or little increase in $K_M$ and not much of a change in the relative activities. The data strongly suggest that this site is not the ubiquinol-1 substrate binding site, $Q_L$. 
Fig 3.1 Overall structure and comparison to previous reported structure. A) Observed dimer structure. B) Comparison of the current structure to the previously reported structure showing the newly observed residues. C) Side view of cyt bo$_3$ with the ubiquinones bound to the enzyme at Q$_H$ and Q$_L$ site. (yellow) D) The red residues indicate the known Q$_H$ site residues, and the ubinonine is bound nearby those residues. The Q$_L$ site ubinonine is bound perpendicular to the Q$_H$ site ubinonine. The head group is facing the cytoplasm.
**Fig 3.2** Residues around Q<sub>L</sub> site for site-directed mutagenesis. Black TM helixes represent subunit I, green TM helixes represent subunit III, and blue subunit IV.

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<td>D&lt;sup&gt;III&lt;/sup&gt;36V</td>
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**Table 3.2** Site-directed mutagenesis for possible Q<sub>L</sub> site and its relative activities at V<sub>max</sub>.
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Table 3.3 $K_M$ assays of specific mutants from Table 3.2.

5. Discussion and Conclusions

Several membrane proteins that utilize quinone substrates have been crystallized and their structures determined without the bound quinone or bound with quinone analogues [9-11]. The initially reported structure of *E. coli* cyt bo$_3$ does not have any ubiquinol bound to the enzyme, but the subsequent structure determined by the Stowell group, using a different detergent, as part of our collaboration does have two long chain quinones bound. Most of the protein can be accounted for in the current structure, a substantial improvement over the previous structure [1]. Portions of the protein are clearly not in the native conformation (e.g., protein helices pointing perpendicular to the rest of the protein) when compared to the structures of homologous cytochrome $c$
oxidases [1, 12, 13]. and the overall resolution (3.8 Å) is still far from desired. Nevertheless, the data have been modeled to indicate a bound ubiquinone-8 with the headgroup where it is expected in the Q_H site and additional ubiquinone-8 bound opposite side of the membrane. The ubiquinone at the Q_H site has most of the isoprene tail units outside the protein, sticking out into what would be the lipid bilayer in the native environment. If representative of what is found in the membrane, this suggests that the head group and first few isoprene units (our of 8) are responsible for the binding affinity of ubiquinol-8.

Site-directed mutagenesis was performed based upon the new structure to observe the Q_L binding site functionality. The data clearly do not support the identification of this as corresponding to the Q_L low affinity ubiquinol binding site. The K_M was measured for WT cyt bo_3 to be 50 μM, which is the same as previously reported [2, 3, 14]. The K_M of the mutants showed essentially no change and, moreover, the activity of the mutants were also similar to the WT. Furthermore, our collaborators determined that the distance between the headgroups of the bound quinones at the Q_H and Q_L sites is substantially too large (37 Å) to be compatible with the experimental electron transfer rate from Q_L to Q_H [15-17]. A plausible explanation is that what has been modeled as a ubiquinone at the Q_L site is actually a phospholipid or detergent. This is suggested by the fact that in the structure of the homologous cytochrome c oxidase from Rhodobacter sphaeroides (resolution of 2.3 Å, 1M56.pdb) there is a well defined lipid site that overlaps well with the putative Q_L site of cyt bo_3. (Fig 3.3) We suggest this binding site is more likely to be a lipid site rather than a ubiquinone binding site.
Overall, the essence of this part of our study is that the new structure helps with filling in the missing residues and moreover shows a natural substrate bound to the enzyme at the $Q_{H}$ site. However, we still have not been able to identify the low affinity ubiquinone binding site. The advantage of having a second ubiquinone binding site is to function as a transiently bound ubiquinone that is able to deliver electrons to the bound ubiquinone at the $Q_{H}$ site. Therefore, the second ubiquinone binding site should be very close to the $Q_{H}$ site. It is not necessary for the ubiquinone to bind tightly to the second site or to stabilize the semiubiquinone form in order to deliver two electrons to the bound ubiquinone at the $Q_{H}$ site [2, 18].
**Fig. 3.3** Structural comparison of top view between (left) cytochrome \( \text{aa}_3 \) from *Rhodobacter sphaeroides* (1M56.pdb) and (right) cytochrome \( \text{bo}_3 \) from *E.coli*. First ubiquinone (yellow) is well bound in the area known for the high affinity site (Q\(_H\)). In cytochrome \( \text{aa}_3 \) the lipid is bound at the same site where in cyt \( \text{bo}_3 \), ubiquinone is bound. The color is coded by the subunits. (black as subunit I, red as subunit II, pink as subunit III, and blue as subunit IV)
6. References


8. Hellwig, P., et al., *Identification of the residues involved in stabilization of the semiquinone radical in the high-affinity ubinquinone binding site in cytochrome


13. Han, D., J.E. Morgan, and R.B. Gennis, G204D, a Mutation that Blocks the Proton-conducting D-channel of the aa3-type Cytochrome c Oxidase from Rhodobacter sphaeroides. Biochemistry, 2005. 44(38): p. 12767-74.


IV. TESTING BIOINFORMATICS-BASED SEQUENCE MOTIFS
PREDICTED TO DESIGNATE THE Q_H AND Q_L UBIQUINOL BINDING SITES

1. Introduction

After enzyme purification using dodecyl maltoside (DDM) there is always a tightly bound ubiquinone at the high affinity site (Q_H) [1-6]. For the enzyme to turnover, either this bound ubiquinone (UQ) should rapidly exchange with ubiquinol (UQH_2) in the ubiquinone pool (msec time scale) or a ubiquinol binds to the enzyme at a second site that is in rapid equilibrium with the quinone pool in the membrane and delivers the two electrons to the tightly bound ubiquinone.

There has been an ongoing debate of whether there are two binding sites or only one [1-3, 7-12]. The high affinity binding site is well studied via sequence analysis (well conserved throughout the quinol oxidases) and by site-directed mutagenesis. The new structure shows a ubiquinone bound to this Q_H site. However, there is no clear evidence where the second binding site is located. It has been suggested that this site is in subunit II [2, 8, 10]. Subunit II was most strongly implicated by using azidoquinone, a photoaffinity cross-linking reagent that was found to selectively bind to subunit II of cyt bo_3 [8]. These results were viewed initially as sensible insofar as this quinol binding site would “replace” the site for binding cytochrome c which is in subunit II of the cytochrome c oxidases [8]. The subsequently determined structure of the enzyme, however, indicated that subunit II is far from the surface of the lipid bilayer, making it more challenging to determine how the quinol could bind to a site within this subunit. It
is noted that the recently determined structure of Complex I from *Thermus thermophilus*, which is an NADH:quinol oxidoreductase, contains a long channel allowing the quinone headgroup to bind to residues far above the surface of the bilayer [13]. However, the structure of cyt *bo*<sub>3</sub> shows no such channel.

Additional support for two ubiquinone binding sites originates from studies with quinone-analogue inhibitors of cyt *bo*<sub>3</sub> such as HQNO and Aurachin. Kinetics studies showed that some inhibitors exhibit a competitive pattern and others are noncompetitive. The data were interpreted as having one group of inhibitors binding to the presumed Q<sub>L</sub> site and others to the Q<sub>H</sub> site [2, 3, 11]. In addition the binding of inhibitors HQNO and Aurachin was demonstrated to abolish the EPR signal from the bound quinone at the Q<sub>H</sub> but did not displace the bound quinone from the enzyme [2]. All these data are consistent with the existence of two adjacent sites for binding ubiquinone.

A recent paper [14] used sequence alignments, bioinformatics and molecular modeling to identify sequence motifs to identify both the Q<sub>H</sub> and Q<sub>L</sub> sites in the quinol oxidases. The residues identified as the Q<sub>H</sub> binding motif included those already confirmed to be critical for enzyme activity and for stabilizing the semiquinone species by EPR spectroscopy, R71, H98 and Q101. Additional residues were suggested to also be important for quinone binding. A motif proposed to represent the Q<sub>L</sub> binding site adjacent to the Q<sub>H</sub> site was also identified. We used the residues identified in this bioinformatics study [14] to guide site-directed mutagenesis to test the predictions for both the Q<sub>H</sub> and Q<sub>L</sub> sites.
2. Methods

Site-directed mutagenesis

The same methods were used as in Chapter 2. All oligonucleotides were ordered from IDT (Integrated DNA technologies). (Table 4.1) PCR was used for site-directed mutagenesis using Agilent (Santa Clara, CA) kit. The sequence verification of mutagenesis products was performed at University of Illinois Urbana-Champaign (UIUC) Biotechnology Center. All chemicals of the purest grade available were used.
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**Table 4.1** List of primers used in the Site-directed mutagenesis.
3. Results

The Q_H binding motif: First, we will see if Q_H has a ubiquinone binding motif as the Pierri group mentioned [14]. (Fig 4.1) The paper indicated that the Q_H ubiquinone binding motif is from residue R71 - Q101. In the high affinity ubiquinone binding motif, five residues that are aromatic, charged or hydrophilic residues were indicated to be potentially involved in the ubiquinone binding was chosen: R71, F93, P75, H98 and Q101. Previous models of the Q_H site have included D75 which is not included in the list and which mutagenesis studies demonstrate is critical for function [2, 15-19]. Residues F93, and P75, previously not considered, are predicted to play an important role in quinol interactions at the Q_H site. From both X-ray structures of cyt bo3 we know that P75 is involved in a turn [20] and not likely to directly interact with the bound ubiquinol. Mutations have previously demonstrated the importance of R71, D75, H98 and Q101. Even thought it wasn’t chosen to interact with the quinone, Q82, which is in the Q_H motif, in the sequence alignment shows to be conserved. Mutations were made to test the importance of both Q82 and F93 to the Q_H site.

As shown in Table 4.2, replacing Q82 by an alanine has little effect on either the quinol oxidase activity or the \(K_M\) for ubiquinol-1. Hence, there is no support for a direct interaction of this residue with ubiquinol at the Q_H site. Both the F93Y and F93A mutations were also studied. Each of these mutations does increase the \(K_M\) for ubiquinol-1 from 50\(\mu\)M (WT) to 72 \(\mu\)M and 91 \(\mu\)M respectively. In addition, the activity of the F93A mutant is substantially reduced to 37\% of the WT activity. Hence, a role of F93 in
the Q_H site does appear to be plausible based on these data. Further studies with the F93 mutants will be discussed in Chapter 5.

**Fig. 4.1** A) Side view of cyt $bo_3$. The high affinity binding site (Q_H) motif is shown in orange and low affinity binding site (Q_L) motif in purple. The residues depicted as direct binding is shown in the figure. The neon green is D75, which wasn’t chosen for the Q_H binding motif, but from previous study it is well indicated as a binding site. B) Top view of the ubiquinone binding motifs of Q_H site and Q_L site. C) Zoomed view of the Q_H residues. *(IFFT,pdb)*
<table>
<thead>
<tr>
<th>Relative activity (%)</th>
<th>$K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100</td>
</tr>
<tr>
<td>*Q82A</td>
<td>88</td>
</tr>
<tr>
<td>*F93Y</td>
<td>81</td>
</tr>
<tr>
<td>*F93A</td>
<td>37</td>
</tr>
<tr>
<td>E164A</td>
<td>28</td>
</tr>
<tr>
<td>E164K</td>
<td>54</td>
</tr>
<tr>
<td>F165A</td>
<td>37</td>
</tr>
<tr>
<td>Q167A</td>
<td>57</td>
</tr>
<tr>
<td>Q167K</td>
<td>75</td>
</tr>
<tr>
<td>T168A</td>
<td>118</td>
</tr>
<tr>
<td>L171A</td>
<td>79</td>
</tr>
<tr>
<td>S177A</td>
<td>91</td>
</tr>
</tbody>
</table>

**Table 4.2** The ubiquinol-1 oxidase activity and $K_M$ values for mutants within either the Q$_H$ or Q$_L$ site motifs within subunit I, as designated in [14]. (*Q$_H$ motif residues.)

**Fig. 4.2** Figure of Subunit I only with site-directed mutagenesis residues from Table 4.2. The red residues indicate the Q$_H$ residues (R71, D75, H98, and Q101). *(IFFT.pdb)*
The Q<sub>L</sub> binding motif: Site-directed mutagenesis was performed to alter most of the residues in the Q<sub>L</sub> ubiquinone binding motif within subunit I (G163 – Y173) except for glycine and alanine residues. E164 and Q167 were predicted [14] to have a direct interaction with the ubiquinone/ubiquinol moiety. S177 was not depicted as a Q<sub>L</sub> site residue, but due to the location nearby the Q<sub>L</sub> motif, the S177A mutant was also made. (Fig 4.2) Results are summarized in Table 4.2. None of the mutations resulted in a substantial increase in the \( K_M \) of ubiquinol-1. Neither E164 nor Q167 are essential for function though mutations in each lead to lower oxidase activity. E164A has a perturbed heme absorption spectrum (not shown) and only 28% of the activity of the WT enzyme. However, the more drastic mutation E164K does not result in any spectroscopic perturbation and retains 54% of the activity. In summary, these mutations provide no support for residues in the putative Q<sub>L</sub> motif being important for substrate binding.

Fig 4.3 (Left) Side view of cyt bo<sub>3</sub> with site-directed mutagenesis residues shown. The subunit I residues are the Q<sub>H</sub> site, and the subunit II residues are the possible Q<sub>L</sub> site. (Right) top view of the two possible residues for Q<sub>L</sub> site. The Q<sub>L</sub> site residues are right on top of the Q<sub>H</sub> site. (IFFT.pdb)
Several mutations were also made in subunit II to examine possible effects on the interaction of ubiquinol with the enzyme. As shown in Fig 4.3, W$^{II}$136 and E$^{II}$259 are just above of the Q$_{H}$ site. At one point, W$^{II}$136 was suggested to be part of the Q$_{L}$ site [10] though subsequent examination indicated this was not likely [2]. We tested the hypothesis that the region adjacent to and just above the Q$_{H}$ site might provide room for a second ubiquinol to bind (i.e., the Q$_{L}$ site). We characterized the following mutants: W$^{II}$136A, W$^{II}$136K, E$^{II}$259A and E$^{II}$259K. The results, summarized in Table 4.3, show that these mutations have virtually no effect on either the oxidase activity or the $K_M$ of ubiquinol-1.

<table>
<thead>
<tr>
<th>Relative activity (%)</th>
<th>$K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100</td>
</tr>
<tr>
<td>W$^{II}$136A</td>
<td>89</td>
</tr>
<tr>
<td>W$^{II}$136K</td>
<td>105</td>
</tr>
<tr>
<td>E$^{II}$259A</td>
<td>135</td>
</tr>
<tr>
<td>E$^{II}$259K</td>
<td>97</td>
</tr>
</tbody>
</table>

Table 4.3 Site-directed mutagenesis of subunit II residues for possible Q$_{L}$ site.

There was no change in the $K_M$ after mutating either to alanine or lysine. This was shown before, and we have verified again that it isn’t the case [2].
4. Discussion and conclusions

Many membrane enzymes use quinone as a substrate. To identify the quinone binding site a number of approaches have been used including X-ray crystallography with small quinone analogues soaked into the crystals [21], using photoreactive quinone analogues and determining the binding site(s) by protein analysis [10, 22-25], and by site-directed mutagenesis combined with steady state kinetics with both substrates and inhibitors to determine changes in $K_i$ or $K_M$ values [2, 3, 6, 11, 12, 23-31]. Key residues in the QH site of cyt bo3 have been defined by crystallography and mutagenesis [1, 2, 7, 18, 19], and a second site has been inferred to exist based the use of a photoreactive quinone analogue and steady state kinetic patterns. The initial suggestion for the existence of two ubiquinone binding sites derives from the fact that preparations of the enzyme could be obtained with 2 equivalents of ubiquinone, though no evidence showed that both were actually bound to the enzyme as opposed to co-purifying along with other lipids [3].

In this chapter we have used site-directed mutagenesis to locate the QL site, guided by a recent bioinformatics analysis [14] that identified motifs in the quinol oxidases predicted to designate both the QH and QL sites. Extensive analysis of mutations altering virtually all of the residues suggested to comprise the QL site gave no evidence in support of this claim. In addition, mutations which would be expected to disrupt a second quinol binding site above the QH site at the interface with subunit II, revealed no evidence for a separate QL site at this location in the protein. In Fig. 4.4 every residues that was used to search for QL site is shown. Our studies leave us with the question; where is the QL site and does it even exist?
Further tests were made to examine the importance of residues predicted to comprise the $Q_H$ site. Besides the residues already known to interact with the semiquinone stabilized at the $Q_H$ site, only one additional residue, F93 was implicated in quinone binding at the $Q_H$ site. Additional important information on the properties of the F93Y and F93A mutants is shown in the next Chapter.
Fig 4.4 The figure shows all the residues that have been mutated in this study. Grey as subunit I, blue as subunit II, orange as subunit III, and red as subunit IV. The $Q_H$ site residues are written in red colors. (*IFFT.pdb*)
5. References


V. REINVESTIGATION OF $Q_H$ UBIQUINONE BINDING SITE

1. $Q_H$ ubiquinone binding site

1. Introduction

The number of ubiquinone binding sites in cyt $b_o$ has been an ongoing question. The high affinity site $Q_H$ site was identified from sequence homology and from the X-ray structure [1] and confirmed by site-directed mutagenesis [1-4]. The residues are well conserved and using site-directed mutagenesis, steady state enzymatic activities were measured, and moreover different biophysical methods such as EPR, FTIR, redox potential, and other methods were used to identify the core residues at this site [1, 3-12]. Although a careful and detailed study was performed to identify the second ubiquinone binding site, the data summarized in the previous Chapter suggests that perhaps there may be no separate $Q_L$ site. As mentioned by Iwata and Wikström group, there may be only one ubiquinone binding site, which will be sufficient as long as the quinone bound at this site could rapidly exchange with the pool of free quinone in the membrane [1, 11, 13]. If the $Q_H$ site is the only quinone binding site then it would be expected that some mutations at this site would alter the $K_M$ for ubiquinol-1 and also perturb the binding of quinone-analogue inhibitors such as HQNO, thought to interact at the $Q_L$ site [14-17]. As shown, these properties are observed, leading to the conclusion that there is only one quinone binding site, defined as the $Q_H$ site.

The $Q_H$ site core is composed of four residues: R71, D75, H98, and Q101. (Fig 5.1) Any site directed mutagenesis in R71 and H98 makes the enzyme inactive and makes kinetic assays impossible. Among the most studied residues in the $Q_H$ site is D75 [2-5, 9, 10, 18]. The one exception is D75E, which retains activity and has been studied [2]. So far, the last $Q_H$ site
residue, Q101, has not been extensively studied. Q101N was shown by the Wikström group [1] to retain about 24% activity with an increase in the $K_M$ (four-folds) for ubiquinol-1. The same mutant was examined by Yap et al. [2] who observed only 5% residual activity and the absence of a ubisemiquinone EPR [1, 2]. In addition to these core residues, we observed that mutations of F93 also alter the ubiquinol-1 oxidase activity as well as the $K_M$. F93 was selected from the proposed $Q_H$ sequence motif by the Pierri group [19]. The location of F93 in relation to the other residues in the $Q_H$ site is shown in Figure 5.1. In this chapter, we will study a new set of Q101 mutants along with further studies on the mutations of F93.

**Fig. 5.1** $Q_H$ site residues with F93. Subunit I as black, subunit II as red, subunit III as cyan, and subunit IV as blue. (*IFFT.pdb*)
2. Methods

Site-directed mutagenesis

The same methods were used as Chapter 2. All oligonucleotides were ordered from IDT (Integrated DNA technologies). (Table 5.1) PCR was used for site-directed mutagenesis using Agilent (Santa Clara, CA) kit. The sequence verification of mutagenesis products was performed at University of Illinois Urbana-Champaign (UIUC) Biotechnology Center. All chemicals, the purest grade available were used.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q101Afor</td>
<td>CCT CAC CAC TAC GAT GCG ATC TTT ACC G</td>
</tr>
<tr>
<td>Q101Arev</td>
<td>CGG TAA AGA TCG CAT CGT AGT GGT GAG G</td>
</tr>
<tr>
<td>Q101Nfor</td>
<td>CCT CAC CAC TAC GAT AAT ATC TTT ACC GCG CAC</td>
</tr>
<tr>
<td>Q101Nrev</td>
<td>GTG CGC GGT AAA GAT ATT ATC GTA GTG GTG AGG</td>
</tr>
<tr>
<td>Q101Tfor</td>
<td>CCT CAC CAC TAC ATG ATC TTT ACC GCG CAC</td>
</tr>
<tr>
<td>Q101Trev</td>
<td>CGG TAA AGA TCG TAT CGT AGT GGT GAG G</td>
</tr>
<tr>
<td>Q101Lffor</td>
<td>CCT CAC CAC TAC GAT CTG ATC TTT ACC GCG CAC</td>
</tr>
<tr>
<td>Q101Lrev</td>
<td>GTG CGC GGT AAA GAT CAG ATC GTA GTG GTG AGG</td>
</tr>
<tr>
<td>Q101Mfor</td>
<td>CCT CAC CAC TAC GAT ATG ATC TTT ACC GCG CAC</td>
</tr>
<tr>
<td>Q101Mrev</td>
<td>CGT GCG CGG TAA AGA TCA TAT CGT AGT GGT GAG G</td>
</tr>
</tbody>
</table>

Table 5.1 List of primers used in the Site-directed mutagenesis.
Steady state enzymatic activity assay

The steady state oxygen reduction activity of cyt bo$_3$ was measured at 25°C using Unisense Oxygen MicroRespiration sensor (Aarhus, Denmark) that has an oxygen electrode chamber of 1 ml. The temperature was cooled by water that flows around the chamber. The reaction mixture was made using 50 mM Kpi, pH 7.0, 0.05% DDM with 2 mM dithiotherol and ~300 µM ubiquinone-1 (Q1, Sigma, Saint Louis, MO) with constant stirring. For the ubiquinone-2 (UQ$_2$, Sigma) measurement, concentration of 250 µM was used with the same condition as others. The oxygen saturated buffer at 25 °C is estimated to be around 250 µM [20]. With all substrates added, the reaction was initiated as soon as pM amount of enzyme was added into the mix. Turnover number (e$^{-1}$/sec/enzyme) of the enzyme was calculated based on the consumption of oxygen slope and subtracting the background slope prior to the addition of the enzyme. The 2-n-Heptyl-4-hydroxyquinoline N-oxide (HQNO) inhibitor (Enzo Life Sciences, Farmingdale, NY) was used for measurement of inhibition with saturated amount of substrate Q$_1$ (300 µM) with DTT.
Fig 5.2 Quinone analogues structures A) ubiquinone-1 B) 2-n-Heptyl-4-hydroxyquinoline N-oxide (HQNO) C) ubiquinone-2 D) ubiquinone-n structure E) Aurachin (AC-10)

Preparation of EPR samples

The preparation of the sample was made the same as previously reported [2]. The concentrated enzyme was dialyzed against 50 mM KPi buffer, pH 8.3, 0.05% DDM, 10% glycerol and 10 mM EDTA. The enzymes were concentrated up to ~300 μM and 110 uL was used. The enzyme was anaerobically reduced under an argon atmosphere with 500 × excess sodium ascorbate, incubated for four hours and then the reduced sample were transferred into argon-flushed EPR tube, with rapid freezing in liquid nitrogen.
3. Results

**Steady state kinetics: \( k_{\text{cat}} \) and \( K_M \) of ubiquinol-1:** The \( Q_H \) site was well studied by the previous group member Lai Lai Yap [2]. Several mutations were repeated for comparison with new mutants at the \( Q_H \) site. The current work goes beyond previous studies by measuring the \( K_M \) of ubiquinol-1 for each mutant in the \( Q_H \) site, shown in Table 5.2. The relative activities of the \( Q_H \) site mutants in the current work are similar to what has been reported [2]. From the collection of mutants in R71, D75 and H98, only D75E had activity (48%), but the \( K_M \) for ubiquinol-1 was not increased.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Relative activity (%)</th>
<th>( K_M (\mu M) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>D75E</td>
<td>48</td>
<td>16</td>
</tr>
<tr>
<td>D75H</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>R71Q</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>H98N</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>H98T</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 5.2** \( Q_H \) site mutations with relative activity at \( V_{\text{max}} \) and \( K_M \) measurement.

Q101 was mutated to various amino acids. (Table 5.3) Most of the Q101 mutants have high activity compared to the other \( Q_H \) site mutants confirming that Q101 is not essential for oxidase function. However, the Thr, Asn, and Leu mutants show increased \( K_M \) for ubiquinol-1, which is consistent with what we expect for a \( Q_L \) site mutant. Neither Q101A nor Q101M show an increase in the \( K_M \). For Q101A the mutant may behave like the WT oxidase due to the small
size of Ala, allowing the water that might able to bind between the ubiquinone and the residue, without changing the interaction with the ubiquinone and the enzyme. For Q101M, the side chain is the same length as that of glutamine and perhaps the sulfur can form a H-bond to the ubiquinone. The mutations of F93 also had lower ubiquinol-1 oxidase activity and, similar to the results with the Q101 mutants, the $K_M$ was also significantly increased, as reported in Chapter 4.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Relative activity (%)</th>
<th>$K_M$ ($\mu M$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Q101A</td>
<td>26</td>
<td>30</td>
</tr>
<tr>
<td>Q101N</td>
<td>10</td>
<td>72</td>
</tr>
<tr>
<td>Q101T</td>
<td>27</td>
<td>200</td>
</tr>
<tr>
<td>Q101L</td>
<td>11</td>
<td>141</td>
</tr>
<tr>
<td>Q101M</td>
<td>51</td>
<td>24</td>
</tr>
<tr>
<td>F93Y</td>
<td>81</td>
<td>72</td>
</tr>
<tr>
<td>F93A</td>
<td>37</td>
<td>91</td>
</tr>
</tbody>
</table>

Table 5.3 Q101 residue with different site-directed mutagenesis and its relative activity at $V_{\text{max}}$ and $K_M$ measurement.

**Inhibition by the $Q_L$-site inhibitor HQNO:** Each active mutant was also studied for the inhibition by HQNO. (Table 5.4) As shown, the apparent $K_i$ of UQ$_1$H$_2$ of WT is 0.68 $\mu M$. The $K_i$ of HQNO for Q101A is also in the same range, 0.86 $\mu M$. Each of the other active $Q_{H1}$-site mutants, Q101M, Q101N, Q101L, Q101T and D75E, show a substantial increase in the $K_i$ of HQNO, as much as 8-fold increase. This is also observed for both of the mutants which do not show an increased $K_M$ for ubiquinol-1, Q101M and D75E. Furthermore, the extent of inhibition
by HQNO at saturation levels is significantly reduced, particularly in the Q101L and Q101N mutants. Similar results were also shown for both the F93Y and F93A mutants, though the magnitude of the increase in the $K_i$ was not as great as with the other mutations shown in Table 5.4. These new data with HQNO provide independent evidence that the residues which are known to stabilize the semiquinone at the $Q_H$ site also are important for interactions expected at the putative $Q_L$ site. In sum, the data are consistent with only one site for quinone binding.

<table>
<thead>
<tr>
<th>Residue</th>
<th>$K_{app}$ ($\mu M$)</th>
<th>Max % of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.68</td>
<td>99</td>
</tr>
<tr>
<td>Q101A</td>
<td>0.86</td>
<td>86</td>
</tr>
<tr>
<td>Q101N</td>
<td>5.44</td>
<td>63</td>
</tr>
<tr>
<td>Q101T</td>
<td>4.77</td>
<td>88</td>
</tr>
<tr>
<td>Q101L</td>
<td>3.97</td>
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</tr>
<tr>
<td>Q101M</td>
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<td>89</td>
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<tr>
<td>D75E</td>
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<td>90</td>
</tr>
<tr>
<td>F93Y</td>
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<td>108</td>
</tr>
<tr>
<td>F93A</td>
<td>2.21</td>
<td>99</td>
</tr>
</tbody>
</table>

Table 5.4 Q101 mutants with inhibitor HQNO and its $K_{app}$ measurement with relative inhibition.
Fig 5.3 EPR spectra of the semiquinone formed by UQ₈ bound at the enzyme.

**Bound ubisemiquinone-8 and the stabilization of the ubisemiquinone:**

Previously it was shown that mutants in R71, D75 and H98 which inactivate the enzyme do not result in the loss of the bound ubiquinone-8 [1, 2]. However, most of these mutants (exceptions being D75E and D75H) [2] do not stabilize the semiquinone at the Q_H site. The same measurements were made for the mutations in Q101 and it is shown that in all cases the content of bound ubiquinone-8 remains the same as in the WT enzyme (Table 5.5), and that most of these mutants also stabilize the semiquinone, albeit with a perturbed EPR signal. As reported previously [2], Q101N, which in our hands has very low activity, fails to stabilize the semiquinone. As shown in Fig 5.3, for all of the other Q101 mutants the semiquinone was formed. Q101A shows a spectrum identical to that of the WT enzyme. Q101M shows a large
increase in the intensity of the EPR signal, whereas the signal is attenuated for Q101L and Q101T. In addition, the EPR spectrum of Q101L and Q101T has an altered shape due to much smaller methyl hyperfine coupling [21] which is observed as the shoulder in the high field portion of the spectrum of the WT, Q101A and Q101M mutant. Both the F93Y and F93A mutants also stabilized the semiquinone at the Q$_H$ site and the spectra each have the shoulder indicating the methyl hyperfine coupling as in the WT enzyme. The EPR spectra of the F93 mutants are shown in Figure 5.4 along with that of the WT enzyme. It is apparent that the magnitude of the EPR signal is increased, particularly for the F93Y which is similar to Q101M. The results are organized in Table 5.6.

<table>
<thead>
<tr>
<th>Cyt $b_{59}$</th>
<th>Relative activity (%)</th>
<th>Quinone content</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100</td>
<td>1.1</td>
</tr>
<tr>
<td>D75E</td>
<td>48</td>
<td>1.7</td>
</tr>
<tr>
<td>D75H$^*$</td>
<td>4</td>
<td>0.9</td>
</tr>
<tr>
<td>D75N$^*$</td>
<td>~0</td>
<td>1.3</td>
</tr>
<tr>
<td>R71D$^*$</td>
<td>~0</td>
<td>1.4</td>
</tr>
<tr>
<td>R71K$^*$</td>
<td>~0</td>
<td>1.3</td>
</tr>
<tr>
<td>R71Q</td>
<td>3</td>
<td>1.1</td>
</tr>
<tr>
<td>H98S$^*$</td>
<td>2</td>
<td>1.4</td>
</tr>
</tbody>
</table>

**Table 5.5** Q$_H$ mutants relative activity at $V_{max}$ and its quinone content measured by HPLC. (* Reported as [2].)
Fig 5.4 The EPR spectra of WT, F93A and F93Y. F93Y has more than 3 fold of increase in intensity corresponding to strong semiquinone formation.

<table>
<thead>
<tr>
<th>Residue</th>
<th>EPR signal</th>
<th>Methyl hyperfine coupling</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Q101A</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Q101N</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Q101T</td>
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<td>No</td>
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<td>Q101M</td>
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<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>F93A</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 5.6 The EPR signal of semiquinone formation and methyl hyperfine coupling.
Steady state kinetics with ubiquinol-2: The steady state kinetics measurements reported so far, were made with soluble ubiquinone analogue, UQ₁. However, it is well known that soluble ubiquinone analogue, UQ₂, with a longer isoprenoid chain can also be used as a substrate [22-25]. Table 5.7 shows the relative activity using UQ₂ at 250 μM. Generally, the quinol oxidase activity with UQ₂ is less affected by the Q₁₁-site mutants compared to the WT than the comparable measurements using UQ₁. This is likely due to the longer isoprenoid chain of UQ₂ increasing the binding affinity to the enzyme [12, 22, 23]. The Q101 mutants that had low activity with UQ₁, e.g., Q101L, Q101T and Q101N, retained up to 60 % activity using UQ₂, while the Q101A has 80 % and Q101M has 70 % of the WT activity. D75E, F93A and F93Y have virtually no loss of activity when using UQ₂ as the substrate. Unfortunately, activity with UQ₂ was often biphasic (Fig 5.5), and previous results from other groups indicate substrate inhibition at high concentrations [22, 23]. Therefore, obtaining consistent and reliable $K_m$ values was not possible.
Table 5.7 The relative activity using UQ$_2$ at 250 $\mu M$. For specific mutants, a biphasic curve was seen.

<table>
<thead>
<tr>
<th></th>
<th>Relative activity (%) UQ$_2$</th>
<th>Relative activity (%) UQ$_1$</th>
<th>biphasic</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100</td>
<td>100</td>
<td>Yes</td>
</tr>
<tr>
<td>R71Q</td>
<td>8</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>H98N</td>
<td>10</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>H98T</td>
<td>9</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>D75E</td>
<td>88</td>
<td>45</td>
<td>Yes</td>
</tr>
<tr>
<td>Q101A</td>
<td>82</td>
<td>26</td>
<td>Yes</td>
</tr>
<tr>
<td>Q101N</td>
<td>61</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Q101T</td>
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<td>No</td>
</tr>
<tr>
<td>Q101L</td>
<td>58</td>
<td>11</td>
<td>No</td>
</tr>
<tr>
<td>Q101M</td>
<td>72</td>
<td>51</td>
<td>Yes</td>
</tr>
<tr>
<td>F93Y</td>
<td>107</td>
<td>81</td>
<td>Yes</td>
</tr>
<tr>
<td>F93A</td>
<td>102</td>
<td>37</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Fig. 5.5 Oxygen utilization due to UQ$_2$ oxidase activity. As seen, two phases can be identified. All the measurements for relative activity were done using the slow phase. The two phases do not appear in the same timeframe.
2. Residue F93 – is this a ubiquinone binding site?

1. Results

As mentioned from previous chapter, F93 was mentioned to be one of the Q$_H$ motif site. Since we have studied a new method to identify the Q$_H$ site residues, the steady state kinetics will be measured upon this residue. As shown in Fig. 5.1, F93 is located on the loop close to the Q$_H$ site. Residue F93 is about 70 % conserved including Tyr. This residue has never been studied before. Table 5.8 shows results for the steady state enzyme kinetics of the $K_M$ and apparent $K_i$ for F93, as studied before with Q101. The F93 mutants are as active as WT. The $K_M$ for both mutants has 1.5 to 2 fold increase. The apparent $K_i$ was measured using HQNO and UQ$_1$. This measurement shows an elevation of $K_i$ of 3~4 folds. The $K_i$ is not as high as the other Q101 residues. The % of inhibition is complete.

<table>
<thead>
<tr>
<th>Relative activity (%)</th>
<th>$K_M$</th>
<th>$K_{app}$</th>
<th>Max % of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100</td>
<td>50</td>
<td>0.68</td>
</tr>
<tr>
<td>F93Y</td>
<td>81</td>
<td>72</td>
<td>2.69</td>
</tr>
<tr>
<td>F93A</td>
<td>37</td>
<td>91</td>
<td>2.21</td>
</tr>
</tbody>
</table>

Table 5.8 Mutation of residue F93 and its relative activities with $K_M$ and $K_{app}$.

Next, EPR was measured. (Table 5.9) EPR shows that the semiquinone is formed and has methyl hyperfine coupling. As Fig 5.4, both the mutants have higher semiquinone yield...
compared to the WT. When measuring the steady state kinetics using soluble UQ\textsubscript{2}, the mutants have high activity without any decrease. As shown in the other Q101 mutants, D75E, and WT, the F93 mutants also show biphasic character.

<table>
<thead>
<tr>
<th></th>
<th>EPR Signal</th>
<th>Methyl hyperfine coupling</th>
<th>Relative activity (% UQ\textsubscript{2})</th>
<th>biphasic</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Yes</td>
<td>Yes</td>
<td>100</td>
<td>Yes</td>
</tr>
<tr>
<td>F93Y</td>
<td>Yes</td>
<td>Yes</td>
<td>107</td>
<td>Yes</td>
</tr>
<tr>
<td>F93A</td>
<td>Yes</td>
<td>Yes</td>
<td>102</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 5.9 The EPR measurements were performed on the mutants. Both we can see the semiquinone formation and methyl hyperfine coupling from the mutants. The different soluble quinone analogue UQ\textsubscript{2} was used for steady state kinetics.

3. Discussion and conclusions

In this Chapter it is demonstrated that some mutations in Q\textsubscript{H}-site residues show phenotypes expected of Q\textsubscript{L}-site mutants: an increase in the $K_M$ of ubiquinol-1 and an increase in the $K_i$ of the inhibitor HQNO. In addition, many of the new mutations reported here show a perturbed EPR signal from the semiquinone, which defines the quinone bound at the Q\textsubscript{H} site. The increase in the $K_M$ of Q101N was initially reported from Wikström group in 2000 [1]. In their preparation, they had used two types of enzymes: ubiquinone depleted version (Triton X-100 purified enzyme) and ubiquinone bound version (DDM purified enzyme). As mentioned in chapter 2, their preparation had many problems including mixed heme ratio between \textit{oo}$_3$ and \textit{bo}$_3$. 

Therefore, the measurement was not accurate. These problems of heme content and the resulting heterogeneity of the enzyme preparations have been resolved and the WT and all mutants showed the expected ratio of heme $b$ : heme $o$ of 1:1. Data presented in the previous chapter included an exhaustive search for residues defining an independent $Q_L$-site, and the results yielded nothing. For this reason, in this chapter we pursue the possibility that the $Q_H$-site is the only quinone binding site in cyt $bo_3$. We obtained a set of mutants among the core residues of the $Q_H$-site which retained significant oxidase function and used steady state kinetics with ubiquinol-1 to determine the $K_M$ for this substrate as well as the $K_i$ of the $Q_L$-site inhibitor HQNO. Increases in these two parameters provide independent phenotypes expected for mutants that perturb the substrate binding site.

Whereas most previous studies of $Q_H$-site mutants focused on inactive R71 and H98 residues, our new focus was to obtain mutants that largely retained activity. In addition to D75E, previously shown to be highly active [2], we determined that Q101 as well as F93 could be mutated without eliminating function. Previous pulsed EPR measurements showed that Q101 interacts with the semiquinone that is stabilized at the $Q_H$-site [2]. The current work, however, shows that mutations at this site have effects expected for perturbing the $Q_L$-site. The results include substantial increases in the $K_M$ for ubiquinol-1 and, even more dramatically, an increase in the $K_i$ of HQNO by up to 9-fold over the value of the WT enzyme. Furthermore, the Q101L and Q101N were inhibited by only about 60% even at saturating concentrations of HQNO.

Whereas the Q101 and F93 mutants exhibit “$Q_L$-site” phenotypes, they also show perturbed EPR signals from the ubisemiquinone that is located at the $Q_H$-site, leading to the obvious conclusion that there may only be one quinone binding site and that involves the residues identified as the $Q_H$-site. This still leaves us with a puzzle of how to explain the data.
previously used to justify the two-site model. The strongest data are those that demonstrated that
1) One equivalent of HQNO can bind to the purified enzyme without displacing the “tightly
bound UQ₈; and 2) Catalytic turnover of UQ₁ did not displace the ubiquinone-8 bound to the
purified enzyme. These appear to necessitate at least one additional binding site in addition to
that where UQ₈ is bound at the Q₇-site. We are now forced to re-evaluate the previous
interpretation of these data.

There appear to be two plausible explanations.

Model 1: Two quinone binding sites that share interactions with some

critical residues. One explanation is that there are two quinone binding sites but that they
share critical interactions with the same set of residues, including at least Q101 and D75. One
site binds the “tightly bound” quinone and stabilizes the semiquinone. The second site overlaps
the first but can bind a second quinone as well as inhibitors such as HQNO without displacing
the quinone from the first site.

Model 2: One quinone binding site. A one-site model would require that the
“tightly bound” quinone that is isolated with the enzyme is primarily bound through hydrophobic
interactions with the isoprenoid “tail” of the quinone. This would allow the small soluble UQ₁
and HQNO to displace the headgroup of the bound ubiquinone-8 but would not displace the
bound quinone. This model is supported by the fact that none of the mutations in R71, D75,
Q101 or H98 result in dislodging the bound quinone although the ability to form the
semiquinone is usually eliminated. Previous data showed virtually no change in the binding of
HQNO to the purified cyt bo₃ due to the presence of the bound ubiquinone-8, which would
require that very little if any of the binding free energy results from interactions with the headgroup.

It might be expected that displacing the UQ$_8$ from the isolated enzyme in a detergent micelle would be difficult due to the size and hydrophobicity of the long isoprene tail of UQ$_8$. However, the mutations in the Q$_{H}$-site residues must be bound to UQ$_8$ within the $E.~coli$ membrane, which is a very different environment from the small detergent micelle. Hence, it is reasonable to conclude that the binding of the UQ$_8$ to cyt bo$_3$ is primarily through hydrophobic interactions with at least part of the isoprene tail and not due to strong interactions with the quinone headgroup.

The fact that the mutations in Q101 and as well as F93A and D75E exhibit much less effect on the activity using UQ$_2$ in place of UQ$_1$ is also consistent with this conclusion. Since UQ$_2$ has a longer and more hydrophobic tail, it would be expected to bind more strongly to the quinone binding site and, perhaps, be better able to overcome the effect of these mutations which do not apparently block essential steps in the catalytic mechanism further downstream from the initial binding. However, the presumed stronger binding through the isoprene tail would also necessitate that UQ$_2$ cannot bind without displacing the bound UQ$_8$ that is bound in the same site if there is only one-site. This is examined in the next chapter
4. References


VI. EXCHANGE OF THE BOUND UBIQUINONE-8 WITH EXOGENOUSLY ADDED QUINONES

1. Introduction

After purification using the detergent DDM there is a “tightly bound” ubiquinone at the $Q_H$ site of cyt $bo_3$. This “tightly bound” quinone was thought to be a cofactor by experiments that showed that even in the presence of excess substrate $UQ_{1H2}$ the bound $UQ_8$ remains bound to the enzyme [1]. In an experiment in which buffer containing excess $UQ_{1H2}$ was flowed through the Ni-NTA resin to which cyt $bo_3$ was bound, there was no displacement of the bound quinone with the soluble quinone. [1] In addition, the binding of the inhibitor HQNO occurs in the presence of bound $UQ_8$ with the same binding constant and stoichiometry [1]. These experiments supported the view that the bound quinone does not exchange with exogenous ubiquinones or quinone analogues. On the other hand, once the $UQ_8$ is removed from the enzyme, such as by treatment with Triton X-100 or by isolating the enzyme from a strain of $E. coli$ that does not synthesize ubiquinone [2, 3], small quinones such as $UQ_1$ or analogues bind readily and can even form stable semiquinones at the $Q_H$-site [2, 4-6]. However, quinones with long isoprenoid chains such as $UQ_8$ or $UQ_{10}$ require overnight treatments, at best, to occupy the vacant $Q_H$ site [2, 3].

Quinones with short isoprenoid chains (or none) such as $UQ_1$ are much more water-soluble than the natural substrates, $UQ_8$ or $UQ_{10}$. The natural substrates present significant experimental problems with membrane enzymes that are characterized in detergents such as DDM. To a large extent it is not possible to simply add $UQ_8$ in a detergent micelle to the enzyme, also in a detergent micelle. This is why $UQ_1$ is routinely used (along with decyl-ubiquinone, which has an alkyl chain in place of the isoprene side chain) as the preferred
substrate for *in vitro* studies. The long and short chain quinones are very different in this regard. Even within the lipid bilayer, the long and short chain quinones behave differently as they partition between the middle and surface of the bilayer [7, 8]. Depending on the length of the quinones, shorter chain quinones preferably locate near the surface, while the longer chain quinones spend longer times at the midplane [8]. It has been shown that the tails are associated with the binding of the enzymes; therefore using short tails might not be an accurate measurement for quinone binding measurements [9-13].

One approach which has been used to avoid these difficulties is to examine the membrane enzymes in reconstituted lipid bilayer or within the natural membrane. One example is CymA, a tetraheme quinol dehydrogenase which is a monotopic membrane enzyme that normally sits on the surface of the membrane bilayer and oxidizes the quinol within the membrane. This enzyme has been studied using reconstituted liposomes containing menaquinone [14]. Using vesicle-bound apo-enzyme it was shown that the water-soluble menaquinol (MK₀) does not bind to the enzyme while the long isoprenoid tail of MK₇ facilitates the guidance of the substrate to the binding site. This study illustrates that the way in which a membrane enzyme interacts with water-soluble analogues of the natural hydrophobic quinone substrate can be very different and exhibit very different kinetics.

Four experiments are described in this chapter in which the exchangeability of the long chain UQ₈ at the Q₇H site is re-examined. Each experiment demonstrated that the UQ₈ bound to the Q₇H site of cyt bo₃ is not irreversibly bound to the enzyme, but could be displaced or replaced at least within minutes. The data make it plausible that the Q₇H site is the only quinone binding site in cyt bo₃.
Experiment 1: UQ\textsubscript{10} was inserted into \textit{E. coli} membranes containing cyt \textit{bo}_3 along with its natural substrate UQ\textsubscript{8}. The enzyme was solubilized in DDM and isolated and the quinone content was determined to see if the exogenously added Q\textsubscript{10} would displace the natural UQ\textsubscript{8} in the Q\textsubscript{H} site.

Experiment 2: Purified cyt \textit{bo}_3 containing one equivalent of UQ\textsubscript{8} was reconstituted with phospholipids to form proteoliposomes without any additional quinone species. The enzyme was solubilized and re-isolated and the quinone content was measured. The purpose of this experiment is to see if the bound UQ\textsubscript{8} would partition into the lipid bilayer and deplete the occupancy of the Q\textsubscript{H} site due to this dilution.

Experiment 3: The purified cyt \textit{bo}_3 was reconstituted into proteoliposomes which contained UQ\textsubscript{10}. After re-isolation of the enzyme, it was determined if UQ\textsubscript{10} had replaced UQ\textsubscript{8} at the Q\textsubscript{H} site. In addition, the monotopic membrane enzyme NDH-2 from \textit{E. coli} [15, 16] was used to reduce the pool of UQ\textsubscript{10} in the reconstituted liposomes to measure the ability of the reduced UQ\textsubscript{10} to be re-oxidized by cyt \textit{bo}_3. This was determined by measuring the depletion of O\textsubscript{2} in the solution.

\[
2\text{NADH} + 2\text{H} + Q \leftrightarrow 2\text{NAD}^+ + Q\text{H}_2
\]

Experiment 4: Purified, recombinant cyt \textit{bo}_3 was bound to the Ni-NTA column in the presence of the detergent DDM. Several column volumes of buffer containing either UQ\textsubscript{2} or UQ\textsubscript{4} were rapidly flowed over the bound enzyme, which was immediately eluted from the column. This repeats the same experiment previously performed using UQ\textsubscript{1}, which did not displace the UQ\textsubscript{8}. The quinone content of the enzyme was determined to determine if quinone species with longer isoprenoid side chains, but more soluble than UQ\textsubscript{8} or UQ\textsubscript{10}, could displace UQ\textsubscript{8} even in detergent micelles.
2. Methods

Fusing UQ_{10}-containing liposomes with *E. coli* membranes

Unilamellar UQ_{10} containing liposomes were prepared with 2 g of asolectin (mixed soybean phospholipid) and 13.5 mg of UQ_{10} dissolved in 6 ml of chloroform [5, 17]. The mix was evaporated to dryness under nitrogen gas. (Around 2 hours.) After drying the mixture, 11 ml of 50 mM Tris-Cl (pH 8.0), 0.1 mM EDTA, 200 mM NaCl, 10 % glycerol buffer was added and resuspended till the mixture was well hydrated. (Hydration took around 3 hours.) Sonication was performed 10 times with a 30 s period at 4 °C. The liposomes were mixed with 10 ml amount of an *E. coli* membrane suspension (collected from ~ 3L cell culture). Buffer was added to the mixture, bringing it up to 30 ml. This was then incubated for one hour in the shaker. Fusion was accomplished by using the flash-freeze method with liquid nitrogen. After freezing, the membrane-liposome mixture was thawed and then sonicated twice for 30s at 4 °C. Again using liquid nitrogen this process was repeated four more times. The volume was increased up to 45 ml by adding buffer. Ultracentrifugation at 42 K rpm for 2 hours was performed. The pellets were collected and resuspended to a final volume of 50 ml of 50 mM KPi buffer. The membranes were solubilized with 1 % of DDM for 2 hours followed by ultracentrifugation at 40 K rpm for 1 hour at 4 °C to remove insoluble material. The purification of cyt *bo*₃ is the same as previously described.
**Quinone extraction from purified cyt bo₃ and analysis by HPLC**

A known amount of purified enzyme (about 5 nmoles in 500 µl) was aliquoted into glass tubes along with the equimolar amount of standard either MK₇ extracted from *Bacillus subtilis* or purchased UQ₁₀. After mixing, 3 ml of methanol:petroleum ether (3 : 2.3) solvent was added to the mixture followed by vigorous vortex and centrifugation. The upper organic layer containing the ubiquinones was extracted and transferred into an empty vial and was dried under nitrogen gas. The remaining mixture was extracted with 3 ml of the same solvent two more times. This organic solvent containing the extracted ubiquinone was dried and the residue dissolved in pre-filtered solvent used as the mobile phase for HPLC, acetonitrile: methanol: ethanol = 3:3:4. The quinone species were resolved using a microsorb-MV 100-5 C18 reverse-phase HPLC column (Varian, Palo Alto, CA).

**Formation of reconstituted proteoliposomes using DOPC**

10 mg of DOPC lipids with or without UQ₁₀ was transferred into a round-bottomed flask and then small amount of chloroform was added until the lipids were dissolved. The liquid was dried under nitrogen flow and then placed under vacuum for at least 4 hours to completely remove the chloroform. The dried lipids were resuspended in 10 ml of 20 mM Hepes-NaOH, pH 7.4. The lipid suspension was subjected to five cycles of freeze-thaw using liquid nitrogen and then treated with an extruder kit from Avanti (Alabaster, AL) to make unilamellar vesicles of predetermined sizes. Large vesicles (>800 nm) were initially used and then the size was decreased to 200 nm. Extrusion was performed multiple times (over 17) to assure a uniform size.
The distribution of the vesicles. The multilamellar vesicles were frozen -20 °C for further use or, if not used in several weeks, it was stored at -80 °C.

The suspension of multilamellar vesicles (470 µl containing about 470 µg of phospholipid) was combined with 17 µl Na-cholate (20% stock) 1 uM of a solution containing enzyme. The enzyme should be as concentrated as possible so the amount of DDM in the final mixture was minimal. The mixture is incubated at room temperature for 30 min with occasional mixing by tapping the tube. The ~ 500 ul sample was loaded onto a PD10 column and eluted with buffer without any detergent. The enzyme was collected in about 1.5 ml and used without storage.

**Quinone extraction from cyt bo₃ in reconstituted liposomes**

Although cyt bo₃ could be readily solubilized from E. coli membranes using 2% DDM, this was found not to be the case for cyt bo₃ that was reconstituted into proteoliposomes using either asolectin or DOPC regardless of whether UQ₁₀ was also present. This observation is consistent with studies from the Møller group, which examined the effectiveness of different detergents to solubilize phospholipid vesicles [18]. For this purpose DDM is very ineffective, but Triton X-100 is the most efficient detergent. However, we could not use Triton X-100 because this would also strip the quinone bound to the Q₁ site [19]. The protocol that was successful to solubilize cyt bo₃ from reconstituted proteoliposomes required a 2 hr incubation in the presence of 10% DDM. The liposome suspension became translucent by this treatment and the cyt bo₃ was isolated using the Ni-NTA column, washing the column with 0.05% DDM, 50 mM KPi buffer at pH 8.3, followed by elution using 150 mM imidazole in the same buffer.
Steady state enzymatic activity assay using liposomes

The steady state oxygen reduction activity of cyt bo3 liposomes was measured at 25 °C using a YSI model 53 oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) that has an oxygen electrode chamber volume of 1.8 mL. The temperature is maintained by using a circulating water bath. The reaction mixture was made using 50 mM Kpi, pH 7.0, 0.05% DDM with 9 pmol enzyme. The oxygen saturated buffer at 25 °C is estimated to have an oxygen concentration around 250 μM. With all substrates added, the reaction is initiated by the addition of 2 ul of 6 ug/ml of NDH-2 (0.143 nmol). The turnover number (e⁻¹/sec/ enzyme) of the enzyme is calculated based on the rate of oxygen consumption after subtracting the background slope prior to the addition of the enzyme.

Exchange of UQ₈ by UQ₂ using purified enzyme in DDM

Two methods were used.

1. Exposing the enzyme to UQ₂ prior to re-isolation of cyt bo3 with the Ni-NTA column: 2.5 uM (5 nmol) cyt bo3 was added to 1.8 ml of 1% DDM, 50 mM KPi buffer at pH 7. To this was added various amounts of UQ₂ (Sigma) and the mixture was incubated for the stated times. Adding the reductant DTT to reduce UQ₂ and initiate enzyme activity made no difference to the final result. After incubation, the mixture was loaded onto the Ni-NTA column and washed thoroughly using 0.05% DDM, 50 mM KPi buffer at pH 8.3. The enzyme was eluted using 150 mM imidazole, 0.05% DDM, 50 mM KPi buffer at pH 8.3.
2. **Exposing the enzyme to UQ\textsubscript{2} while the cyt bo\textsubscript{3} is bound to the Ni-NTA column.** The second method involves a quick exchange using enzyme already bound to the Ni-NTA column. The enzyme ~2.5 \textit{uM} (5 \textit{nmol}) was added to ~500 \textit{ul} resin. The enzyme-bound Ni-NTA resin was washed with 2 \textit{ml} of 1\% DDM, 50 mM KPi buffer at pH 7 containing different concentrations of UQ\textsubscript{2} and then immediately washed with about 15 \textit{mL} of 0.05\% DDM, 50 mM KPi buffer at pH 8.3. The enzyme was then eluted using 150 mM imidazole, 0.05\% DDM, 50 mM KPi buffer at pH 8.3. Following elution, the buffer was exchanged to eliminate the imidazole.

3. **Results**

**UQ\textsubscript{8} bound to the Q\textsubscript{H} site is exchanged for exogenous UQ\textsubscript{10} for cyt bo\textsubscript{3} in the E. coli membrane**

To identify if exogenous quinone can bind at the Q\textsubscript{H} site, we have fused liposomes containing UQ\textsubscript{10} with \textit{E. coli} membranes containing recombinant His-tagged cyt bo\textsubscript{3}. After purifying the enzyme, the amount of UQ\textsubscript{8} that co-purifies is reduced and UQ\textsubscript{10} is observed (Fig 6.1). This result shows that the exogenous UQ\textsubscript{10} can displace the endogenous UQ\textsubscript{8} normally bound to the enzyme. This is not a quantitative experiment but shows at least that over the course of the several hours duration of this experiment the UQ\textsubscript{8} must dissociate in order to be replaced by UQ\textsubscript{10}. The next set of experiments was designed to examine exchange of UQ\textsubscript{8} bound to purified cyt bo\textsubscript{3} placed in an artificial lipid bilayer.
Fig 6.1 The membrane fusion with liposomes including UQ₁₀. MK₇ was used for standard.

Exchange of UQ₈ bound to cyt bo₃ in reconstituted proteoliposomes.

1. Proteoliposomes without added UQ₁₀: Purified cyt bo₃ was reconstituted into unilamellar vesicles composed of phospholipids, DOPC. The enzyme was then solubilized using excess DDM detergent and re-isolated using a Ni-NTA column, followed by quinone extraction. The purpose of this experiment was to see if the bound UQ₈ would dissociate into the phospholipid bilayer and be diluted out to such an extent that the equilibrium occupancy of UQ₈ in the Qₐ site would be diminished. This is
what was observed. Whereas the bound UQ$_8$ has never been observed to dissociate from the purified cyt $bo_3$ in DDM detergent, the UQ$_8$ is dissociated once the enzyme is placed in a phospholipid bilayer. It was discovered during this experiment that at least 2 h incubation at room temperature with very high concentrations of DDM was required to solubilize the enzyme from the proteoliposomes. Control experiments showed that the bound UQ$_8$ is not removed even by treatment of the enzyme with 10% DDM. Hence, it is the placement within the lipid bilayer that facilitates dissociation of the long chain quinone. Under these conditions, 90% of the bound UQ$_8$ was removed from the purified enzyme (see Fig 6.2)

![Graph](image)

**Fig 6.2** The exchange study using liposomes without substrates.
2. **Reconstitution into proteoliposomes with UQ$_{10}$.** Next, we included UQ$_{10}$ in the liposomes and measured the quinone content bound to the enzyme following re-purification. As shown in Fig 6.3, the bound quinone (UQ$_8$) is nearly completely (97%) replaced by UQ$_{10}$. This clearly demonstrates that within a bilayer UQ$_8$ and UQ$_{10}$ exchange occurs at the Q$_H$ site.

![Graph showing quinone exchange](image)

**Fig 6.3** The exchange study using liposomes with UQ$_{10}$ substrates. MK$_7$ was used for standard.

The steady state kinetics was measured by adding NADH plus purified type-2 NADH:ubiquinone oxidoreductase (NDH-2) to the reconstituted vesicle containing both UQ$_{10}$ and cyt $b_0$. NADH is oxidized by the NDH-2 enzyme with binds to the surface of the
proteoliposome as a monotopic membrane enzyme, and the enzyme reduces the UQ\textsubscript{10} within the phospholipid bilayer (1\% of the total lipid is UQ\textsubscript{10}). The reduced UQ\textsubscript{10} can then be re-oxidized by the cyt \textit{bo}\textsubscript{3} within the bilayer. The reconstituted respiratory chain results in NADH oxidation and the reduction of O\textsubscript{2} to water. Turnover of cyt \textit{bo}\textsubscript{3} was determined to be 500 e\textsuperscript{-}/s, which is comparable to what has been measured using the short chain soluble UQ\textsubscript{1}H\textsubscript{2} as the substrate in DDM detergent. Hence, the enzyme is perfectly capable of rapidly using the exogenously added UQ\textsubscript{10}H\textsubscript{2} as a substrate within the bilayer.

\textbf{UQ\textsubscript{8} bound to cyt \textit{bo}\textsubscript{3} can be readily exchanged for UQ\textsubscript{2} in DDM detergent}

The data from the experiments described above demonstrate at least that UQ\textsubscript{8} can be removed and replaced at least over the course of hours when the enzyme is placed in a lipid bilayer. Shorter times for the exchange could not be observed due to the technical issues of re-solubilizing and isolating the enzyme from the proteoliposomes. Nevertheless, it is evident that the UQ\textsubscript{8} might exchange more rapidly. This was tested by examining whether UQ\textsubscript{2} could displace UQ\textsubscript{8} from the Q\textsubscript{H} site with the enzyme in DDM detergent. UQ\textsubscript{2} is sufficiently water soluble so it can be readily added to detergent solution containing the enzyme, unlike the long chain UQ\textsubscript{10}. Although previous result showed that UQ\textsubscript{8} remains bound the pure cyt \textit{bo}\textsubscript{3} in the presence of a large excess of UQ\textsubscript{1}, it was hoped that perhaps the more hydrophobic UQ\textsubscript{2} might bind and displace UQ\textsubscript{8} from the Q\textsubscript{H} site. The fact that the mutations in the core residues at the Q\textsubscript{H} site do not result in loss of the bound UQ\textsubscript{8} suggests that the most important binding interactions are though the hydrophobic isoprenoid tail. The results show that just a 2 minute exposure of the
purified enzyme to a solution containing UQ$_2$ results in nearly complete removal (>80%) of the UQ$_8$ from the Q$_H$ site. (Table 6.1) Repeating the same experiment with UQ$_1$ does not result in removal of UQ$_8$.

<table>
<thead>
<tr>
<th>UQ$_2$ (μM)</th>
<th>50</th>
<th>125</th>
<th>250</th>
<th>375</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 2min</td>
<td>66%</td>
<td>30%</td>
<td>24%</td>
<td>26%</td>
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**Table 6.1** Different UQ$_2$ concentrations by incubation or flow through methods measuring the UQ$_8$ content still bound.

4. Discussion and conclusions

The UQ$_8$ that copurifies along with the enzyme in DDM detergent is “tightly bound” insofar as it does not dissociate from the enzyme even after considerable washing while bound to a column such as a Ni-NTA column. Hence, this bound has been considered to be a cofactor of the enzyme which does not dissociate significantly and, therefore, cannot serve as the substrate binding site which must exchange with the UQ$_8$ in the quinone pool on a timescale of about 2 ms (steady state $k_{cat}$ of about 500 UQ$_8$H$_2$ per sec). The “tight binding” of UQ$_8$ at this site is reinforced by the lack of any loss of the bound quinone even from mutants which alter the residues that are known to interact with the headgroup of the semiquinone species at the Q$_H$ site [1, 19]. Moreover, this bound quinone does not dissociate when the enzyme is incubated with
large excess of exogenous UQ₁ or UQ₁H₂ or in the presence of the inhibitor HQNO. These data present a strong case in support of a second “substrate binding site” denoted as the low affinity Q_L site.

Most of the previously reported experiments involved characterizing the enzyme in DDM detergent. In the current work we have shown that the UQ₈ bound at the Q_H site does dissociate much more readily when the enzyme is in a phospholipid bilayer, though this is still on an “hours” time scale. Furthermore, in detergent DDM, the bound UQ₈ can be displaced by UQ₂ within a couple of minutes. Hence, at the very least the possibility must be considered that the quinone that is isolated bound at the Q_H site might, within the E. coli membrane, exchange rapidly with pool of UQ₈ within the membrane bilayer.

These data must now be considered in the light of the previously described experiments showing that 1) Despite an extensive search site-directed mutagenesis has not revealed a single residue outside of the Q_H site which indicate that it is part of the mysterious Q_L site; and 2) Mutations at D75, Q101 and F93 have been found that have properties expected for being at the substrate binding site, an increase in the K_M of ubiquinol-1 and an increase in the K_i of the inhibitor HQNO.

The simplest explanation is that there is only one quinone binding site which is defined by the residues at the Q_H site. So far, the implicated residues are R71, D75, F93, H98 and Q101. The extent to which these residues are important may depend on different interactions with the oxidized, fully reduced and semiquinone forms of the substrate. Postulating one site for quinone binding presents the problems that it has been well documented that the UQ₁H₂ acts as a substrate without displacing the bound UQ₈ at what is now postulated to be the only quinone
binding site, and it has also been clearly shown that the inhibitor binds to the enzyme whether or not the UQ\textsubscript{8} is bound to the Q\textsubscript{H} site.

How can two molecules interact simultaneously at the same site (UQ\textsubscript{1} vs UQ\textsubscript{8} and HQNO vs UQ\textsubscript{8})? One possibility is that the “one site” is large enough to bind two molecules simultaneous, each relying on critical interactions with the same residues. The second possibility, which we favor, is that the binding free energy for quinones binding at the Q\textsubscript{H} site is primarily from the hydrophobic isoprenoid tail and very little comes from interactions with the headgroup. In this way, the headgroup of UQ\textsubscript{8} can be effectively dissociated from the region of the protein defined by the R71-Q101 set of residues, while at the same time remaining firmly bound via interactions with the isoprenoid side chain. The short tails on HQNO and UQ\textsubscript{1} do not interfere with the interface that is most important for defining the affinity of UQ\textsubscript{8} to the Q\textsubscript{H} site, but the longer isoprenoid chain on UQ\textsubscript{2} does bind to this same portion of the protein and can displace UQ\textsubscript{8} even when the only place it can go is into the detergent micelle of DDM.

We postulate there is only one binding site for the substrate UQ\textsubscript{8}H\textsubscript{2} which we expect to be able to very rapidly dissociate when the enzyme is within the lipid bilayer of the \textit{E. coli} membrane. Furthermore, we speculate that the headgroup of the natural substrate can be displaced by small quinone analogues (substrates or inhibitors) without displacing UQ\textsubscript{8} when the enzyme is within a detergent micelle.

Further work is needed to confirm elements of this model.
5. References


