THE STUDY OF $AA_3$-TYPE CYTOCHROME C OXIDASE IN RHODOBACTER SPHAEROIDES

BY

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DISSERTATION

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ABSTRACT

Cytochrome c oxidase is the final electron acceptor in the respiratory chain and catalyzes the highly exergonic oxygen reduction reaction to water and forms a transmembrane electrochemical proton gradient. This transmembrane gradient is used by ATP synthase to produce ATP. The oxygen chemistry reaction of the enzyme is coupled to a proton pump, which substantially contributes to the transmembrane electrochemical gradient. Two proton entry pathways, D pathway and K pathway, have been resolved in X-ray crystal structures. But the exit pathway for the pumped proton and its mechanism is not well understood. The work in this thesis presents extensive studies in proton translocation in both the D-pathway and putative exit pathway. The mutations in the highly conserved R481 confirmed that the residue itself and the hydrogen bonds it forms with the heme propionates are not critical for proton pumping ability and the environmental changes of the hemes were detected on the R481 mutant oxidases. The putative exit pathway is very complicated to define due to the network of many water molecules and hydrophilic residues in the area. But clearly, changing the charge status in some of the residues in putative exit pathway affected the function of the oxidases and the environment of hemes. The D-pathway proton translocation study reveals that the waters do not necessarily need to be hydrogen-bonded to conserved serines in the middle of the pathway. However, the serine mutations caused changes in the pKₐ of E286 (branch point for substrate proton and pumped proton), which led to the conclusion that the pKₐ of E286 is not directly related to proton pumping ability.
AKNOWLEDGEMENTS

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To My Parents
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<th>Full Form</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide m-chlorophenylhydrazine</td>
</tr>
<tr>
<td>CcO</td>
<td>cytochrome c oxidase</td>
</tr>
<tr>
<td>CO</td>
<td>carbon monoxide</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DM</td>
<td>n-dodecyl β-D-maltoside</td>
</tr>
<tr>
<td>E. coli</td>
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<td>HEPES</td>
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</tr>
<tr>
<td>Kan</td>
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</tr>
<tr>
<td>mL</td>
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<td>microliter</td>
</tr>
<tr>
<td>MW</td>
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<td>reduced nicotinamide adenine dinucleotide</td>
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<td>NAD⁺</td>
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</tr>
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</tr>
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<td>nanometer</td>
</tr>
<tr>
<td>P. d</td>
<td><em>Paracoccus denitrifican</em></td>
</tr>
<tr>
<td>Pi</td>
<td>phosphate</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
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<tr>
<td>--------</td>
<td>------------------------------------------</td>
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<tr>
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</tr>
<tr>
<td>R. s</td>
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<tr>
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<td>spectinomycin</td>
</tr>
<tr>
<td>Sm</td>
<td>streptomycin</td>
</tr>
<tr>
<td>Tet</td>
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<tr>
<td>TMPD</td>
<td>N,N,N′,N′-tetramethyl-p-phenylenediamine</td>
</tr>
<tr>
<td>Tricine</td>
<td>N-tris[(hydroxymethyl)methyl]glycine</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>Val</td>
<td>valinomycin</td>
</tr>
<tr>
<td>τ</td>
<td>time constant</td>
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CHAPTER 1: INTRODUCTION

1.1 Energy Metabolism

Living organisms can be divided into autotrophs and heterotrophs by the way they take the carbon from the environment. Autotrophs (plants and photosynthetic bacteria) obtain the carbon from CO$_2$ in the atmosphere, whereas heterotrophs obtain carbon by the degradation of nutrients from autotrophs, not from CO$_2$ in the atmosphere. During catabolic metabolism the energy-containing nutrients such as carbohydrates, fats, and proteins are degraded into smaller, energy-depleted products such as CO$_2$, H$_2$O, and NH$_3$. The energy released by these processes is conserved in the form of ATP and reduced electron carriers (NADH, NADPH, and FADH$_2$) as shown in Figure 1.1. The rest is lost as heat. Catabolism consists of sequential metabolic pathways including glycolysis, citric acid cycle, and finally to oxidative phosphorylation to produce useful chemical energy currency, ATP (Figure 1.2).

In glycolysis, a molecule of glucose, which is the source of electrons is degraded by a series of enzyme-catalyzed reactions to produce two molecules of pyruvate and this is coupled to exergonic electron flow toward O$_2$. During glycolysis, some of the free energy is conserved in the form of ATP and NADH. The final product of glycolysis, pyruvate, is utilized in the next metabolic pathway citric acid cycle (Kreb cycle, TCA cycle). The compounds derived from the breakdown of carbohydrate, fats, and proteins are oxidized to CO$_2$ during this cycle and most of the energy from the oxidation is kept in the electron carriers, FADH$_2$ and NADH.

1.2 Oxidative phosphorylation and respiratory chain

Oxidative phosphorylation is a highly efficient energy-converting processes. This final stage of cellular respiration converges all oxidative events that produce the energy required for
living organisms in ATP. Oxydative phosphorylation involves the reduction of O₂ to H₂O with electrons donated by NADH and FADH₂. The electron flow takes place in the inner membrane of mitochondria in eukaryotes or the cytoplasmic membrane in prokaryotes. The free energy generated by electron flow in the electron transport chain is conserved in form of a pH gradient, the transmembrane electrochemical potential, which is subsequently utilizes by ATP synthase to produce ATP. ATP synthesis is based on the chemiosmotic theory, introduced by Peter Mitchell in 1961 (1), where the energy extracted from biological oxidation reactions generates a transmembrane differences in proton concentration.

The electrons pass through the respiratory chain (Fig. 1.3) which consists of a series of membrane-bound carriers. Complex I and complex II catalyze electron transfer to ubiquinone from electron donors such as NADH (complex I) and succinate (complex II). Complex III transfers electrons from ubiquinol to cytochrome c. Complex IV ends the sequence by transferring electrons from reduced cytochrome c to O₂. Complex I, III, and IV couple electron transfer with proton translocation across the membrane from the matrix to the intermembrane space. This proton pumping utilizes the energy conserved by the oxidation of NADH and succinate. For each pair of electrons transferred to O₂, four protons are pumped out by complex I, four protons by complex III, and two protons by complex IV. The vectorial equation for the processes is therefore, NADH + 11H⁺ + 1/2O₂ -> NAD⁺ + 10H⁺ + H₂O.

The energy stored in such a gradient is called proton-motive force and consist of two different potential energies. The chemical potential energy due to the different concentration of H⁺ in the matrix and intermembrane space separated by the membrane, and the electrical potential energy due to the separation of charge when proton moves across the membrane without a counterion.
The electrochemical energy in the proton gradient finally drives the production of energy currency of living organisms, ATP by ATPase.

1.3 Heme-copper oxidase superfamily

The terminal heme-copper oxidase superfamily is defined by the presence of subunit I, which contains the catalytic center, composed of the oxygen-binding heme and Cu\textsubscript{B}. Subunit I also has low-spin heme ligated to two histidines, which facilitates the transfer of electrons from Cu\textsubscript{A} to catalytic center (2).

The three types of hemes, heme B, heme O, and heme A in subunit I of the heme-copper oxidases are shown in Figure 1.4. Heme O differs from heme B by the addition of a hydroxyethylfarnesyl side chain. Heme A has a formyl group instead of methyl group which is presented in heme B and heme O. Mitochondrial oxidase has two hemes A and referred to as \textit{aaa\textsubscript{3}}-type cytochrome oxidase. Heme \textit{a\textsubscript{3}}, the high-spin heme is coordinated to a single histidine and has oxygen-binding site. Whereas heme \textit{a} is coordinated with two histidines and is called low-spin heme. The variation in heme-copper oxygen reductases is due the differences in the active site and proton channels. The A- and B-families need either an \textit{o} or \textit{a} heme in the active site (the hydroxyl group on the tail is part of the K-channel), whereas the C-family utilizes \textit{b} heme in the active site (they use a conserved tyrosine in place of the tail).

1.4 Cytochrome \textit{c} oxidase in \textit{Rhodobacter sphaeroides}

\textit{Rhodobacter sphaeroides} can be grown aerobically, anaerobically, or photosynthetically and has three distinct respiratory oxidases as shown in Figure 1.5. The genome encodes two cytochrome \textit{c} oxidases and a heme-copper quinol oxidase. Under high O\textsubscript{2} tension the \textit{aaa\textsubscript{3}}-type
cytochrome $c$ oxidase predominates, whereas the $cbb_3$-type oxidase is present when grown under microaerophilic conditions and as well as under photosynthetic conditions. The quinol oxidase exists in aerobic condition.

The mammalian cytochrome $c$ oxidase has 13 subunits. Subunit I, II, and III are encoded within the mitochondrial genome and the rest of subunits are encoded in the nuclear genome (3), whereas in bacterial system CcO exists as a simpler form of four subunits, but still transfers electrons and pump protons as the mitochondrial CcO does. Comparison of the mitochondrial and bacterial complexes suggests that the three subunits are critical for function of oxidases. The simpler structure of bacterial oxidases made it convenient to manipulate the oxidases for many studies.

The X-ray crystal structure of bovine cytochrome $c$ oxidases has been resolved to 1.8 Å (4-7) and bacterial $aa_3$-type oxidases from Paracoccus denitrificans (8, 9) and Rhodobacter sphaeroides have good crystal structures. Additional structure was resolved in the two subunit version of R. sphaeroides which presented bound lipids, detergent, Cd$^+$, and water in the enzyme (10-12). Based on the structures, there are two proton-uptake pathways leading from negative side of membrane toward the buried heme $a_3$-Cu$_B$ catalytic site site. There are four redox-related cofactors. Subunit II contains two Cu ions and subunit I contains heme $a$, heme $a_3$ and Cu ion (Cu$_B$). Heme $a_3$ and Cu$_B$ form the catalytic center where the oxygen molecule binds and is reduced. Electrons are transferred from reduced cytochrome $c$ and sequentially transfer to the Cu$_A$ center, heme $a$, the heme $a_3$-Cu$_B$ center, and finally to O$_2$ as shown in Figure 1. 6. For every four electrons transferred from reduced cytochrome $c$ four substrate protons are transferred to the active site for oxygen reduction and another four protons are pumped across the
membrane. The overall reaction catalyzed by Complex IV is 4 Cyt c (reduced) + 8H_N^+ + O_2 -> 4 Cyt c (oxidized) + 4H_P^+ + 2H_2O

1.5 Proton pathways of aa_3-type oxidases

Based on the X-ray crystal structures and mutagenesis studies, two proton entry pathways have been resolved. D pathway and K pathway, named after the conserved residue aspartic acid and lysine residues in each channel, respectively. For the proton exit pathways, an H pathway was proposed in the bovine oxidase (13, 14), but the pathway could not be accepted universally for both eukaryotes and prokaryotes (15, 16).

The D pathway is known to transfer both substrate protons for oxygen reduction chemistry and pumped protons. In D pathway, there are 10 hydrogen-bonded water molecules aligned from the entry point of the pathway (D132) facing the negative side (cytoplasmic side) of the membrane to E286, located 26 Å away from D132 and 10 Å from the binuclear center. E286 is highly conserved among the oxidases and has functional importance as a branch point of substrate protons and pumped protons. Between E286 and the active site, there is hydrophobic cavity with no crystallographic water molecules (17-20, 22-25). For proton transfer to the binuclear center, putative water molecules have been suggested for transient connection between E286 and binuclear center (3, 21, 22). Mutagenesis studies showed that replacing E286 by either alanine or glutamine inactivated the enzyme in *Rhodobacter sphaeroides* cytochrome c oxidase.

The other major proton entry pathway is called the K pathway, named after the highly conserved lysine 362. It is suggested that E101 in subunit II is the entry point for proton transfer in the K pathway (23). When K362 was substituted by methionine, the binuclear center was not able to be reduced implying that the proton required for the O to R transition is supplied by K pathway (24).
Only two waters in the K pathway were resolved in the X-ray crystal structure and complete connectivity of the proton along the K pathway is not clear, however computational studies have suggested positions for the waters in K pathway (25).

The exit pathway is presumed to be located above the two hemes where there is large number of water molecules but it is not known. Two highly conserved arginines, R481 and R482, are located just above the two hemes and have a hydrogen-bonding networks with the propionates of the hemes. Mutagenesis studies on R481 and R482 showed that these residues themselves are not directly acting as a proton-loading site for pumped protons (45). The R481/R482/heme D ring propionates carboxylate cluster and heme propionates themselves were suggested to act as an acceptor for pumped protons (26-30). In addition, electrostatic calculation proposed that one of the CuB ligand, H334, could be the proton loading site for the exit pathway (31).

1.6 Catalytic cycle

During turnover, four electrons are utilized to reduce O_2 to H_2O followed by the sequential transfer from reduced cytochrome c to Cu_A, heme a, and finally to catalytic site (heme a-Cu_B). Each of the transitions between the intermediates accompanies proton and electron transfer to catalytic site and proton pumping, typically in the microsecond time scale. Characterizing the details of each intermediate is important to understand the proton pumping mechanism. The catalytic cycle and the nature of intermediates have been defined by a series of time-resolved spectroscopic methods (32-38). The flow-flash technique has been frequently used. In the flow-flash method, the oxidase is first fully reduced and a CO ligand is bound to ferrous heme a_3, at the site where O_2 binds at the catalytic site. Then, the anaerobic-CO solution
is mixed with an O₂-containing solution. Upon a few nanosecond laser flash the CO ligand is removed, producing O₂ bound reduced enzyme, which is linked to oxygen reduction.

The catalytic cycle (Figure 1.5) initiates with fully oxidized enzyme, O state, where Cu_B in the catalytic site has high electron affinity and produces the one-electron reduced enzyme by forming the E state. During this step, one electron is taken up along with 2 protons from the negative side (N-side). Next, the E state to R state transition translocates a second electron to the binuclear center and two protons are taken up as well.

The reduced catalytic center now binds an O₂ molecule in the ferrous form of heme a₃ forming the A state in a time scale of 10 µs. After the A state either P_M or P_R states can be formed based on the availability of a third-electron. P_M state is formed (300 µs) when there is no electron available. The third electron is transferred from an external electron donor to the catalytic site. The electron is coupled to proton transfer. The O-O bond is broken in the P_M state and the active tyrosine is thought to form a neutral radical (41). This step is not coupled to proton pumping. The P_R state is formed by rapidly accepting the third electron from heme a with the time constant of 30-50 µs, which is faster than that of the proton transfer to the catalytic site (100 µs) and forms F state. The final transition, F state to fully oxidized O state takes a proton from bulk solution and pumps one proton in time scale of 1.2 ms (Figure 1.7) (39, 40).

1.7 Proton pumping

The proton motive force built by CcO is a utilized universally and coupled with energy-generating processes in eukaryotes and prokaryotes. There are two basic ways to couple redox reactions to generate proton motive force: “direct coupling”, also called “oxidoreduction loop”, in which the proton carrier is involved in proton translocation in both oxidation and reduction (1).
The other way is “indirect coupling”, in which exergonic reaction supports the endergonic proton translocation across the membrane. Cytochrome c oxidase is a redox-driven proton pump. In P. denitrificans and R. sphaeroides, they pump 1 proton per electron. The oxidases separate charges across the membrane. 8 protons cross the membrane per O\textsubscript{2} and one proton is pumped for each electron used in oxygen reduction chemistry. Overall reaction is as follows: 4 Cyt c (reduced) + 8H\textsubscript{N}\textsuperscript{+} + O\textsubscript{2} -> 4 Cyt c (oxidized) + 4H\textsubscript{P}\textsuperscript{+} + 2H\textsubscript{2}O.

The proton pumping mechanism proposed by Brzezinski suggested that the pumped protons are not necessarily synchronized with the electron transfer from heme \textit{a} to the catalytic site (42). When electrons and protons are transfer to the catalytic site, E286 becomes deprotonated and this lead to local structural change, involving M107 and W172. The distance between the D-propionate of heme \textit{a\textsubscript{3}} and R481 increases and results in water movement between the two groups. This structural change also affects the pK\textsubscript{a} of heme propionate and reprotonates E286, the structure then relaxes and pumped proton get released to positive side (P-side) of the membrane.

Proton pumping requires a gating mechanism that can guide proton input and output directions. Siegbahn and Blomberg suggested that there are kinetic barrier to control the proton translocation directed by the charge distribution in different steps of catalytic cycle (43). Wikstrom’s recent model points out pumped protons are delivered from N-side of the membrane to stabilize the electron delivered to the active site and upon proton transfer to active site proton at the pump site is pumped to P-side of membrane due to electrostatic repulsion. As for the directionality of pumped protons, E286 is suggested to act as a gate and prevent backflow of the proton from the pump site to the negative side of the membrane through D pathway (44).
1.8 Scope of thesis

This thesis presents the study of proton translocation in the $aa_3$-type cytochrome $c$ oxidase of *Rhodobacter sphaeroides*. *R. sphaeroides* has the advantage of a simpler structure for genetic manipulation compared to that of the more complicated mammalian cytochrome $c$ oxidase. In chapter 2, site-directed mutations were introduced at the highly conserved R481 residue to find clues for the beginning point of the putative exit pathway. For the characterization of R481 mutant oxidases, steady-state kinetics, proton pumping ability were measured. This part of work is published in Biochemistry, 2009, 48(30): 7123-31. In chapter 3, Resonance Raman spectra were measured to observe the environmental changes of hemes in the R481 mutant oxidases, which resulted in affected oxidation kinetics and shifted UV-vis spectra. This work is published in Biochim. Biophys. Acta., 2009, 1787(10):1272-5. In addition, the vibrational modes of propionates were identified by applying the Resonance Raman spectroscopy and this work is published in Anal. Biochem., 2009, 394(1):141-3. In chapter 4, conserved serine residues in D pathway were targeted for the site-directed mutagenesis in order to study the proton translocation pattern and its relation to the hydrogen-bonded water network with the serines. In chapter 5, several mutations were introduced and characterized in putative exit pathway based on their electrostatic calculation and simulation results. The published studies in this thesis were reproduced with permission from the journals.
1.9 Figures

Figure 1.1 Catabolism release energy in the form of ATP and reduced electron carriers such as NADH, NADPH, FADH$_2$. 
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Figure 1.4 Three types of hemes, heme B, heme O, and heme A.
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Figure 1.6 Overall structure of *Rhodobacter sphaeroides* with all four subunits and two core subunit I and II showing cofactors, electron and proton pathways.
Figure 1.7 The catalytic cycle.
1.10 References


CHAPTER 2: PROPERTIES OF ARG481 MUTANTS OF THE AA₃-TYPE CYTOCHROME C OXIDASE FROM RHODOBACTER SPHAEROIDES SUGGEST THAT NEITHER R481 NOR THE NEARBY D-PROPIONATE OF HEME A₃ IS LIKELY TO BE THE PROTON LOADING SITE OF THE PROTON PUMP

2.1 Introduction

Cytochrome c oxidase is the terminal enzyme of the aerobic respiratory chains of most prokaryotes as well as all eukaryotic mitochondria. The enzyme couples the chemistry of reduction of O₂ (to 2 H₂O) to proton translocation across the membrane, generating a transmembrane electrochemical potential (1-10). The proton electrochemical gradient produced is then used to drive many energy-requiring processes, including the synthesis of ATP by the ATP synthase. A number of substantial questions about the mechanism of the proton pump remain to be answered, including identifying the exit pathway(s) of pumped protons and the site or sites, which must bind and release protons during the catalytic cycle. Of particular interest in the current work is the potential protonation site formed by the ion pair between a highly conserved arginine, R481 (in the Rhodobacter sphaeroides oxidase) and the D-propionate of heme a₃. This arginine/propionate ion pair has been suggested to play critical roles in the exit pathways for both pumped protons and water (11-15).

In the R. sphaeroides and related oxidases, all of the pumped protons are transferred from the bacterial cytoplasm through the D-channel (16-19) to a highly conserved glutamate (E286) which is near the active site (Figure 2.1). From E286, the pumped protons are transferred
to the exit channel and then to the bacterial periplasm. The question of the pathway used by pumped protons beyond E286 remains unknown, although a number of studies have addressed this question (12, 15, 20-27). The proton pump requires at least one protonatable site, which during each electron transfer, binds a proton from the N-side of the membrane and then releases it to the P-side. Several candidates have been suggested as the “proton loading site” (19, 28-35), including the A-propionate of heme $a_3$, W280, Cu$_{B}$-ligands H333 and H334, and any of a cluster of interacting residues consisting of the D-propionate of heme $a_3$, R481, R482 and W172. The candidates that are closest to E286 are the D-propionate of heme $a_3$, along with R481 and W172, which each hydrogen bond to the D-propionate of heme $a_3$. The location of these residues, just “above” E286 has focused attention on these groups, either as the proton loading site or, at least, as water binding components within the exit pathway of pumped protons.

A set of mutations of R481 has been examined in the cytochrome bo$_3$ quinol oxidase from *Escherichia coli* (12, 36). It was concluded (12) that proton pumping requires stabilization of the D-propionate of heme $o_3$ (equivalent to heme $a_3$) in the anionic state. The mutations of the *E. coli* oxidase indicate that although a positively charged side chain in the R481 position is not absolutely required for proton pumping, stabilization by hydrogen bonding of the deprotonated carboxylate of the D-propionate of heme $o_3$ is critical to pumping (12). The R481Q mutation is 70% active and pumps protons, whereas R481L (about 40% active) did not pump protons.

The main purpose of the current work is to examine the equivalent mutations of R481 in the oxidase from *R. sphaeroides* to determine whether the previous conclusions can be universally applied. The results show that neither the positive charge at position 481, nor the capacity for hydrogen bonding to the adjacent D-propionate of heme $a_3$ are absolutely required.
for proton pumping. Neither R481 nor the D-propionate of heme $a_3$ is a viable candidate as the proton loading site in the mechanism of the proton pump.

### 2.2 Materials and Methods

#### 2.2.1 Mutagenesis

The Quik-Change mutagenesis kit from Stratagene was used to introduce the mutations. PJS3-SH plasmid (37) was used as the template for the mutations and then pRK415-1 plasmid (38) was used as expression plasmid. The expression plasmid with the mutation was transferred into S-17-1 cells by electroporation. The plasmid was transferred into *R. sphaeroides* JS100 strain by conjugation. Restriction enzymes are from Invitrogen. Sequencing was performed by UIUC Biotech Center.

#### 2.2.2 Protein Purification

Cells were grown in Sistrom's minimum media with 50 µg/mL spectinomycin, 50 µg/mL streptomycin, and 1 µg/mL tetracycline at 30 °C until early stationary phase.

His-tagged wild type and mutant enzymes were purified by histidine affinity chromatography. Cell pellets were homogenized in 50 mM potassium phosphate buffer at pH 6.5 with final concentration of 1 mM EDTA, 8 mM MgSO$_4$, DNase, and protease inhibitor cocktail. In order to break the cell, the suspended cell mixture went through a microfluidizer 5 times at 20,000 psi. Cell debris was spun down at 8000 rpm for 30 min, and the supernatant was ultracentrifuged at 40,000 rpm for at least 5 h to pellet the cell membrane. The membrane was homogenized with 50 mM potassium phosphate buffer at pH 8.0, and solubilized with 2% (final concentration) dodecyl maltoside (DM), stirring for two hours at 4 °C. The solubilized
membrane was then ultracentrifuged at 40,000 rpm for 1.5 h, and supernatant was added to the Ni-NTA resin and stirred for 2 h at 4 °C. The amount of Ni-NTA resin used was 1 mL resin per mg of cytochrome oxidase estimated to bind (approximately 1 mg per liter of growth medium). The resin with bound enzyme was loaded into the column. The column was washed until the flow-through is colorless, usually around 30 column volumes, with 50 mM potassium phosphate buffer at pH 8.0, 10 mM imidazole, and 0.1% DM. The oxidase was eluted with 50 mM potassium phosphate buffer at pH 8.0, 150 mM imidazole, and 0.1% DM. The enzyme was concentrated to about 20 mg/mL with a 50 kD-cutoff concentrator (Millipore) and the concentrated enzyme was desalted with a PD10 column (Amersham). The protein was aliquoted, fast frozen in liquid nitrogen and stored at −80 °C.

For the proton pumping assay, the enzyme was further purified by anion exchange chromatography. The concentrated protein (5 – 10 mg) eluted from the Ni-NTA column was diluted at least 10-fold to a final volume of no more than 4 mL with buffer A (10 mM potassium phosphate buffer, 1 mM EDTA, and 0.2% DM at pH 7.2) and loaded onto tandem DEAE-5PW column (Toso-HaaS) attached to FPLC system (Amersham, model ÄKTA Basic). The column was washed with buffer A and then eluted using a gradient with buffer B (buffer A plus 1 M KCl). The enzyme elutes when the gradient is about 20% Buffer B. The eluted protein was then concentrated, aliquoted, fast frozen in liquid nitrogen and stored at −80 °C.

### 2.2.3 UV-vis Spectroscopy

A Shimadzu UV-Vis-2101PC spectrophotometer was used to obtain the spectra of the enzyme (1.5 μM) in 50 mM potassium phosphate buffer at pH 8.0, 0.1% DM. The concentration of the oxidase was determined from the dithionite reduced-minus-air oxidized spectrum using
the following relationships. Concentration of oxidase (mM) = \[A_{605}(\text{red-ox}) - A_{630}(\text{red-ox})\]/24 mM\(^{-1}\) cm\(^{-1}\) or Concentration of oxidase (mM) = \[A_{606}(\text{red}) - A_{640}(\text{red})\]/40 mM\(^{-1}\) cm\(^{-1}\).

2.2.4 Steady-state Activity Measurements

The steady-state activity of the enzyme was determined by the rate of oxygen consumption, monitored polarographically using a YSI model 53 oxygen meter equipped with a water-jacketed and stirred-glass measuring vessel. The reaction chamber was filled (1.8 mL) with 50 mM potassium phosphate buffer pH 6.5, 0.1% DM, 10 mM ascorbate, 0.5 mM TMPD, and 30 µM horse heart cytochrome c. The enzyme was added to initiate the reaction.

2.2.5 Reconstitution of Oxidase into Phospholipid Vesicles

Cytochrome c oxidase vesicles (COVs) were used to measure the proton pumping stoichiometry (protons pumped per electron). Asolectin (soybean) (80 mg/mL), 2% cholic acid, and 100 mM HEPES-KOH at pH 7.4 were mixed and sonicated using a model W-375 sonicator (Heat Systems-Ultrasonics, Inc.). The solution was sonicated under a stream of Argon gas for at least 5 cycles (until the sonicated mixture becomes clear), each cycle consisting of 1 min on followed by 1.5 min off. The oxidase was added to the sonicated lipid/cholate mixture to a final concentration of 0.9 µM. The detergent was slowly removed by the addition of aliquots of Bio-Beads (Bio-Rad). The Bio-Beads (66 mg/mL) were added every 30 min for 4 h at 4 °C. After this, the sample was diluted 1.5-fold with the 100 mM HEPES-KOH buffer, pH 7.4. Additional Bio-Beads were added at room temperature: 133 mg/mL, every 30 min for 2 h and, finally, 266 mg/mL every 30 min for 1 h. The solution was then dialyzed overnight against 60 mM KCl.
These treatments result in the incorporation of the enzyme into small unilamellar vesicles. The lipid/protein ratio is selected to result in an average of less than 1 oxidase molecule per vesicle.

2.2.6 Proton-pumping Assay

Two different methods were employed to determine the stoichiometry of proton pumping, and both techniques yielded compatible results.

In the stirred-cell method, proton pumping is directly measured with a pH electrode. The stirred-cell (1.5 mL) was filled at 25 °C with the enzyme solution, containing 60 mM KCl, 40 μM horse heart cytochrome c, 10 μM valinomycin, and 0.4 μM oxidase. Most of the O₂ was removed from this solution by stirring under the stream of water-saturated argon gas. At this point, ascorbate was added to a final concentration of 300 μM. After equilibration, in which the final remnants of O₂ were removed by the oxidase, the reaction was initiated by injection of 10 μL of air-saturated water, equilibrated at 25 °C. The oxygen-saturated solution contains approximately 2.5 nmol O₂, which is promptly consumed. Protons released or consumed are recorded by the pH electrode. After each determination, the system was calibrated by adding 10 μL of anaerobic 1 mM HCl solution (10 nmol H⁺).

Each of these experiments was repeated again in the presence of protonophore, CCCP (10 μM) to equilibrate the protons on the inside and outside of COVs. In all cases, the data indicated 1 proton consumed per electron.

Proton-pumping was also measured using a pH-sensitive dye using a stopped-flow method (39) with an SX.17-MV model stopped-flow spectrophotometer from Applied Photophysics equipped with diode array detector. Proton pumping of the COVs was measured by monitoring the absorption changes of phenol red at 557 nm, the isosbestic point of reduced
and oxidized cytochrome c (39). Stopped-flow measurements were done by mixing a solution containing 60 mM KCl, 5 µM valinomycin, and 0.4 µM COVs at pH 7.4 with a solution containing 60 mM KCl, 10 µM reduced cytochrome c, and 40 µM phenol red at pH 7.4. The experiment was repeated with 10 µM CCCP added to the COV solution. The data analysis was done using SPLINE function of MATLAB (The Mathworks, Inc). Evaluation of the proton pumping is done by comparing the proton consumption determined in the presence of CCCP to the proton release, determined in the absence of CCCP, but with valinomycin present to discharge any membrane potential.

2.2.7 Stopped-flow Kinetics

The rate of reduction of the fully oxidized enzyme (reduction kinetics) was measured using the SX.17-MV model stopped-flow spectrophotometer from Applied Photophysics. The enzyme was freshly oxidized to avoid problems associated with the resting vs pulsed forms of the enzyme (40). A solution containing 50 mM Tricine at pH 8.0 and 0.1% DM with 5 µM oxidase was placed in syringe barrel at the loading position of the stopped-flow system. Argon gas was applied to remove the oxygen in the syringe barrel. 10 mM ruthenium (III) hexamine and 30 mM dithionite were added to fully reduce the enzyme. In the second syringe, air-saturated 50 mM Tricine at pH 8.0 and 0.1% DM was loaded. Upon mixing, the reaction of the reduced enzyme with O₂ is rapid, but the excess reductant present then re-reduces the enzyme. This re-reduction is monitored spectroscopically.
2.2.8 Preparation of Fully Reduced CO-bound Cytochrome c Oxidase

The buffer was exchanged to 100 mM HEPES, 0.1% DM and 50 µM EDTA at pH 7.5 using an Amicon Ultra (Millipore, Billerica, MA). The sample with a final enzyme concentration of 5-10 µM was transferred to an anaerobic cuvette and the atmosphere was exchanged to N₂ on a vacuum line. The anaerobic sample was reduced with 1-2 mM ascorbate plus 0.5-1 µM ruthenium (III) hexamine. The atmosphere was then exchanged to CO.

2.2.9 Optical Flow-flash Measurements

Fully reduced CO-bound oxidase, at a concentration of 5-10 µM in a buffer composed of 100 mM HEPES, 0.1% DM, 50 µM EDTA at pH 7.5 was mixed 1:5, in a modified stopped-flow apparatus (Applied Photophysics, Surrey, UK), with an O₂-saturated buffer of the same composition. About 200 ms after mixing the CO ligand was dissociated by an 8-ns laser flash at 532 nm (Quantel, Brilliant B) and the enzyme reaction with O₂ was monitored optically as absorbance differences at single wavelengths. Data were analyzed using the ProK software (Applied Photophysics).

2.2.10 Electrochemistry

The FTIR difference spectra were obtained using the techniques previously described (41). A 3-bounce attenuated total reflectance (ATR) attachment with a 3 mm diamond prism (SensIR now Smiths Detection) was used with a BioRad (now Varian Inc.) FTS-575C FTIR spectrophotometer equipped with a liquid-nitrogen cooled MCT detector. A thin film containing the enzyme was adhered to the surface of the diamond prism. The initial step is to remove the detergent from the purified enzyme and pellet the enzyme. 10 µL of 150 µM enzyme solution
was diluted 300-fold with water. The solution was concentrated using an Amicon 50 K membrane concentrator to a final volume of 500 μL. This dilution and concentration was repeated. The final suspension of enzyme was pelleted using a bench-top centrifuge. The pellet was re-suspended in 10 μL of water and could be stored at −80 °C.

To prepare the protein film, 6 μL of this sample was pipetted onto the ATR diamond prism and air-dried for a few minutes. This caused the protein to stick firmly to the crystal surface. The presence of residual phospholipids in the preparation appears to help stabilize the enzyme and assist in the adherence to the surface. The protein film was rehydrated by the first humidifying the air around the film until a stable FTIR spectrum is recorded. Then a 1 mL solution of the titration buffer (30 mM HEPES, 100 mM KCl, 5 mM MgCl₂, pH 7.5, in H₂O) is put on the film in order to re-wet the sample. The protein concentration is estimated to be approximately 300 μM. The sample was sealed with an acrylic lid, designed to allow the space above the film to be perfused with buffer of any composition. In this way, the redox status of the enzyme was altered, as previously described (41), to obtain the fully reduced and fully oxidized states. Upon changing the buffer composition, the state of the enzyme in the film was monitored by visible spectroscopy using a home-built apparatus with an Ocean Optics USB2000 spectrometer. The absorption spectrum in the visible was obtained by reflectance off the surface of the sample on the diamond ATR crystal. Thus, one can record the visible spectrum simultaneously with the infrared spectrum as the applied potential is changed. In general, the sample was equilibrated with a buffer by flowing the solution over the sample for about 2 h. A peristaltic pump (Cole-Parmer, Masterflex C/L) is used for the flow of the buffer. All experiments were performed at 22 °C with a flow speed of 0.33 mL/min.
In order to achieve the electrochemical titration, a potentiostat (CV-27, BAS) is connected to the flow-electrochemical cell that is mounted on the ATR unit. The flow-electrochemical cell is designed as previously described (41), with gold particles (1-2 mm) as the working electrode and platinum plated titanium electrode as the counter electrode. These two electrodes are separated by two ion-exchange membranes and a compartment that is filled with 400 mM phosphate buffer and continuously bubbled with argon gas in order to prevent any oxygen diffusion into the titration buffer. Three mediators are used to equilibrate the potential of the protein film with the titration buffer; potassium ferrocyanide (+420 mV vs NHE), ruthenium (III) hexamine (+50 mV vs NHE) and menadione (-12 mV vs NHE). An Ag/AgCl reference electrode is located on the ATR unit close to protein film.

Titrations were repeated with at least two different sample films. Each high potential step was followed by equilibration at -292 mV (vs NHE) to record a background and also reduce any oxygen that might have leaked into the sample compartment. Equilibration times for each point were about 30 min.

Since there are 4 redox-active centers in the enzyme, a quantitative fit to the data was not attempted. The results were analyzed qualitatively in order to observe whether the mutations influenced the electrochemical properties of the hemes.

2.3 Results

The following mutants were made: R481H, R481N, R481Q and R481L. Each mutant oxidase was purified to homogeneity and characterized.
2.3.1 UV-Vis Spectra

The wild type \textit{aa}_3\text{-type oxidase} from \textit{R. sphaeroides} has a characteristic absorption spectrum with a Soret band at 424 nm and an \(\alpha\) band at 600 nm in the oxidized state. Upon reduction by adding dithionite, the Soret band and \(\alpha\) band shift to 445 nm and 605 nm, respectively. The Soret band at 445 nm is contributed by both low-spin heme \(a\) and high-spin heme \(a_3\). In the \(\alpha\) band, heme \(a\) mainly contributes up to 80\% of the peak (42).

In spectra of the dithionite-reduced enzymes, both the Soret band and \(\alpha\) band are shifted for all of the mutants. In R481H, the absorption peaks are shifted to 442 nm and 603 nm, respectively and, for the rest of mutants, the two peaks are shifted slightly more, to 441 nm and 602 nm for the Soret band and \(\alpha\) band, respectively (Figure 2.2). In other words, the data indicate that the environments of heme \(a\) and heme \(a_3\) are perturbed by all of the R481 mutations.

2.3.2 Steady-state Activity

All the mutants exhibit lower steady state activity than the wild type oxidase (Table 2.1). R481H, which can maintain a salt bridge and hydrogen bond to the carboxyl of the D-propionate has 18\% of the oxidase activity. R481N, R481Q and R481L each have about 5\% of the wild type oxidase activity. These values compare to the activity of the equivalent mutations in the \textit{E. coli bo}_3\text{-type quinol oxidase} (12): R481N (~60\%), R481Q (~50\%) and R481L (~40\%).

2.3.3 Electrochemical Titrations

Electrochemical titrations of the wild type oxidase were obtained using the enzyme deposited as thin film on a diamond ATR, perfused with buffer at pH 7.5 (Figure 2.3). The
titrations were obtained by monitoring the absorbance at 602 nm and varying the electrochemical solution potential using a potentiostat. The data could be roughly viewed as exhibiting two redox steps with a high potential step at +452 mV (NHE) and a low potential step at 136 mV (NHE). FTIR difference spectra, recorded simultaneously (not shown), displayed the same two steps and confirmed that each of the two steps contains contributions from both heme $a$ and heme $a_3$.

The same two-step titration is observed with the R481L mutant, but with the midpoints shifted to 419 mV (NHE) and 78 mV (NHE). The shape of the redox titration curve for the wave at 78 mV is steeper than the equivalent redox process with the wild type oxidase, suggesting cooperativity. However, this is an artifact due to incomplete equilibration with the mutant. The data should not be considered quantitatively, therefore, but the trend is clear. The leucine substitution for arginine results in destabilizing the reduced form of the hemes, reducing the midpoint potentials by about 35 mV and 60 mV for the high and low potential steps, respectively. The shifts are in the expected direction based on removing a positive charge from the vicinity of the hemes.

### 2.3.4 Respiratory Control and Proton Pumping

To measure proton pumping of the isolated enzymes, both wild type and mutant enzymes were reconstituted in the vesicles. The extent to which the vesicles are able to maintain a proton motive force is indicated by the respiratory control ratio (RCR). This is the ratio of the steady state oxidase activity of the vesicle-reconstituted enzyme in the presence of uncoupler (uncontrolled activity) to the activity in the absence of uncoupler (controlled activity). The controlled oxidase activity is limited if a proton motive force is generated across the vesicle.
membrane, and this activity is increased upon the addition of CCCP (protonophore) and valinomycin (ionophore), which collapse the proton electrochemical gradient. Each of the mutant enzymes exhibits an RCR substantially greater than 1 (Table 2.1), indicating that each enzyme is generating a proton-motive force.

The proton pumping assay was performed using the stir-cell method with a pH-sensitive electrode and stopped-flow method (Figure 2.4) using the enzyme-reconstituted vesicles. The results of both methods are essentially the same.

Using a stopped-flow method, the pH changes in solution are monitored with a pH-sensitive dye. After mixing the solutions containing the enzyme and excess oxygen, the reaction that ensues is limited by the amount of reduced cytochrome c that is present. The data from the stopped-flow definitively show that the wild type pumps protons (Figure 2.5A) and that R481L (Figure 2.5B) also pumps protons but at a lower stoichiometry. There is no indication of proton pumping by R481Q (Figure 2.5C) or by R481N (not shown). R481H pumps protons with about the same stoichiometry as does R481L (not shown). In all cases, in the presence of the protonophore CCCP, net alkalinization was observed due to proton consumption from the reduction of oxygen to water. Note that for the wild type enzyme, the rate of alkalinization is considerably faster than the rate of proton pumping (Figure 2.5A). This reflects the fact that in the presence of the uncoupler, the enzyme specific activity increases 10-fold (see Table 2.1).

2.3.5 Stopped-flow Reduction Kinetics

To determine the extent to which the mutations in R481 alter the rate of reduction of the fully oxidized enzyme, a stopped-flow spectrophotometer was used. The fully oxidized enzyme was mixed with ruthenium (III) hexamine. The reduction rate of the hemes (not resolved into
heme $a$ and heme $a_3$) was monitored using the Soret band at 445 nm. The wild type and R481H mutants are reduced by ruthenium (III) hexamine at about equal rates ($\sim 160$ s$^{-1}$), and the greatest influence on the rate of reduction, observed for the R481Q mutant is slower by only a factor of 2 ($\sim 80$ s$^{-1}$) (Table 2.2).

**2.3.6 Flow-flash Measurement of the Oxidation of the Fully Reduced Enzyme**

To determine how the R481 mutants slow down the rate of oxidation of the fully reduced enzyme, the flow-flash assay was utilized to compare the wild type enzyme with the R481H, R481Q and R481L mutants. In this assay, the reaction is initiated by photolysis of the CO-adduct of the fully reduced enzyme in the presence of $O_2$. Since the reaction is not rate-limited by the process of mixing solutions, fast processes can be time-resolved. Earlier experiments with the wild type enzyme showed that there are 4 sequential steps (43) (Table 2.3). 1) $R \rightarrow A$, formation of the initial complex of the reduced enzyme with $O_2$; 2) $A \rightarrow P_R$, reaction splitting the $O-O$ bond to form the oxoferryl form of heme $a_3$ with the concomitant oxidation of heme $a$, but without proton transfer into the active site; 3) $P_R \rightarrow F$, proton transfer from E286 to the active site, converting $-OH$ to $H_2O$ associated with CuB (II); 4) $F \rightarrow O$, coupled electron transfer from heme $a$ and proton transfer from E286 to the active site. The two latter reactions are linked to proton pumping (44, 45). The reaction of fully reduced R481H with $O_2$ is nearly the same as the wild type oxidase, except that the $F \rightarrow O$ step is slowed by about 4-fold (Figure 2.6, Table 2.3). This correlates with the 18% steady state turnover of the R481H mutant.

The reaction of fully reduced R481Q with $O_2$ is much more perturbed (Figure 2.6, Table 2.3). The initial combination of $O_2$ with heme $a_3$ proceeds at the same rate as does the wild type, indicating that the structure of the active site and pathway for $O_2$ are not altered. The rate of the
A→P step is slowed by about 6-fold. The steps following, P→F and F→O, are not clearly resolved in part because of the spectroscopic changes due to the mutation. However, it is estimated that the rate of the P→F rate is about 100-fold slower than the wild type. The reaction to form the O state may not be complete on the 1 second time scale. These data indicated substantial perturbation, perhaps due to slow proton transfer to the active site that is required for both the P→F and F→O steps of the reaction. This mutant does not pump protons.

The most interesting mutant, R481L, displays even greater indications of perturbation (Figure 2.6, Table 2.3). The rate of binding of O₂ is about 3-fold slower, likely a perturbation to the structure of the active site or of the normal pathway used by O₂ to reach heme a₃. The rate of the A→P step is substantially slower than the wild type (25-fold), indicating a slower electron transfer rate from heme a to the active site. As with the R481Q mutant, the steps following the formation of the P state are not clearly resolved, though it is evident that there is a very large decrease in the rates of these steps. Most remarkably, despite the large perturbations evident from the R481L mutation, the mutant oxidase still pumps protons.

2.4 Discussion

The motivation for the current work was to test the importance of the ion pair of R481 and the D-propionate of heme a₃ in the mechanism of the proton pump of the heme-copper oxidases. Previous work (12) on the cytochrome bo₃ quinol oxidase from E. coli indicated that proton pumping requires stabilization of the anionic form of the carboxylate of the D-propionate of heme a₃. Most notably, with the E. coli oxidase, R481Q (60% active after isolation), which can hydrogen bond to the D-propionate can pump protons, whereas R481L (40% active after purification), which cannot hydrogen bond to the D-propionate, does not pump protons. The
most important result from the current work is that the R481L mutant oxidase from the \textit{aa}$_3$-type oxidase from \textit{R. sphaeroides} can pump protons. This result virtually rules out R481 as well as the associated D-propionate of heme \textit{a}$_3$ as being the proton loading site in the proton pump mechanism. Assuming a common mechanism, this conclusion should apply generally to all the proton pumping heme-copper oxidases.

Previously, the R481K mutant has been characterized in both the \textit{R. sphaeroides} oxidase (15, 46-48) as well as in the \textit{P. denitrificans} oxidase (49). Under most circumstances, these enzymes behave as do the wild type oxidases. The R481K mutation in the \textit{R. sphaeroides} oxidase resulted in lowering the midpoint potentials of heme \textit{a} and heme \textit{a}$_3$ by 40 mV and 15 mV, respectively. It was also deduced that the R481K mutation alters the pK$_a$ of the heme \textit{a}$_3$ D-propionate. This propionate is hydrogen bonded in the wild type oxidase by R481 and also by W172, and the protonation state of this cluster appears to modulate the rate of internal electron transfer during catalytic turnover. The D-propionate cluster (including R481 and W172) has also been suggested to be the proton acceptor for pumped protons (15). Computational studies have also indicated that either the D-propionate of heme \textit{a}$_3$ or R481 or W172 could function as the proton loading site proposed in the mechanism of the proton pump (46).

Computational approaches have also been used to examine a plausible mechanism of proton transfer from E286 to the D-propionate of heme \textit{a}$_3$ (14, 21, 25, 46). There is a hydrophobic cavity that can accommodate water molecules between the presumed proton donor (E286) and the proton acceptor. These water molecules would provide a pathway for rapid proton transfer. Furthermore, the orientation of the water molecules between E286 and the D-propionate of heme \textit{a}$_3$ has been computationally shown to depend on the charge distribution on the hemes. The water orientation within this cavity could act as a kinetic valve or gate, allowing
pumped protons to exit but not to leak backward (14). Conformational changes of W172 and/or the collapse and formation of the water chain between E286 and the D-propionate of heme $a_3$ have also been suggested as mechanisms to gate the proton pump (46). The dynamics of R481/D-propionate ion pair has been examined using molecular dynamics methods and the movement of these residues is proposed to be a key factor in the water-mediated transfer of pumped protons as well as the transport of water out of the enzyme (11). In addition, such dynamics could also regulate the pK$_a$ values of the D-propionates of both heme $a$ and heme $a_3$ (47).

The equivalent of both the R481K (49) and W172F (13) mutants (R473K and W164F) have been characterized in the oxidase from *P. denitrificans*. The R481K mutant perturbs the FTIR reduced-minus-oxidized difference spectrum, and this was used to help assign the FTIR bands of the different heme propionates. It was concluded that the D-propionate of heme $a$, but not that of heme $a_3$, is protonated upon reduction of the enzyme and, by extension, proposed to be protonated/deprotonated during the catalytic cycle (49).

The W164F mutant of the oxidase from *P. denitrificans* (equivalent to W172 in *R. sphaeroides*) retains 40% of steady state oxidase activity and has reduced proton pumping (0.5 proton/electron) (13). In single turnover (flow flash) experiments, the W164F mutation appears to result in a delay in reprotonating E286 (E278 in *P. denitrificans*) after the glutamate has donated its proton to the active site forming the P$_R$ state, thus slowing down the P$_R$→F transition. The FTIR spectra of the W164F mutant indicate perturbation of the heme propionates. In addition, the midpoint potential of heme $a_3$ is decreased by about 50 mV by the W164F mutation.
The current work confirms that R481 is important for the optimal function of cytochrome c oxidase. Even the replacement of this residue by a lysine lowers the midpoint potential of both hemes, although both oxidase activity and proton pumping remain unaltered (47). The R481H mutant results in lower oxidase activity (18%) and the stoichiometry of the proton pump is about half that of the wild type. This is somewhat similar to the phenotype reported for the W164F oxidase of *P. denitrificans*. Proton pumping is not abolished but, rather, the stoichiometry is reduced.

The less conservative mutations of the *R. sphaeroides* enzyme, examine in the current work, R481N, R481Q and R481L, all reduce the oxidase turnover to about 5% of the wild type. The reduced turnover rate is accounted for by inhibition of steps in the reaction of the reduced enzyme with O₂. For the R481H mutant, the mutation appears to selectively lower the rate of the last step in the reaction sequence, the F→O transition. Since the previous step, Pₐ→F is due to proton transfer from E286 to the active site, it is concluded that this proton transfer is not altered by the R481H mutation. Possibly, the slower F→O rate is due to a change in the electrochemical properties of the hemes, though this is pure speculation. The R481Q mutant clearly inhibits the rate of electron transfer from heme *a* to the binuclear center, measured by the A→Pₐ transition. More drastic, however, is the inhibition of steps following the formation of the Pₐ state (Pₐ→F and F→O), suggesting the proton transfer from E286 is also be influenced by the mutation. This is also observed for the W164F mutant of the oxidase from *P. denitrificans*, where the Pₐ→F step is also inhibited (49).

The most perturbed structural variant examined in the current work was the R481L mutant oxidase. Oxidase activity is similar to R481Q (about 5%), but the flow-flash single turnover study indicates that even the initial formation of the O₂ complex is delayed compared to
the wild type. This suggests some conformational alteration limiting access to the active site by 
O₂ diffusing from the external medium. Once the initial complex with the fully reduced enzyme 
and O₂ is formed, each electron and proton transfer step leading to the fully oxidized enzyme is 
strongly inhibited.

Electrochemical titrations of the wild type and R481L mutant oxidases were performed 
(Figure 2.3). Results for the wild type were qualitatively similar to results reported for the 
oxidase from *P. denitrificans*, showing two waves corresponding to the oxidation of the 
interacting hemes (41). The midpoints of the two waves were shifted lower for the R481L 
mutant by 33 mV and 58 mV, respectively. These values do not represent the midpoint 
potentials of heme *a* and heme *a₃*, since both hemes are represented in each wave, and a specific 
model would be required to obtain further detail. Furthermore, the shapes of the titration curves 
of the mutant indicate that complete equilibration was not achieved over the entire redox range. 
For the purposes of this work, the observation that the R481L mutant results in lower midpoint 
potentials of the hemes is sufficient, and is qualitatively similar to what has been reported for the 
R481K mutant of the *R. sphaeroides* oxidase (47) and the W164F mutant of the *P. denitrificans* 
oxidase (13). Each of these residues hydrogen bonds to the D-propionate of heme *a₃*.

Remarkably, despite the evidence for alterations of each step in the oxidation of the *R. sphaeroides* oxidase by the R481L mutation (Figure 2.6, Table 2.3), the enzyme still functions 
as a proton pump, albeit with about half the stoichiometry of the wild type. The slow rate of 
binding by O₂ (R→A), slow electron transfer (A→Pᵣ), slow proton transfer (Pᵣ→F) and slow 
coupled electron/proton transfer (F→O) exhibited by this mutant do not completely disable the 
proton pump. It is, therefore, difficult to imagine that the dynamics of the R481/D-propionate 
ion pair plays a critical part of the mechanism of proton pumping. Similarly, it is also very
unlikely that any member of the cluster containing R481, R482, the D-propionate of heme $a_3$, and W172 is the proton loading site, essential for the proton pump to function. Replacing R481 by a leucine should have a major influence on the pK$_a$ values of all of these residues.

It is still quite likely, and seemingly unavoidable, that pumped protons must be transferred from E286 through the region of the protein occupied by R481 and the D-propionate of heme $a_3$. In the wild type, this might well involve waters hydrogen bonded to any or all of the residues in this cluster. Such pathways must also exist in the R481L mutant. The driving force for the pumped proton, however, must be provided by formation of strong proton binding site outside of the cluster represented by R481, R482, W172 and the D-propionate of heme $a_3$. The best candidates for the proton loading site that remain are the A-propionate of heme $a_3$ and one of the histidine ligands to Cu$_B$. 


2.5 Figures and Tables

Figure 2.1 Structure of *R. sphaeroides* cytochrome *c* oxidase, defining the residues discussed.

Three redox active metal centers: heme *a* and the heme *a*$_3$-Cu$_{II}$ binuclear center are in subunit I; Cu$_A$ is in subunit II. The water molecules defining the D-channel used for the input of all the pumped protons from D132 to E286 is also shown. The figure was produced by using VMD from crystal structure 1M56 (12, 15, 20-27).
Figure 2.2 UV-visible spectra of the dithionite-reduced oxidases: wild type, R481H, R481N, R481L, and R481Q. Both the Soret band at 445 nm and α band at 605 nm are shifted in all of the mutants.
Figure 2.3 Electrochemical titrations of the *R. sphaeroides* wild type and R481L mutant oxidases. The solution potential (vs NHE) is plotted versus the absorbance at 602 nm. The lines are simply a polynomial fit and not based on any model. The estimated midpoints of the two waves are indicated. The midpoints are shifted to lower values in the R481L mutant. The steep curve for the titration of the R481L mutant is due to incomplete equilibration, so the value of the midpoint (419 mV) should be considered to be an estimate.
Figure 2.4 Proton pumping assay with the wild type and R481H, R481N, R481L, and R481Q mutant oxidases. Proton pumping was directly measured with pH-electrode. The traces on the right show that in the presence of the protonophore CCCP, rapid alkalinization is observed. $H_0^+$ denotes the pH change in the absence of CCCP, $H_C^+$ denotes the pH change in the presence of CCCP. The bar indicates $\Delta p$H of 0.001 unit.
Figure 2.5 Proton pumping determined by monitoring the absorbance change of phenol red at 557 nm using a stopped-flow spectrophotometer. An absorbance increase indicates alkalinization, which is observed in the presence of the protonophore CCCP. In the presence of valinomycin, without CCCP, the change of the phenol red absorbance is downward, indicating acidification due to proton pumping for both (A) the wild type oxidase and (B) the R481L mutant. In contrast, the acidification is not observed with (C) the R481Q mutant oxidase.
Figure 2.6 Flow-flash reaction of fully reduced wild type, R481H, R481Q and R481L mutant oxidases. Panel A: reaction is monitored at 445 nm. Panel B: reaction is monitored at 595 nm.

Experimental conditions: 5-10 µM fully reduced and CO-bound oxidase were mixed 1:5 with an oxygen saturated buffer containing 100 mM HEPES, 0.1% DM, 50 µM EDTA at pH 7.5. Traces are scaled to 1 µM of reacting enzyme.
Table 2.1
Comparison of activities of wild-type and mutant oxidases from *R. sphaeroides* before and after reconstitution.

<table>
<thead>
<tr>
<th>Oxidase</th>
<th>Activity, e⁻ s⁻¹ aa⁻¹</th>
<th>In detergent</th>
<th>In vesicles</th>
<th>RCR&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Controlled&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Uncontrolled&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1100</td>
<td>32</td>
<td>312</td>
<td>10</td>
</tr>
<tr>
<td>R481H</td>
<td>201</td>
<td>17</td>
<td>150</td>
<td>9</td>
</tr>
<tr>
<td>R481L</td>
<td>54</td>
<td>25</td>
<td>96</td>
<td>4</td>
</tr>
<tr>
<td>R481N</td>
<td>63</td>
<td>25</td>
<td>71</td>
<td>3</td>
</tr>
<tr>
<td>R481Q</td>
<td>43</td>
<td>14</td>
<td>57</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup>measured in the absence of ionophores.
<sup>b</sup>measured in the presence of the protonophore CCCP.
<sup>c</sup>the ratio of the (uncontrolled/controlled) enzyme turnover numbers.
Table 2.2
The rate constants for reduction of the wild type and mutant oxidases by ruthenium(III) hexamine.

<table>
<thead>
<tr>
<th>Oxidase</th>
<th>$k_{\text{red}}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>156</td>
</tr>
<tr>
<td>R481H</td>
<td>160</td>
</tr>
<tr>
<td>R481L</td>
<td>98</td>
</tr>
<tr>
<td>R481N</td>
<td>136</td>
</tr>
<tr>
<td>R481Q</td>
<td>82</td>
</tr>
</tbody>
</table>
**Table 2.3**

Time constants ($\tau = 1/k$) for the four steps time-resolved for the reaction of the fully reduced oxidase with $O_2$.

<table>
<thead>
<tr>
<th>Oxidase</th>
<th>Sample</th>
<th>R→A</th>
<th>A→P</th>
<th>P→F</th>
<th>F→O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau$ (μs)</td>
<td>$\tau$ (μs)</td>
<td>$\tau$ (ms)</td>
<td>$\tau$ (ms)</td>
<td></td>
</tr>
<tr>
<td>WT$^a$</td>
<td>8</td>
<td>40</td>
<td>0.1</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>R481H</td>
<td>6</td>
<td>40</td>
<td>0.1</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>R481L</td>
<td>26</td>
<td>1000</td>
<td>10$^b$</td>
<td>10$^b$</td>
<td></td>
</tr>
<tr>
<td>R481Q</td>
<td>9</td>
<td>250</td>
<td>10</td>
<td>10$^b$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Data for the wild type are from (43).

$^b$Not resolved. Assumed to correspond to the F→O transition.
2.6 References


CHAPTER 3: INVESTIGATIONS OF HEME ENVIRONMENT OF CYTOCHROME C OXIDASE FROM RHODOBACTER SPHAEROIDES USING R481 MUTANTS

3.1 Introduction

In this work, Resonance Raman spectroscopy was applied to investigate how extensively the structural properties of cytochrome c oxidases in *Rhodobacter sphaeroides* are disturbed by the R481H, R481L and R481Q mutations, and to address how the disturbed structural properties account for the perturbed functionality of the mutants by looking at the heme environment. Arginine 481 is highly conserved residue in the heme-copper oxidase families of enzymes (1-5). It is located just above the two hemes and hydrogen bonded to heme propionates. The R481 residue has been speculated to take part in proton loading site or act as a beginning point of the unknown exit pathway. The conservative mutation of R481 to Lys was introduced in *R. sphaeroides* does not affect the activity and proton pumping ability (6). Additional mutagenesis of R481 in the same system were substituted to His, Leu, and Gln resulted in the activity drop to ~18% (R481H) or 5% (R481L and R481Q) of that of the wild type oxidase. And proton pumping efficiency of both R481H and R481L decreased to ~ 40% of wild type oxidase (32). R481Q did not pump proton. The functional data confirmed the importance of R481, although the molecular mechanism underlying the perturbed functionalities, especially the remarkable partial retention of the proton pumping efficiency of the H481L mutant (in which the Leu substituent is incapable of forming H-bond with the D propionate of heme $a_3$) remains unclear. Particularly, the altered UV-Vis spectra in all of R481 mutants and decreased mid-point potential of R481L mutant (32) draw the attention to the heme environment of the mutant oxidases. In addition, structural
markers for the propionate groups were established by making the distinction between the modes from the propionates of heme $a$ and heme $a_3$, as well as those from the propionates on pyrrole rings A and D in each heme on the basis of H$_2$O-D$_2$O isotope substitution experiments combined with wavelength-selective resonance enhancement (for bovine CcO) or mutagenesis studies (for $R$. sphaeoides CcO).

The general principle of Raman scattering is when a molecule is subjected to radiation with a frequency $\nu_0$, most of the photons are scattered as elastic or Rayleigh scattering from the molecule without a frequency change ($\nu' = \nu_0$) (Fig. 3.1(a)). But a small fraction of the photons (1 in $10^7$ photons) are scattered by losing (Stokes Raman scattering) or gaining (anti-Stokes Raman scattering) a quantum of vibrational energy ($\nu' = \nu_0 \pm \nu_i$) as illustrated by the red and magenta lines in Fig. 3.1(b). The Raman shift, $\nu_i$, refers the energy of an internal vibrational mode of the molecule. The scattering event occurs in $10^{-14}$ seconds or less. The frequencies of the Stokes and anti-Stokes bands are equally displaced with respect to the frequency of the incident light (Fig. 3.2(a)). The intensities of the anti-Stokes lines are typically much weaker than those of the Stokes lines because the anti-Stokes scattering originates from molecules in excited vibrational states (e.g., $\nu = 1$ in Fig. 3.1(b)), and depending on the Boltzmann distribution, the populations of the vibrational excited states are typically very small at room temperature. Due to the weak nature of the anti-Stokes lines, only Stokes lines are considered in most applications of biological systems (7, 8).

The intensity and frequency of Resonance Raman lines are modulated by the protein environment surrounding the heme and provide useful structural information for heme protein. There are several advantages of Resonance Raman spectroscopy for studying heme-containing proteins:
(1) The water background in the Raman spectrum is very weak, which is optimal for studying biological molecules.

(2) As an extremely sensitive spectral probe for hemeproteins; Resonance Raman measurement requires only a minute amount of sample (typically 50–100 µL of 10–50 µM samples).

(3) The selectivity of the probe enhances the vibrational modes of individual chromophore in proteins and makes it very convenient in studying multiple chromophores heme proteins.

(4) Soret excitation is exquisitely sensitive to small structural changes at the heme site, typically the active center, hence the interactions between endogenous or exogenous heme ligands with the surrounding protein matrix can be explored in depth.

(5) By employing pulsed laser systems, dynamic properties of a hemeprotein can be examined. The heme group is composed of a porphyrin macrocycle with conjugated double bonds and an iron atom that is coordinated by four nitrogen atoms of the porphyrin. The $aa_3$-type of cytochrome $c$ oxidase in *Rhodobacter sphaeroides* contains two hemes $a$. The major difference between the heme $a$ and the other heme species are that the vinyl group is substituted by hydroxy farnesyl and a methyl group is oxidized to a formyl group. The carbonyl stretching modes from the formyl groups on both heme $a$ and heme $a_3$ (in the 1600–1680 cm$^{-1}$ region) are resonance-enhanced and are useful probes of the conformation near the hemes. Also, the porphyrin in-plane vibrational modes in the high-frequency region (1300–1700 cm$^{-1}$) of the Resonance Raman spectra of heme proteins are sensitive to the electron density in the porphyrin macrocycle and to the oxidation state, coordination, and spin states of axially coordinated ligands of the heme iron atom (9-12).

Other Resonance Raman lines include, the $v_2$ mode, in the region between 1550 and 1600 cm$^{-1}$, is sensitive to the iron spin state. The $v_3$ mode in the 1475–1520 cm$^{-1}$ region is
sensitive to both the axial coordination and spin state of the iron. The strong \( \nu_4 \) mode in the 1350 and 1400 cm\(^{-1} \) region is sensitive to the \( \pi \)-electron density of the porphyrin macrocycle and therefore the oxidation state of the iron. The frequency and intensity of these Raman lines are further modulated by the protein environment surrounding the heme and, therefore, provide useful structural information for hemeproteins (13).

3.2 Materials and Methods

3.2.1 Mutagenesis and purification

R481H, R481L and R481Q were constructed and purified as described in 2.2.1 and 2.2.2.

3.2.2 Raman measurements

The Resonance Raman spectra were taken at Albert Einstein College of Medicine (Bronx, New York) in collaboration with Tsuyoshi Egawa. The Resonance Raman spectra were carried out as previously described (13). Briefly, the 413.1 nm excitation from a Kr ion laser (Spectra-Physics, Mountain View, CA) was focused to a \(~30\ \mu\text{m}\) spot on the spinning quartz cell rotating at \(~1,000\ \text{rpm}\). The scattered light, collected at a right angle to the incident laser beam, was focused on the 100 \(\mu\text{m}\)-wide entrance slit of a 1.25 m Spex spectrometer equipped with a 1200 grooves/mm grating (& Lomb, Analytical Systems Division, Rochester, NY), where it was dispersed and then detected by a liquid nitrogen-cooled CCD detector (Princeton Instruments, Trenton, NJ). A holographic notch filter (Kaiser Optical Systems, Ann Arbor, MI) was used to remove the laser line. The Raman shift was calibrated with indene. The laser power was kept <4 mW for all measurements to avoid photo-damage to the protein.
3.2.3 H₂O-D₂O difference spectra measurements

To identify the propionate structural marker lines in bovine CcO (bCcO), we have obtained resonance Raman spectra of the deoxy enzyme (a^2+, a_3^2+) in H₂O versus D₂O (ca. 85 % deuterium content) with 441.6 nm excitation from a He-Cd laser. To avoid protein conformational changes induced by long exposure of the protein to D₂O (14, 15), the resonance Raman spectra were measured within an hour following the exposure of the samples to the D₂O buffer. The bCcO, isolated from bovine hearts (16), was prepared in 0.1 M Tris pH (pD) 8.5 with 0.1 % n-decyl-β-maltoside (16) and reduced by sodium dithionite in an argon atmosphere. For the Raman measurements, the bCcO samples were placed in a spinning cell; the scattered light from the samples was dispersed by a Spex 1.25 m polychromator and detected by a charge-coupled device detector (17).

3.3 Results

3.3.1 Steady-state activity

All the mutants showed lower steady state activity than the wild type oxidase. R481H, which can maintain a salt bridge and hydrogen bond to the carboxyl of the D-propionate has 18% of the oxidase activity. R481N, R481Q and R481L each have about 5% of the wild type oxidase activity (see Table 2.1).

3.3.2 Proton pumping measurement

Proton pumping measurement done by using pH probe and stopped flow apparatus both results showed that R481H and R481L pumped proton with 40% efficiency of wild type oxidase. But R481Q did not pump proton (see Figure 2.4 and 2.5).
3.3.3 UV-Vis spectra

The wild type $aa_3$-type oxidase from $R. sphaeroides$ has a characteristic absorption spectrum with a Soret band at 424 nm and an $\alpha$ band at 600 nm in the oxidized state. In spectra of the dithionite-reduced enzymes, both the Soret band and $\alpha$ band are shifted for all of the mutants. In R481H, the absorption peaks are shifted to 442 nm and 603 nm, respectively and, for the rest of mutants, the two peaks are shifted slightly more, to 441 nm and 602 nm for the Soret band and $\alpha$ band, respectively. In other words, the data indicate that the environments of heme $a$ and heme $a_3$ are perturbed by all of the R481 mutations. The Soret band at 445 nm is contributed by both low-spin heme $a$ and high-spin heme $a_3$. In the $\alpha$ band, heme $a$ mainly contributes up to 80% of the peak (18).

3.3.4 Resonance Raman Spectroscopy of Wild type oxidase

The resonance Raman spectra of the wild type enzyme were measured as references for the R481H, R481L, and R481Q mutants of $Rhodobacter sphaeroides$ CcO. The resonance Raman spectra of the wild type enzyme in fully oxidized and reduced states in the high frequency region (~1300-1800 cm$^{-1}$) are shown in figures 1a and 2a, respectively. The vibrational modes observed in this high frequency region of the spectra are sensitive to the electronic and structural properties of the hemes, such as the electron density in the porphyrin macrocycle and the spin- and coordination-states of the heme iron atoms, as well as the environment/orientation of the formyl group attached to the hemes. The assignments of these well-established vibrational modes, including the totally symmetric porphyrin skeletal vibrational modes (19, 20) and the formyl stretching modes ($\nu_{C=O}$) (21), are indicated in Figures
3.3 and Figure 3.4. The frequencies of the electron density marker line ($\nu_4$) in the oxidized and reduced spectra are at 1370 and 1357 cm$^{-1}$, respectively.

### 3.3.5 Resonance Raman Spectroscopy of R481 mutants

The oxidized forms of all the R481 mutants (R481H, R481L and R481Q) exhibit similar spectra in Figure 3.3b-d. They comprise porphyrin skeletal vibrational modes akin to those of the wild type oxidase, although the intensity ratios of the 1573 cm$^{-1}$ band (the high-spin $\nu_{2,a3}$ mode) to the 1585 cm$^{-1}$ band (the low-spin $\nu_{2,a3}$ and $\nu_{2,a}$ modes) are significantly reduced, as manifested in the difference spectra (e-g). The data indicate that the mutations induce a partial conversion of the spin-state of heme $a_3$ from high to low-spin, implying that in some population of each mutant the distal site of the heme $a_3$ iron is occupied by a stronger field ligand. However, the variations in the spin sensitive lines preclude a quantitative assessment of the degree of change. In addition to the spin-state change, differences are apparent in the 1620-1690 cm$^{-1}$ spectral region, containing the formyl vibrational modes of both heme $a$ and $a_3$. As shown by the difference spectra (e-g), the mutations introduce two new bands at ~1640 and ~1660 cm$^{-1}$ at the expense of the formyl vibrational modes of hemes $a$ and $a_3$ at ~1646 and ~1671 cm$^{-1}$, respectively, implying the perturbation in the conformations of the formyl groups.

As the spectral assignments of the formyl modes in this spectral region are complicated by the presence of the $\nu_{10}$ porphyrin skeletal vibrational mode at 1640 cm$^{-1}$, which is associated with the low-spin component of heme $a_3$ (2I-23), to analyze the difference spectra curve-fitting method was used. As shown in the right panel of Figure 3.3, the R481H-wt difference spectrum can be fitted with two positive formyl modes centered at 1653 and 1662 cm$^{-1}$, two negative formyl modes at 1649 and 1673 cm$^{-1}$, and a $\nu_{10}$ mode at 1640 cm$^{-1}$. The data demonstrated that
the mutations causes an upshift and downshift in the formyl vibrational frequencies of heme $a$ and $a_3$, respectively.

The reduced forms of all the mutants, like the oxidized forms, exhibit similar spectra (Figure 3.4b-d). As compared to the wt enzyme, the porphyrin skeletal vibrational modes are mostly unchanged, except that the relative intensity of the 1566 cm$^{-1}$ band (the high-spin $\nu_{2,a3}$ mode) is slightly reduced, indicating a partial conversion of the high-spin heme $a_3$ to a low-spin species which is most pronounced in the R481Q mutant. The difference spectra (e-g) in the 1560-1700 cm$^{-1}$ region show that the mutations introduced new broad band(s) at $\sim$1633 cm$^{-1}$ at the expense of the formyl vibrational modes of hemes $a$ and $a_3$ at $\sim$1610 and $\sim$1662 cm$^{-1}$, respectively. Curve fitting of the R481H-wt difference spectrum show that the formyl vibrational modes of hemes $a$ and $a_3$ of the wt are at 1611 and 1664 cm$^{-1}$, respectively; they shift to 1633 and 1636 cm$^{-1}$, respectively, in the mutants. The data indicate that the formyl modes of heme $a$ and heme $a_3$ are upshifted and downshifted, respectively, in the mutants, as in the case of the oxidized mutants.

### 3.3.6 H$_2$O-D$_2$O difference spectra measurements of R481H mutant

As shown in Fig. 3.5A, in the H$_2$O-D$_2$O difference spectra several spectral features are apparent in the 1100 -1400 cm$^{-1}$ region, which we assigned to the CH$_2$ bending modes of the propionate groups. It is important to note that as there are no exchangeable protons on the CH$_2$ groups, these isotope-dependent shifts are not the usual H/D isotope shifts; instead, they are a result of conformational changes originated from the alteration in the H-bonding network linked to the carboxylate groups of the propionates. To distinguish the contribution of each of the two hemes, we have measured the spectra of the CO-bound enzyme ($a^{2+}, a_3^{2+}$-CO) in H$_2$O and D$_2$O,
as with 441.6 nm excitation in the CO-bound enzyme only the modes from heme $a$ are resonance enhanced, whereas in the deoxy enzyme all the modes from both heme $a$ and $a_3$ are enhanced (24). On this basis, the modes at 1215/1251 and 1179/1233 cm$^{-1}$ are assigned to the propionate modes of heme $a$ and $a_3$, respectively, as the former modes in the deoxy and the CO-bound forms are similar, whereas the intensities of the latter modes are greatly diminished in the CO-bound form as compared to the deoxy species, as manifested in the double difference spectrum shown at the bottom of Fig. 3.5A.

In the crystallographic structure of bCcO (Fig. 3.5B), the propionate groups attached to the D-rings of hemes $a$ and $a_3$ form strong H-bonds with R438 (R481 in $R.s$ numbering). We hypothesize that the H$_2$O/D$_2$O associated changes in the CH$_2$ bending modes of the propionate groups shown in Fig. 3.5A are a result of the perturbation in this H-bonding network. To evaluate this hypothesis and to differentiate the propionate group on the D-ring from that on the A-ring, we examined the resonance Raman spectra of the wild-type (wt) and R481H mutant of *Rhodobacter sphaeroides* CcO (RsCcO). As the R481 residue in RsCcO is equivalent to the R438 in bCcO (Fig. 3.5B-C); the R481H mutation is expected to eliminate the H-bonding interaction. The wt and R481H proteins were prepared as previously described (25) and handled in the same fashion as the aforementioned protocol applied to the bCcO samples. The H$_2$O-D$_2$O difference spectrum for the wt deoxy RsCcO exhibits the same spectral pattern in the 1160-1280 cm$^{-1}$ region (Fig. 3.6A, bottom trace) as that observed in bCcO (Fig. 3.5A, trace c). Hence the propionate modes were assigned in the same manner as those in bCcO, in which the 1218/1248 and 1178/1233 cm$^{-1}$ lines are attributed to heme $a$ and $a_3$, respectively.

One of the two propionates modes from heme $a_3$, that at 1233 cm$^{-1}$, disappeared completely in the R481H mutant (Fig. 3.6B, bottom trace). It is therefore assigned to the D-ring
propionate mode of heme $a_3$, whereas the remaining line at 1178 cm$^{-1}$ is assigned to an A-ring propionate mode of heme $a_3$. On the other hand, when the deoxy spectrum of the R481H mutant (in H$_2$O) was compared with that of the wt protein, significant line broadening was evident at 1248 cm$^{-1}$ (Fig. 3.6C). As the R481H mutation is expected to only affect the D-ring propionates and the 1233 cm$^{-1}$ line has been assigned to the D-ring propionate mode of heme $a_3$, the 1248 cm$^{-1}$ is assigned to the D-ring propionate of heme $a$. It is important to note that, although in the mutant the H-bonding interactions with R481 are not present, the H$_2$O/D$_2$O-sensitivity of the 1248 cm$^{-1}$ line is plausibly a result of a new H-bonding interactions between the D-ring propionate of heme $a$ and a nearby residue, such as R482 (Fig. 3.5C). The remaining mode at 1218 cm$^{-1}$ is assigned to the A-ring propionate of the heme $a$.

**3.4 Discussion**

There are extended hydrogen-bonding networks mediated by R481, R482, the D-propionates of the two hemes, two water molecules and H334 (one of the Cu$_B$ ligands), which link heme $a$, heme $a_3$, and the Cu$_B$ center as shown in Figure 3.7. The mutations introduced in R481 (R481H, R481L and R481Q) result in the changes in their functionalities. The steady state activity of mutants decreased to ~5-18% of the wild type and the proton pumping is abolished in R481Q, but is retained at a ~40% level in both R481H and R481L (32). Moreover, the mutants showed the patterns of disturbed hemes in UV-Vis spectra with shifted bands. In order to investigate further, Resonance Raman spectroscopy was applied for the characterization of the changes in heme environment in R481 mutant oxidases. In addition, distinction of the modes from the propionates of heme a and heme a3 were made by comparing the wild type and R481H mutant oxidase on the basis of H2O-D2O isotope substitution.
The vibrational modes detected in high frequency region (~1300-1800 cm⁻¹) of spectra are sensitive to the electronic and structural properties of the hemes, such as spin-state of heme iron atoms and the environment/orientation of the formyl group of heme. And earlier Raman studies indicated that three major factors can modify the formyl vibrational mode of the a-type heme: (1) the redox state of the heme iron, (2) the electronic coupling between the formyl group and the heme macrocycle controlled by the relative orientation of the formyl group with respect to the heme plane, and (3) the hydrogen bonding interactions between the formyl group and its surrounding protein matrix (21, 26).

All of the R481 mutants (R481H, R481L, and R481Q) indicated partial conversion of the high-spin heme a₃ to low-spin species and also perturbed the formyl groups in both oxidized and reduced states. This implies that the existence of the hydrogen-bond between the R481 mutant and the propionates of two heme does affect the state of the formyl group as mentioned in the third case of the major factor above.

It is well-established that in *Rhodobacter sphaeroides* CcO, the heme a formyl group is strongly H-bonded with the surrounding protein matrix, as indicated by its low formyl νC=O frequency at 1610 cm⁻¹ (26). And this is consistent with the crystallographic data in bovine cytochrome oxidase by showing a strong H-bond between R52 and the formyl group of heme a (Figure 3.7). This movement of the hemes interrupts the H-bonding interaction between the formyl group of heme a and R52, thereby accounting for the increase in the formyl nC=O frequency to values similar to that of the isolated heme a in an aqueous environment (Table 3.1). In contrast to heme a, the formyl group of heme a₃ in the wt RsCcO is in a hydrophobic environment, as indicated by a very high formyl nC=O frequency in both oxidation states. Current Resonance Raman data indicate that the heme movement induced by the R481 mutations also
causes the exposure of the formyl group of heme $a_3$ to a new environment capable of donating stronger H-bond(s) to it, accounting for the downshift of the formyl n$_{C=O}$ frequency to that observed in an isolated heme $a$ in an aqueous solvent.

On the basis of the studies of the mammalian CcO, Yoshikawa and coworkers proposed that the H-bonding interaction between the formyl group of heme $a$, located in the H channel, and R38 (equivalent to R52 in RsCcO) is essential for proton pumping (27, 28). They hypothesize that the oxidation state of heme $a$ alters the H-bonding interaction between R38 and the formyl group of the heme $a$, thereby mediating proton translocation. The observation that the R481L mutant of RsCcO still pumps protons although the H-bonding interaction between R52 and the formyl group of heme $a$ in this mutants is disrupted, demonstrates that the proton pumping model proposed by Yoshikawa and coworkers is not operative in RsCcO and supports the concept that the mammalian and bacterial enzymes follow distinct proton pumping mechanisms.

In addition to the movement of the heme $a$ and $a_3$, as reported by the formyl n$_{C=O}$ modes, the Raman data show that the mutations also induce partial conversion of the high-spin heme $a_3$ to a low spin species. This suggests that the induced movement of the heme $a$ and $a_3$ is associated the rearrangement of the Cu$_B$ center (due to the disruption of the H-bonding network involving H334 shown in Fig.), possibly leading to the dissociation of one of its histidine ligands and the subsequent coordination of the ligand to the heme $a_3$ iron or leading to the coordination of an exogenous strong field ligand such as a hydroxide ion.

The Resonance Raman lines detected and assigned of the four propionate groups of heme $a$ and heme $a_3$ in CcO. As the propionates have been postulated to play critical roles in both the electron transfer and proton translocation events (29-31), the establishment of these well-defined
marker lines reporting the structural information of each of the four propionates of heme $a$ and heme $a_3$ forms an important basis for the future studies of the catalytic process of CcO.

3.5 Figures and Tables

![Diagram](image)

Fig. 3.1 Schematic illustration of vibrational Resonance Raman effect.
Fig. 3.2 Schematic illustration of a typical scattering spectrum containing the Rayleigh, Stokes and anti-Stokes bands (a). A typical optical absorption spectrum of heme a protein (b).
Fig. 3.3 Resonance Raman spectra of oxidized forms of the wild type and R481 mutant oxidases of RsCcO. The identities of the spectra and the associated difference spectra are as indicated. Right Panel: Lorentzian curve fitting of the difference spectrum (e); the positive and negative bands are attributed to R481H and wild type, respectively. For the fitting, the intensities, frequencies and widths were free-floating. The frequencies from the best-fit are listed in Table 3.1. The excitation wavelength was 413.1 nm.
Fig. 3.4 Resonance Raman spectra of reduced forms of the wild type and R481 mutant oxidases of RsCcO. The identities of the spectra and the associated difference spectra are as indicated.

Right Panel: Lorentzian curve fitting of the difference spectrum (e); the positive and negative bands are attributed to R481H and wt, respectively. For the fitting, the intensities, frequencies and widths were free-floating. The frequencies from the best-fit are listed in Table 3.1. The excitation wavelength was 413.1 nm.
Fig. 3.5 Resonance Raman spectra of deoxy and CO-bound forms of bovine CcO (bCcO) in H₂O and D₂O with 441.6 nm excitation (A) and the X-ray crystallographic structures of the hemes α and α₃ of bCcO (B) and *Rhodobacter sphaeroides* CcO (RsCcO) (C). In panel A, the dotted and solid lines in traces a and b are those obtained in H₂O and D₂O, respectively, whereas traces c to e are the various difference spectra as indicated. All of the difference spectra are scaled up by sixfold with respect to the parent spectra. The dotted lines in panels B and C show H-bonding interactions between R438 (R481) and the propionate side chains. The PDB codes for the crystallographic data are 2OCC (bovine) and 1M56 (*R. sphaeroides*).
Fig. 3.6 Resonance Raman spectra of the wild type and R481H mutant oxidases of deoxy *R. sphaeroides* CeO (RsCcO) in H$_2$O and D$_2$O with 441.6 nm excitation. In panels A and B, the dotted and solid traces on the top are those obtained in H$_2$O and D$_2$O, respectively, whereas the bottom traces are the associated H$_2$O–D$_2$O difference spectra. In panel C, the dotted and solid traces on the top are those associated with the wt and R481H mutant, respectively, whereas the bottom trace is the associated wild type–R481H difference spectrum. The difference spectra in panels A and B are scaled up by fourfold as compared with the parent spectra.
Fig. 3.7 Extended hydrogen-bonding network in the catalytic site of RsCcO (PDB: 1M56). The two hemes, their formyl groups and the critical residues are as indicated. The two red spheres represent water molecules. The figure was rendered with PyMol, DeLano Scientific LLC.
Table 3.1

The frequency shifts in the formyl modes of heme \( a \) and \( a_3 \) induced by the mutations in the R481 residue in RsCcO.

<table>
<thead>
<tr>
<th>Oxidation state</th>
<th>Heme ( a )</th>
<th>Heme ( a_3 )</th>
<th>Heme ( a ) model</th>
<th>Heme ( a ) model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced</td>
<td>1611 → 1633 (+22)</td>
<td>1664 → 1636 (−28)</td>
<td>1635</td>
<td>1644</td>
</tr>
<tr>
<td>Oxidized</td>
<td>1649 → 1653 (+5)</td>
<td>1673 → 1662 (−11)</td>
<td>1660</td>
<td>1667</td>
</tr>
</tbody>
</table>

The modes of a heme \( a \) model in aqueous and \( \text{CH}_2\text{Cl}_2 \) environment are listed as references (33).
3.6 References


CHAPTER 4: INVESTIGATIONS OF PROTON TRANSLOCATION IN D-PATHWAY IN AA3-TYPE CYTOCHROME C OXIDASE

4.1 Introduction

Crystal structures of CcOs and mutagenesis studies revealed two proton-uptake pathways in bacterial aa3-type CcOs; the D and K pathways. For each O2 that is reduced to water, the K pathway is used to transfer two protons upon initial electron transfer to the catalytic site, while the D pathway is used to transfer the remaining six protons, i.e. two protons that are used for O2 reduction and all four protons that are pumped. The D pathway starts at Asp132 (D132) at the CcO surface on the N side of the membrane and leads to Glu286 (E286), via about 10 water molecules that are resolved in the X-ray crystal structures. The E286 residue is buried within the membrane-spanning part of the protein, and from this site protons are transferred either to the catalytic site or to an acceptor of protons that are pumped, located closer to the P-side (1-8). Because the D pathway is used to transfer both substrate protons and protons that are pumped across the membrane, the mechanism of proton transfer through this pathway has attracted much interest. As indicated above, E286 has been implicated as the branching point from which the trajectories from substrate and pumped protons lead in different directions (9). In addition, the water structures around the residue and in the D pathway determine the proton-transfer kinetics and the apparent pKₐ of the residue. These water molecules are stabilized in a hydrogen-bonded network to conserved residues in the D pathway. Replacement of some of these residues, using site-directed mutagenesis, has resulted in uncoupling of proton pumping from the oxygen reduction (these mutant CcOs are referred to as "uncoupled mutants"). There are two categories of such uncoupled mutants (2, 10) (i) those in which proton transfer through the D pathway is

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slowed (11) and (ii) those in which the proton transfer kinetics is not altered or slowed only to a small degree (12-19). The first case (i), the uncoupling could be explained by their slowed proton transfer because proton pumping most likely requires rapid (compared to electron transfer and proton transfer to the catalytic site) protonation of a "pumping site". In the second case (ii) the uncoupling must be due to specific modifications of components of the proton-pumping machinery. One illustrative example of the type (ii) category is the Asn139Asp (N139D) mutant CcO in which proton transfer through the D pathway is slightly faster as in the wild-type CcO, which proton pumping is completely impaired (15). The only functional alteration of this mutant CcO was an increase in the E286 pK_a from 9.4 in the wild-type CcO to ~11 in the mutant CcO. The reason for the elevated pK_a is not known, but one possibility is a local change in the structure of water molecules in the D pathway (20, 21). The reasons for uncoupling have also been discussed based on theoretical calculations (22, 23).

Results from another study showed that replacement of Ser197, which is hydrogen bonded to water molecules close to E286 (~7 Å), by Ala did not result in any changes in function, which indicates that there is plasticity in the water coordination of the D pathway (18). However, in this case replacement of the residue by Asp resulted in a lower overall activity, uncoupling of proton pumping from O_2 reduction and a change in the pH dependence of the proton-transfer rate (18).

Collectively, these results indicate that understanding the design of the proton-pumping machinery of CcO requires understanding of the detailed interactions between amino-acid residues and water molecules within the D pathway. A region that is of particular interest in this respect is a water cluster consisting of W112, W141, W148 and W149, near two serine residues, Ser200 (S200) and Ser201 (S201). This cluster has been proposed to define a “proton trap”
region (23-25) ~7 Å from E286. Early studies of the Ser201Ala (S200A) mutantCcO showed that it displays 50 % activity compared to wild-typeCcO and that the proton-pumping stoichiometry was essentially unaffected (26). To further investigate the role of the water cluster, in this study we introduced the single and double mutations Ser200Ile (S200I), Tyr33Phe (Y33F), Ser200Val/Ser201Val (S200V/S201V) and Ser200Val/Ser201Tyr (S200V/S201Y) (Figure 4.1). Furthermore, to investigate only internal proton transfer in these mutantCcOs, they were combined with the Asp132Asn (D132N) mutation, which blocks proton uptake from solution near the N-side surface of the protein. The S200I, Y33F, S200V/S201V and S200V/S201Y structural modifications resulted in lower catalytic activities, but only in the Ser200Val/Ser201Tyr mutantCcO proton pumping was impaired. In both the S200V/S201V and S200V/S201Y mutantCcO the slowed activities could be explained by dramatically slowed proton transfer through the D pathway during O2 reduction. Furthermore, the apparent E286 pKa was elevated from 9.4 in the wild-typeCcO to >12 in the S200V/S201V mutantCcO. The results show that the structural details of the water hydrogen bonding is important for maintaining rapid proton transfer rates, however, the water structure within the D pathway is not critical for proton pumping.

4.2 Materials and Methods

4.2.1 Preparation and characterization of the mutantCcOs

See 2.2.1, 2.2.2, and 2.2.3

4.2.2 Steady-state activity measurement

See 2.2.4
4.2.3 Reconstitution of oxidase into vesicles and proton pumping assays

See 2.2.5 and 2.2.6

4.2.4 Stopped-flow oxidation and reduction kinetics

To determine the oxidation kinetics 5 μM CcO (final concentration) was solubilized in 50 mM Tricine at pH 8.0, 0.1 % DM, ~100 μg/mL catalase (from bovine liver, Sigma). The solution was saturated with Argon gas and 400 μM dithionite was added to reduce the enzyme. The reduced CcO was mixed with oxygen-saturated 50 mM Tricine pH 8.0 buffer in the stopped-flow apparatus (see above). In this experiment excess dithionite was rapidly oxidized by oxygen, and the reduced CcO was oxidized by the excess oxygen.

Reduction kinetics was measured first by oxidizing reduced CcO and then following re-reduction as a function of time in a stopped-flow apparatus. CcO at a concentration of 5μM was added to 50 mM Tricine at pH 8.0 and 0.1 % DM was loaded in one driving syringe. The buffer containing 10 mM ruthenium (III) hexamine was mixed with 30 mM dithionite and loaded into the other driving syringe. Upon mixing, the reaction of the reduced enzyme with O2 is rapid, but the excess reductant present then re-reduces the enzyme. This re-reduction is monitored spectroscopically.

4.2.5 Flow-flash kinetics - optical detection

Purified CcO at a concentration of 7-20 mM was solubilized in 100 mM HEPES (pH 7.5) or 100 mM CAPS (pH 10.0, 10.5 11.0) or 100 mM potassium phosphate (pH 12), and 0.05% DDM. Complete reduction of the enzyme was obtained by adding 2-3 mM ascorbate and 1-1.5
mM hexaammineruthenium (II) chloride in an anaerobic cuvette under N₂ atmosphere. After reduction the atmosphere was exchanged for CO, which results in formation of the fully reduced CcO-CO complex.

All optical flow flash measurements were performed using a flow-flash setup described in more detail in (43). Briefly, the reduced, CO bound CcO is rapidly mixed (1:5) with an oxygen-saturated buffer (same composition as that in which the enzyme is kept). About 300 ms after mixing the reaction was initiated by removing the blocking CO ligand by a ~10 ns laser flash at 532 nm. The oxidation of CcO was monitored by absorbance changes at various wavelengths (Figure 4.5).

Proton uptake during O₂ reduction was studied as described above, except that unbuffered solutions were used: 100 mM KCl (set to pH 7.8), 0.05% DDM, 100 mM EDTA, and 40 mM phenol red. Changes in phenol red absorbance were monitored at 560 nm.

### 4.2.6 Flow-flash kinetics - electrical detection

Membrane potential generation during reaction of the reduced CcO with O₂ was monitored as originally described in (33). The set-up used in our measurements is described in (35). Briefly, a thin Teflon film, separating two compartments, was soaked in lipids (100 mg of soybean IIS lecithin/ml of n-decane). Liposome reconstituted CcO was fused to the surface by incubation in 0.1 M MOPS, pH 7.5, and 12 mM CaCl₂. Subsequently, the compartments where carefully washed to remove the liposomes that had not fused to the measuring membrane. The solution was replaced by a mixture of equal amounts of Bis-Tris Propane, CAPS and CHES buffer (total concentration: 0.1 M, pH 7.5) and Ag/AgCl electrodes (DRIREF-2SH, World Precision Instruments) where inserted on each sides of the measuring membrane. After changing
the atmosphere, first to N\textsubscript{2} and then to CO, 50 mM glucose, 0.12 mg/ml glucose oxidase, 75 µg/mL catalase and 50 µM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) was added to both compartments. The reaction was initiated by flashing off the CO-ligand bound to the catalytic site of CcO, using a laser, 0.2 s after injecting 50 µl of O\textsubscript{2}-saturated buffer in direction towards the measuring membrane.

### 4.2.7 FTIR/Electrochemical Analysis

The spectroelectrochemical cell (44) was used for FTIR measurements in the 2000 to 1000 cm\textsuperscript{-1} spectral region equipped with calcium fluoride windows. A gold grid was used as the working electrode; it was chemically modified with, 2 mM Cysteamine for 1h and then washed with distilled water (45). The counter and reference electrodes were a Pt wire and an Ag/AgCl sat 3M KCl electrode, respectively. The working electrode was then set in the cell, and pushed against the window to form a thin layer of solution (about 10 µm). A potential step of −0.5 V to 0.5 V was applied (vs. Ag/AgCl/3 M KCl); add +0.208 V for the standard hydrogen electrode (SHE\textsuperscript{–}) potentials. The sample solutions were prepared in 50 mM Phosphate buffer, pH 8, in the presence of 0.1 % DM and KCl added to a final concentration of 100 mM. In order to accelerate the redox reactions, a mixture of 19 mediators was added at sub-stoichiometric concentration of 40 µM each to the protein solution (45). The measurements were recorded with the FTIR spectrometer (Bruker IFS 28), DTGS as detector. Scanner velocity was 10 kHz and 6 times 256 scans were averaged at a resolution of 4 cm\textsuperscript{-1}. The measurements were performed at 5\textdegree C, using a water thermostat. Electrochemically induced difference spectra were recorded and processed as previously described by Hellwig et al. (45).
4.3 Results

4.3.1 Steady State Activities

All investigated mutant CcOs displayed lower steady-state activities compared to the wild-type CcO (see Table 4.1). Removing a single hydroxyl group in S200 upon replacement of the residue by an isoleucine (S200I mutant CcO) resulted in a relatively high steady-state activity (83 % of wild-type) and Y33F had next highest steady-state activity (54 %) followed by S200I. In the S200V/S201V mutant CcO the steady state activity decreased to 37 % of that of the wild-type CcO, while introduction of a Tyr residue at position 201 (S200V/S201Y) resulted in a further decrease in the activity (11 % of the wild-type CcO).

4.3.2 Respiratory Control and Proton Pumping

To determine the proton-pumping stoichiometries, the mutant CcOs were reconstituted into lipid vesicles and, proton uptake and release were studied. We first determined the respiratory-control ratios (RCRs), defined as the ratio of uncontrolled (i.e. with the H\(^+\) and K\(^+\) ionophores (CCCP and valinomycin) added to the vesicles) and controlled (with only valinomycin added) activities to verify that the CcO-containing vesicles were tight. The mutant CcOs displayed RCRs >2 (see Table 4.1, where an RCR of >1 is indicative of an intact membrane). As observed previously (27) structural variants of CcOs carrying the Asp132Asn (D132N) mutation displayed reversed RCRs (<1) indicating that protons could be provided from the (wrong) positive side of the membrane.

Proton pumping was measured by monitoring pH changes outside of the vesicles after addition of a small amount of O\(_2\) to the anaerobic vesicle-CcO solution using a pH-sensitive electrode. With the wild-type CcO a rapid acidification of the external medium, due to proton
release, was observed. It was followed by a slow alkalization of the medium owing to proton leakage back into the vesicles. In the presence of the proton ionophore CCCP alkalinization was detected due to the consumption of protons during the oxygen reduction. This signal was used to normalize the proton-pumping stoichiometry as (for a given amount of O$_2$); the total signal corresponds to one proton consumed per electron transferred to O$_2$. The results from the proton-pumping measurements are shown in Figure 4.2 and the stoichiometries are given in Table 4.1.

The S200I mutant CcO pumped protons with almost the same stoichiometry as the wild-type CcO. The S200V/S201V double mutant CcO displayed a proton pumping efficiency of 46 % of that of the wild-type CcO while the S200V/S201Y double mutant did not pump protons. Furthermore, addition of the D132N mutation in all studied mutant CcOs resulted in impaired proton pumping (the D132N single mutant does not pump protons (11)).

The proton-pumping measurements were also repeated using a different assay based on the use of a pH-sensitive dye to monitor proton release (Figure 4.3). These studies gave the same results as those described above.

### 4.3.3 Oxidation and Reduction Kinetics

To determine the reduction kinetics, the fully oxidized enzyme was mixed with a solution containing ruthenium (III) hexamine (reducing agent) in a stopped-flow apparatus, after which absorbance changes were monitored at 445 nm. The oxidation kinetics was monitored at the same wavelength after mixing the reduced enzyme with an O$_2$-containing solution in a stopped-flow apparatus. For the S200I mutant CcO the decrease in the oxidation rate (53 % of that obtained with the wild-type CcO) was larger than that of the reduction rate (87 %). Y33F also showed decreased rate in both oxidation (10 %) and reduction (35 %). The small decrease in
these rates is consistent with the decrease in the overall activity of this mutant CcO. As seen in Table 4.2 both the reduction and oxidation rates of the S200V/S201V and S200V/S201Y double mutant CcOs were slowed. However, the oxidation rates were slowed to a much larger degree than the reduction rates indicating that the decreased overall activities of these mutant CcOs were due to slowed oxidative part of the reaction.

4.3.4 Flow-flash kinetics - optical detection

To determine rates of internal proton-transfer reactions and transitions between specific intermediate states that are formed during oxidation of the reduced CcO, we investigated this reaction using the flow-flash technique.

Before we discuss the results with the mutant CcO, we briefly summarize results from earlier studies of the wild-type CcO (Figures 4.4 and 4.5A). The PR → F transition, which involves proton uptake through the D pathway, is seen as an increase in absorbance at 580 nm with a time constant of ~100 μs at pH 7.4. It is followed by a decrease in absorbance with a time constant of ~1 ms, associated with the F → O transition, which involves electron and proton transfer to the catalytic site. The oxidation reaction is linked to proton uptake from solution with time constants coinciding with those of the PR → F and F → O transitions, and a stoichiometry of 0.7-0.8 H+ per CcO molecule taken up during each transition (28). Furthermore, changes in the protonation state within the D pathway can be indirectly detected by monitoring electron transfer from CuA to heme a (absorbance changes at 830 nm) (29). In the wild-type CcO the electron initially residing at CuA equilibrates with heme a (~50 % oxidation of CuA) during the PR → F transition. If this transition is not linked to proton uptake from solution and a negative
charge is left behind within the D pathway, this electron transfer does not take place.

With the S200V/S201V mutant CcO, at pH 7.5 the $P_R \rightarrow F$ and $F \rightarrow O$ transitions displayed time constants of 7.7 ms and 29 ms (Figure 4.5B), respectively, i.e. slowed by factors of 77 and 15, respectively, compared to the wild-type CcO. As indicated above, the $P_R \rightarrow F$ transition can in principle take place without accompanying proton uptake from solution; the proton needed to form the F state is then transferred internally from the D pathway (30, 31). As seen in Figure 4.5D, both the $P_R \rightarrow F$ and $F \rightarrow O$ transitions were linked to proton uptake from solution. Furthermore, as in the wild-type CcO, with the S200V/S201V mutant CcO the $P_R \rightarrow F$ transition was linked to electron transfer from Cu$_A$ to heme $a$ (Figure 4.5C, an increase in absorbance at 830 nm indicates oxidation of Cu$_A$), which also indicates that the reaction does not result in changes in the charge state of the D pathway (protons are transferred all the way from solution to the catalytic site during both the $P_R \rightarrow F$ and $F \rightarrow O$ transitions).

With the S200V/S201Y mutant CcO (data not shown) the $P_R \rightarrow F$ and $F \rightarrow O$ transition time constants were 14 ms (140 times slower than with the wild-type CcO) and 200 ms (200 times slower than with the wild-type CcO), respectively, i.e. both transitions were even slower than with the S200V/S201V mutant CcO.

Figure 4.6 shows the pH dependence of the $P_R \rightarrow F$ and $F \rightarrow O$ transition rates with the S200V/S201V mutant CcO. As seen in the figure, the rate of the $P_R \rightarrow F$ transition was essentially pH independent in the range 7.5 - 12, while the $F \rightarrow O$ transition rate displayed a small pH dependence, where the rate decrease by a factor of ~2 upon increase of the pH from 7.5 to 12.

Introduction of the D132N mutation (blocks proton uptake from solution) in the S200V/S201V mutant CcO (i.e. a S200V/S201V/D132N triple mutant CcO) yielded a $P_R \rightarrow F$
time constant of \(~5\) ms, i.e. similar to that observed with the S200V/S201V mutation alone. This result is consistent with earlier studies showing that the F state can be formed without proton uptake from solution if the proton needed to form the F state can be taken internally from E286 in the D pathway (30, 31). The next transition, \(F \rightarrow O\) was much slower (\(~0.2\) s) in the S200V/S201V/D132N triple than in the S200V/S201V double mutant CcO, which is consistent with earlier results with the D132N mutant CcO (30, 32).

4.3.5 Flow-flash kinetics - Electrometric measurements

Figure 4.7 shows results from measurements of voltage changes upon oxidation of the fully reduced CcO. This is the same reaction as that described above, but in this case the technique is used to detect charge movements within the CcO oriented in a membrane (33). Results from previous studies have shown that with the wild-type CcO the voltage changes reflect the \(P_{R} \rightarrow F\) and \(F \rightarrow O\) transitions and display time constants of \(~100\) µs, 0.5-1 ms/1-5 ms (the \(F \rightarrow O\) transition is biphasic), respectively (34). The observed voltage changes are attributed to charge transfer perpendicular to the membrane surface, i.e. transfer of the substrate and pumped protons, and electron transfer from Cu\(_A\) to heme \(a\). In addition, in a recent study a voltage change that occurs prior to the \(P_{R} \rightarrow F\) transition was identified (35). At neutral pH this voltage change displayed a time constant of \(~30\) µs with the \(P.\ denitrificans\) CcO (c.f. 65 µs with the \(R.\ sphaeroides\) CcO) and an amplitude of \(~15\) % of the total voltage change (35-37), and it was attributed to internal proton transfer from E286 to a loading site for pumped protons (35) or charge transfer within the K pathway as a result of electron transfer to the catalytic site (36).

With the S200V/S201V mutant CcO the first component displayed a time constant of \(~65\) µs (\(~25\) % of the total amplitude) and it is attributed to the charge transfer discussed above,
prior to the $P_R \rightarrow F$ transition. The second and third components displayed time constants similar to those of the $P_R \rightarrow F$ and $F \rightarrow O$ transitions, 6.5 ms ($\sim$40% of the total amplitude) and 40 ms ($\sim$35% of the total amplitude).

With the S200V/S201Y mutant $CcO$ the first component with a time constant of 65 µs comprised $\sim$40% of the total amplitude, i.e. a larger fraction than with the S200V/S201V mutant $CcO$. The remaining part of the trace displayed time constants of $\sim$14 ms ($\sim$13%) and $\sim$200 ms (47%).

### 4.3.6 FTIR/Electrochemical Analysis

Figure 4.8 shows the oxidized minus reduced FTIR spectra for the WT $CcO$ from *R. Sphaeroides* by comparison to those of the S200V/S201V and D132N/S200V/S201V mutant oxidases.

Small differences were observed in the vibrational modes, 1300-1550 cm$^{-1}$; The vibration mode of 1485 cm$^{-1}$ observed in the wild type doesn’t seem to be present in the D132V/S200V/S201V mutant and slightly shifted modes are evidenced for the vibrational bands at 1386 cm$^{-1}$ and 1537 cm$^{-1}$. Other differences were observed in the region of 1700 - 1750 cm$^{-1}$. 4 cm$^{-1}$ of shift in the D132N/S200V/S201V mutant enzyme at 1717 cm$^{-1}$ was observed, which corresponds to $\nu$(C=O) in wild type at 1713 cm$^{-1}$. The vibrational modes of the $\nu$(C = O) stretching mode in protonated COO$^-$ group of E286 were appeared at 1738/1746 cm$^{-1}$ and at 1734/1748 cm$^{-1}$ in D132N/S200V/S201V mutant and S200V/S201V mutant, respectively. The results imply the perturbation of the $\nu$(C = O) stretching mode in E286. The spectra of the
mutant enzymes are not identical to that of the wild type and the small shift of the trough is due to \( v(C=O) \) of the E286 in the oxidized form.

### 4.4 Discussion

The D pathway of *Rhodobacter sphaeroides* *aa*$_3$-type CcO is used to transfer protons that are pumped and substrate protons used for reduction of O$_2$ to water. As described in the Introduction section, in this pathway there is a cluster of water molecules, coordinated by hydrogen bonds, mainly to S200, S201 and Ser157. This water cluster has been suggested to act as a transient proton donor-acceptor within the pathway (23-25), being the primary proton donor to E286 after its deprotonation. In other words, proton transfer was proposed to take place in two steps; deprotonation of E286 and then reprotonation of the glutamic acid from the water cluster followed by immediate proton uptake from solution.

In the present study we set out to investigate the effect of structural alterations of the above-mentioned S200 and S201 residues on proton transfer through the D pathway and proton pumping. We removed the hydrogen bonds between the residues and water by removing the hydroxyl group(s) of the serine(s). In addition, we introduced a bulky residue at one of the serine positions. In all these structural variants the steady state activity was lower than with the wild-type CcO. Furthermore, the reduction rates of the S200V/S201V and S200V/S201Y mutant CcOs were much less affected than the oxidation rates, which is consistent with previous observations that the K pathway is used for proton transfer during reduction and the D pathway is used during oxidation of the CcO. Thus, the slowed overall activity was primarily due to slowed oxidation of the enzyme (see Table 4.2). Replacement of S200 by isoleucine maintained a high activity (~80 % of the wild-type activity) and the mutant CcO pumped protons. Because
introduction of the bulky isoleucine residue is likely to result in perturbation of the water cluster coordinated around the S200 and S201 residues, this result suggests that the water structure in this region is not critical for proton pumping. Furthermore, even though removing the hydrogen bonds to the serine residues (S200V/S201V) did affect the proton pumping stoichiometry (50 % of the wild-type value) the ability to pump protons remained in this structural variant. Most likely the waters in this region are able to relocate and adopt positions that facilitate proton transfer even without the hydrogen bonds to the nearby serine residues. One exception is the S200V/S201Y double mutant CcO, which displayed a very low activity (11 %) and did not pump protons, indicating that in this case the mutation interrupted the proton pathway.

The flow-flash data showed that for both the S200V/S201V and S200V/S201Y mutations proton transfer through the D pathway during the P_R → F and F → O transitions during oxygen reduction were significantly slowed; in the S200V/S201V mutant CcO at pH 7.5 the P_R → F and F → O transitions were slowed by factors of ~80 and ~15, respectively, while in the S200V/S201Y mutant CcO these transitions were slowed by factors of ~140 and ~200, respectively.

Upon blocking proton uptake from solution, by introduction of the D132N mutation, in the S200V/S201V mutant CcO, the P_R → F transition was ~50 times slower than with the wild-type CcO, i.e. the transition was slowed by a factor similar to that observed with the S200V/S201V mutation alone. Results from earlier studies have shown that upon blocking proton uptake from solution by introduction of the D132N mutation, the P_R → F transition takes place with about the same time constant as that observed with the wild-type CcO (~100 μs). The proton needed at the catalytic site to form the F state is then transferred internally from E286 (30, 31) and this proton transfer is the rate-determining step of the P_R → F transition (38). The results
from this study show that the same situation is observed with the S200V/S201V mutant CcO; the
time constants of F formation were ~8 ms without and ~5 ms with the D132N substitution. In
other words, the slowed $P_R \rightarrow F$ transition in the S200V/S201V mutant CcO must be due to
slower proton transfer from E286 to the catalytic site and not due to slowed proton transfer via
the area around residues S200 and S201 (which is found "below" E286, see Figure 4.1).

With both the S200V/S201V and S200V/S201Y mutant CcOs we observed voltage
changes associated with the $P_R \rightarrow F$ and $F \rightarrow O$ transitions, as well as a rapid voltage change,
prior to these transitions, with a time constant of ~65 µs. This voltage change was originally
attributed to internal proton transfer from E286 to a loading site for pumped protons (35), but
more recent results indicate that it is due to charge transfer within the K pathway as a result of
electron transfer to the catalytic site (36). The relatively larger contribution of this voltage
change in the S200V/S201V (~25 % of the total amplitude) and S200V/S201Y (~40 % of the
total amplitude) mutant CcO than in the wild-type CcO (~15 % of the total amplitude) is
consistent with a smaller pumping stoichiometry with the S200V/S201V and lack of proton
pumping by the S200V/S201Y mutant CcO. This is assuming that the 65-µs voltage change has
essentially unchanged amplitude in these structural variants and that the relative contribution of
the remaining voltage changes decreases by one unit charge (one instead of two pumped protons)
for the S200V/S201V and by two unit charges (no pumped protons) S200V/S201Y mutant CcOs.
Furthermore, the observation that this voltage change displayed essentially the same time
constant with the S200V/S201V and S200V/S201Y mutants (where proton transfer from E286 is
significantly slowed) as with the wild-type CcO is not consistent with the proposal that it is due
to proton transfer from E286 (c.f. (35)).

As outlined in the Introduction section structural modifications of residues within the
D pathway may result in uncoupling of proton pumping from oxygen reduction. Results from studies of these mutant CcOs have been important for understanding functional aspects of the proton pumping machinery. The most clear-cut example of such mutant CcOs is the N139D structural variant, which displayed the same \( (Paracoccus\ denitrificans\ CcO,\ (12)\) or even higher activity \( (Rhodobacter\ sphaeroides\ CcO,\ (13)\) as compared to the wild-type CcO. In this mutant CcO the proton-transfer rate through the D pathway was unaltered. Yet, proton pumping was completely impaired. The only detectable alteration was an increase in the E286 pK\(_a\) from 9.4 in the wild type \((38)\) to \(~11\) in the mutant CcO \((39)\). Further replacement of D132 by asparagine in the N139D mutant CcO (i.e. N139D/D132N double mutant CcO) resulted in restoration of the proton pumping activity and a decrease in the E286 pK\(_a\) to \(~9.7\) \((19)\). These results were interpreted to indicate a correlation between a change in the E286 pK\(_a\) and a change in the pumping stoichiometry. Furthermore, because in the structural replacements discussed above, addition of an extra negative charge (N139D) and removal of another negative charge (N139D/D132N double mutant CcO) correlated with changes in the E286 pK\(_a\), one could speculate that the E286 pK\(_a\) changes could be of electrostatic origin. However, the electrostatic effect was found to be a less likely explanation for the altered pK\(_a\) based on both theoretical calculations \((22)\) and more recent experimental results. These results showed that replacement of N139 by threonine also led to uncoupling of proton pumping from O\(_2\) reduction and a change in the E286 pK\(_a\) \((40)\). However, in this case the pK\(_a\) decreased to \(~7.6\). These results pointed to a structural alteration as a likely explanation for the altered pK\(_a\) and impaired pumping \((20)\). This conclusion was also supported by the recently determined structure of the N139D mutant CcO from \(P.\ denitrificans\), which showed changes in the water structure around 286 \((21)\).

The results with the S200V/S201V mutant CcO showed that also for this mutant CcO the
E286 pK\textsubscript{a} was altered. The P\textsubscript{R} → F rate was essentially pH independent while the F → O rate displayed only a very weak pH dependence, which indicates that the E286 pK\textsubscript{a} increased to a value >12. This larger change in pK\textsubscript{a} as compared to that seen e.g. with the N139D mutant CcO, is consistent with the smaller distance from S200/S201 (6-7 Å) than from N139 (~25 Å) to E286. However, in the case of the S200V/S201V mutant CcO the change in the pumping stoichiometry appears not to correlate with the change in the E286 pK\textsubscript{a}; despite the larger pK\textsubscript{a} shift for the S200V/S201V than for the N139D mutant CcO, the former still pumps protons (with a 50 % stoichiometry).

One possible explanation for this apparent discrepancy may be offered when considering that the E286 pK\textsubscript{a} is typically obtained from kinetic experiments where a reaction (P\textsubscript{R} → F) that is linked to proton transfer through the D pathway is considered. According to the model used to derive the pK\textsubscript{a} the rate of this proton-transfer reaction is determined by the protonation state of the glutamic acid (38). However, if the proton transfer also involves a structural reorientation of the E286 side chain then the measured pK\textsubscript{a} value would also reflect the equilibrium constant between the different positions of the side chain (40). In such a case the measured E286 pK\textsubscript{a} would be determined both parameters such as chemical environment of the carboxylate and also by the dynamics and orientation of the side chain. And the perturbation of the carboxylate group of E286 was observed in S200V/S201V and D132N/ S200V/S201V by ν(C=O) stretching mode in the mutant oxidases measured by FTIR/electrochemical analysis. The parameters are likely to change upon altering the water structure, but depending on the details of the change, the effect on the apparent pK\textsubscript{a} value may differ. The different mutations in the D pathway discussed above may contribute to different degrees to the changes in the chemical environment and the
dynamics. Because the pumping stoichiometry is more sensitive to changes in one of the two parameters, it must not necessarily directly correlate with the change in $pK_a$ value.
Figure 4.1 The D pathway in CcO. The pathway starts near D132 and leads to E286 via a number of water molecules that are resolved in the X-ray crystal structure.
Figure 4.2 The results of proton pumping in wild type and mutant cytochrome c oxidase vesicles (COVs). CCCP is a protonophore that equilibrates the protons across the membrane. Without CCCP (H$_6^+$) the pumped protons lead to an acidification, while with the addition of CCCP to the COVs (H$_c^+$) the net uptake of protons by CcO gives an alkalization upon initiation of the reaction. Injecting oxygen-saturated water initiates the reaction showing rapid acidification when the enzymes pump protons as in the case of mutants. The mutants with D132N in the background did not pump proton and showed rapid alkalization.
Figure 4.3 Proton pumping determined using a stopped-flow spectrophotometer by monitoring the absorbance change of phenol red at 557 nm. In the presence of the protonophore, CCCP absorbance at 557 nm increases, indicating alkalinization. In the presence of valinomycin, without CCCP, the change of the phenol red absorbance is downward, indicating acidification due to proton pumping for the wild type oxidase,
Figure 4.4 The schematic illustration of the reaction of reduced CcO with O$_2$.
Figure 4.5 The reaction of the fully reduced CcO with O₂. The reaction is initiated at time = 0. (A) Reaction of the wild-type CcO with O₂ at pH 7.4 and 10.4 at 580 nm. (B) Reaction of the S200V/S201V double mutant CcO with O₂ at 580 nm at pH 7.5 and 10.0. (C) Reaction of the S200V/S201V double mutant CytC with O₂ at 830 nm at pH 7.5 (D) Absorbance changes at 560 nm associated with changes in the protonation state of the pH dye phenol red (an increase in absorbance is due to proton uptake).
Figure 4.6 The pH dependence of the $P_R \rightarrow F$ (A) and $F \rightarrow O$ (B) transitions in the pH range 6.0 to 12.0. Experimental conditions were the same as in Figure 4.2, except different buffers were used in different pH ranges.
Figure 4.7 Voltage changes during reaction of the reduced S200V/S201V and S200V/S201Y mutant CcOs. The inset shows the first 1 ms of the measurements. Experimental conditions: 50 mM glucose, 0.12 mg/ml glucose oxidase, 75 µg/ml catalase and 50 µM TMPD in a buffer mixture of equal amounts Bis-Tris Propane, CHES and CAPS (total concentration of 0.1 M, adjusted to the pH values indicated in the figures). The CO ligand was dissociated by laser flash about 0.2 s after injection of 50 µl of an O2-saturated buffer in a direction towards the Teflon mesh.
Figure 4.8 Fully oxidized-minus-reduced FTIR differences spectra of the wild type, S200V/S201V, and D132N/S200V/S201V CcO from *R. sphaeroides.*
Table 4.1 Comparison of enzyme activities of controlled and uncontrolled state, respiratory control ratio, and proton pumping efficiency of wild-type and mutant oxidases from *R. sphaeroides*.

<table>
<thead>
<tr>
<th></th>
<th>activity, e⁻ s⁻¹ (\text{aa}^3)</th>
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<tr>
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Table 4.2 Oxidation and reduction rate constants.

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<th>Oxidation rate constant (s$^{-1}$) (%)</th>
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<th>$\text{F} \rightarrow \text{O}$ (s$^{-1}$)</th>
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<td>5</td>
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<tr>
<td>Y33F</td>
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<td>63 (10)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D132N/S200I</td>
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<tr>
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<td>5</td>
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<tr>
<td>D132N/Y33F</td>
<td>98 (35)</td>
<td>8 (1)</td>
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</table>
4.6 References


CHAPTER 5: CHARACTERIZATION OF THE MUTANTS IN THE
PUTATIVE EXIT PATHWAY

5.1 Introduction

The X-ray crystal structures of cytochrome c oxidases have been solved for several
organisms and two proton entry D and K pathways have been resolved (1-5). But the structures
do not show any clear proton exit pathway from the active site (6-9). The exit pathway is
presumed to exist above the two hemes connected to the P-side of the membrane. This region
above the hemes is the interface of subunit I and II, where numerous polar and charged residues
form hydrogen-bonded network with many water molecules.

The structural similarity in the main subunits of the cytochrome c oxidases of eukaryotic
and prokaryotic oxidases made the comparison possible in different species. In bovine heart
cytochrome c oxidase, the proton exit pathway (H pathway) was suggested which is composed of
hydrogen bonded network located across the enzyme (10-12). Residue D51 is a proton release
group in H pathway based on its conformational changes of the side chain in different redox
states. But this H pathway in eukaryotes is not conserved for the prokaryotes. Theoretical
predictions were also employed to investigate the clusters of electrostatically strongly coupled
residues participating in exit pathway. From the energy profiles for the proton translocation the
most favorable exit pathway suggested involves K227 residue in subunit II. Recently, the data
from combination of geometrical analysis of internal cavities in the enzyme and the network of
their connections using molecular dynamics simulations and free energy calculations suggested
two putative exit pathways, which are well conserved among different aa3-type oxidases. One is via highly conserved K227/D229 site and the other towards D364 and T337 (13, 14).
In this chapter, we characterized the residues such as K227(II), D229(II), and T337(I) in the putative exit pathway using site-directed mutagenesis and Resonance Raman spectroscopy. In addition, Q228(II) and Q127(II) are also included for the characterization. The targeted residues form a ring shaped gate in the middle of the pathway and Q127 is located at the end of the pathway facing the P-side of the membrane with numerous waters around them (Figure 5.1). The goal of the site-directed mutagenesis was to change the charge status of the residues in order to disturb the proton translocation in the putative proton exit pathway. Positively charged K227 was substitutes to methionine and negatively charged D229 was changed to asparagines. And the residues without charge, Q228 and Q127, positive charged lysine was introduced instead of glutamine. T337 was substituted to two positively charged residues, lysine and arginine. Both T337K and T337R mutant oxidases turned out to be inactive but the other mutants maintained steady-state activity lower than that of wild type oxidase. All the mutant oxidases pumped proton except the ones that were inactive. The results showed that the changing the charge in the residues does not uncouple proton pumping ability even though they showed decreased proton pumping efficiency. The shifted UV-Vis spectra in all the mutant oxidase and the results of Resonance Raman spectra in K227M and D229N support the changes in heme environment upon introducing mutations.

5.2 Materials and Methods

5.2.1. Mutagenesis

For the mutagenesis, Quik-Change mutagenesis kit (Stratagene) was used. pUC plasmid and pMB3073 plasmid was used as template for mutations in subunit I and subunit II, respectively. pRK415-1 plasmid (17) was used for expression. The expression plasmid
containing the mutation was transferred into S-17-1 cells by electroporation. And the plasmid was transferred into *Rhodobacter sphaeroides* JS100 (for subunit I mutations) and YZ200 strain (for subunit II mutations) by conjugation. Sequencing was performed by UIUC Biotech Center.

5.2.2 Protein purification

See 2.2.2.

5.2.3 UV/Vis Spectroscopy

See 2.2.3.

5.2.4 Steady-state activity measurement

See 2.2.4.

5.2.5 Reconstitution of oxidase into phospholipid vesicles

See 2.2.5.

5.2.6 Proton-pumping Assay

See 2.2.6.

5.2.7 Resonance Raman measurements

Resonance Raman spectra was taken at Albert Einstein College of Medicine (Bronx, New York) in collaboration with Tsuyoshi Egawa.
The samples for the Resonance Raman (RR) measurements were solved in 10 mM potassium phosphate, pH 8.0, with 40 mM potassium chloride, and 0.1% DM. Fully reduced forms were made by reducing the samples with sodium dithionite in nitrogen, while the reduced-CO forms were obtained by introducing CO to fully reduced samples. Raman scattering was excited by 413.1 nm line of a Kr\textsuperscript{+} laser (Spectra physics, BeamLock 2080) in a spinning cell, and collected into Spex 1.25 m polychromator equipped with a charge-coupled device detector (Princeton Instrument, 1100PB).

5.3 Results

5.3.1 UV/Vis Spectroscopy

Typically, the oxidized cytochrome c oxidase has Soret band at 424 nm and \(\alpha\) band at 600 nm. And upon reduction of the oxidase by adding dithionite, Soret band and \(\alpha\) band shift to 444 nm and 605 nm, respectively. The Soret band is contributed by both heme \(a\) (\(~35\%) and heme \(a_3\) (\(~65\%), whereas \(\alpha\) band is mostly due to heme \(a\) transition (\(~85\%) (23). The mutants were blue shifted in Soret band in the range of 412 nm to 420 nm when oxidized as shown in Figure 5.2. \(\alpha\) band was not much affected in both oxidized and reduced states in most of the enzymes. When we consider the contribution of two hemes for Soret band and \(\alpha\) band, the shifted spectra of mutant oxidases could be mostly due to the changes in heme \(a_3\).

5.3.2 Steady-state kinetics

The mutant oxidases showed lower steady-state activities compare to wild type oxidase. Both T337K and T337R are essentially inactive, with less than 1% activity of the wild type
oxidase. D229N has 20% of wild type activity. And the rest of mutants have around half the activity of that of wild type oxidase (Table 5.1).

5.3.3 Proton pumping ability

For the proton pumping measurement the enzymes were reconstituted in phospholipid vesicles and once the cytochrome oxidase vesicles (COVs) are formed, respiratory control ratio (RCR) was measured to check the intactness of the enzymes in membrane vesicle. Each of the mutant enzymes exhibited an RCR substantially greater than 1 (Table 5.1), indicating that each enzyme is generating a proton motive force.

The proton pumping was measured by using pH-sensitive electrode (Figure 5.3). The wild type oxidase pumps protons by showing rapid acidification of the external medium upon adding the air-saturated water. And the acidification is followed by slow alkalization due to proton leakage back into the vesicle. All of the mutants pumped protons with less than 50% efficiency of that of wild type oxidase. The proton pumping was measured again by using stopped-flow apparatus by monitoring the pH changes with a pH-sensitive dye (Figure 5.4). The results obtained using stopped-flow system are identical to those obtained by using the pH-electrode.

5.3.4 Resonance Raman spectroscopy

The oxidized form of heme $a_3$, which is at the water bound six-coordinate low-spin state (6chs) in the wild type oxidase, became a mixture of the native 6chs and five-coordinate high-spin state (5chs) forms in K227M. While it was converted almost completely to the 6chs state in D299N. The Resonance Raman (RR) band of formyl modes of heme $a$ and heme $a_3$ are at 1647 and 1673 cm$^{-1}$, respectively (Figure 5.5A). These two bands became weak in both K227M and
D229N compared to wild type oxidase (Figure 5.5B, Figure 5.5C) and a new band arose at 1651 cm\(^{-1}\) (K227M) or 1653 cm\(^{-1}\) (D229N) (Figure 5.5D, Figure 5.5E). The changes in the mutants imply the changes in the environment around the formyl groups of heme \(a\) and heme \(a_3\). But it is unclear whether the new formyl RR band is attributed by heme \(a\) or heme \(a_3\).

In the reduced state, the formyl RR bands from heme \(a\) and heme \(a_3\) are at 1612 cm\(^{-1}\) and 1664 cm\(^{-1}\), respectively (Figure 5.6A). These two peaks became weak in K227M (Figure 5.6.B) and completely disappeared in D229N, and a new band arose at 1624 cm\(^{-1}\) (Figure 5.6C, Figure 5.6D). This again means that the environment around the formyl groups of heme \(a\) and heme \(a_3\) changed upon the mutations. Besides the new formyl band, the mutant proteins showed a new \(\Delta v_2\) band at 1586 cm\(^{-1}\), which is within the frequency range of 6chs ferrous heme, suggesting that the 5chs \(a_3\) heme became the 6chs state upon the mutations (Figure 5.6B, Figure 5.6C).

The CO bound form of oxidase was also analyzed by Resonance Raman spectroscopy. This measurement is also a useful probe for studying the environment of the heme proximity. The results of CO bound forms were similar to those found for the reduced forms of mutants (Figure 5.7). The formyl RR bands from heme \(a\) and heme \(a_3\) (1612 and 1665 cm\(^{-1}\)) (Figure 5.7A) became weak in the mutants and a new band arose at 1633 cm\(^{-1}\). On the other hand, a new \(\Delta v_2\) band showed up at 1590 cm\(^{-1}\), which is within the frequency range of 6chs ferrous heme but shifted from the \(\Delta v_2\) frequency of heme \(a_3\) in wild type at 1585 cm\(^{-1}\), implying that the electronic state of the CO bound \(a_3\) heme of mutants are different from wild type oxidase (Figure 5.7B-E).

In a low frequency region, the CO form of wild type oxidase showed two \(v_{\text{Fe-CO}}\) RR bands at 519 and 491 cm\(^{-1}\), which corresponds to \(\alpha\) and \(\beta\) conformers of the CO bound state, respectively (Figure 5.8A). In the mutant proteins, the RR band of the \(\alpha\) conformer at 519 cm\(^{-1}\) weakened (K227M) (Figure 5.8B) or disappeared (D229N) (Figure 5.8C). Although the RR intensity
around 490 cm$^{-1}$ increased concomitantly in the mutants, the band peak was shifted by 4 cm$^{-1}$ from that of the β form of wild type protein at 491 cm$^{-1}$. These results indicate that the mutation caused substantial changes at the environment of heme $a_3$.

5.4 Discussion

The exit pathway has not defined by crystal structure and the mechanism of proton translocation to P-side of membrane is not understood yet. In bovine heart cytochrome $c$ oxidase, H pathway (exit pathway) was proposed based on relationship of aspartate 51 (bovine numbering) and water molecules that forms the hydrogen bond network within the channel (10, 12). And it was suggested that D51 is involved in proton pumping. But the equivalent residue of D51 in bovine system does not exist in plant and bacterial system.

Possible proton exit pathways for the bacterial systems have been proposed based on electrostatic calculations and simulations (13). Several highly conserved residues were suggested for characterization to understand the water traffic mechanism for pumped protons (14). The suggested residues include K227 and D229 in subunit II and T337 in subunit I. D229 and T337 were also included in possible water channel suggested in bovine heart cytochrome $c$ oxidase from structure resolved at 2.8 Å (15). They pointed out the arrangement of well-conserved hydrophilic residues at the interface between subunits I and II could provide a water channel to P-side with small conformation changes.

In current study, target residues are K227, Q228, D229, Q127, and T337. They are located above two hemes and at the interface of subunit I and subunit II. This region, as mentioned earlier studies, has characteristics that can facilitate the proton transfer to P-side of the membrane. The arrangement of the residues form a ring-shaped gate and many waters are
passing though it and extends to Q127, which faces the P-side of the membrane. The purpose of mutation is to disturb these waters in the region by changing the charge state of the targeted residues and characterize the mutants such as proton pumping abilities. The mutations in subunit II include K227M, Q228K, D229N, Q127K, Q127E, and Q127M. In subunit I, T337K and T337R were introduced.

The results from the functional characterization clearly showed that none of the mutant oxidase selectively impairs proton pumping ability except the ones that are inactive (T337K and T337R). T337 is the closest residue located to the boarder of subunits I and II. The substitutions of T337 to arginine and lysine with long and positive charged side chain could act unfavorably for the conformation of the enzymes and resulted in inactivity.

Even though these residues are not located in the vicinity of the hemes, their optimal functions and the state of the hemes were disturbed upon mutations as shown by shifted UV-Vis spectra and changes in heme environment measured by Resonances Raman spectroscopy. The formyl vibrational mode changes are apparent in both oxidized and reduced K227M and D229N mutant oxidases, which imply the interaction of the heme and the surrounding protein matrix has been modified. In addition, the mutant oxidases showed the new \( \nu_2 \) band at 1586 cm\(^{-1} \), which is within the frequency range of low-spin heme, suggesting that the high-spin heme \( a_3 \) became the low-spin heme upon the mutations. Lastly, the difference of electronic states represented by \( \alpha \) and \( \beta \) conformations of the CO bound \( a_3 \) heme of wild type and mutant oxidases suggested that the environment of the heme proximity is affected in K227M and D229N mutants. \( \text{CcO} \) from \( R.\ sphaeroides \) has been shown to possess two distinct Fe-CO stretching modes at 519 cm\(^{-1} \) and 491 cm\(^{-1} \), which are ascribed to \( \alpha \) and \( \beta \) conformations, respectively. The two conformations are suggested to be the major conformations at the binuclear center. Their functional significance
and the origin for the splitting have not been established but they have been attributed to changes in the distance between the iron atom of heme $a_3$ and Cu$_B$ (16-18).

Collectively, the mutations introduced in putative exit pathway were not able to impair proton pumping selectively by the charge change in a single residue. The mutant oxidases showed decreased activities and proton pumping with lower efficiency than wild type oxidase, which imply the optimal function of the enzymes have been disturbed even though they are not located near the catalytic site but 15 to 20 Å away at the interface of two subunits. The unique characteristic of this region with numerous polar and hydrophilic residues with water molecules not only created the flexibility of proton translocation to achieve protons pumping but also managed to interrupt the remote environment of hemes upon mutations. Due to the sensitivity of the area where mutants are introduced it is hard to completely exclude this area as the exit pathway but it is clear that the region has flexibility for conformational changes and it is unlikely that any of the single residue targeted act as a critical role in the pathway.
Figure 5.1 Structure of *R. sphaeroides* cytochrome *c* oxidase, defining the residues discussed for the putative exit pathway. Both heme *a* and heme *a*$_3$ are located in subunit I (represented as yellow ribbon); Cu$_A$ is in subunit II (represented as pink ribbon). The mutated residues are aligned at the interface of subunit I and subunit II surrounded by many water molecules shown in red spheres. The red arrow designates the direction of the putative exit pathway. Right side figure is the view from P-side, looking into the exit channel where the target residues are forming a shape of channel. The number next to residue indicates the located subunit. The figure is produced by VMD from crystal structure 1M56 (12).
Figure 5.2 UV-visible spectra of the oxidized oxidases of wild type oxidase and the mutants introduced. Soret bands are blue shifted in all cases of mutations. Wild type oxidase shows Soret and α band at 424 nm and 600 nm, respectively. The Soret bands in mutant oxidases are shown from 412 nm to 420 nm depending on mutations.
Figure 5.3 Proton pumping assays of wild type and mutant oxidases. Proton pumping is directly measured by a pH electrode in the stirred-cell containing 60 mM KCl, 40 µM cytochrome c, 300 µM ascorbate, 10 µM valinomycin, and 0.45 µM oxidase in total volume of 1.5 mL. Injecting 10 µL of oxygen-saturated water equilibrated at 25 °C to the sample initiates the reaction. Rapid acidification, indicating proton pumping, is detected on the traces on the left in wild type including all cases of mutations. The traces on the right show that upon the addition of the protonophore CCCP, rapid alkalinization is observed. \( \text{H}_0^+ \) is without the CCCP presence and \( \text{H}_c^+ \) is with the presence of CCCP in reaction.
Figure 5.4 Proton pumping determined by monitoring the absorbance change of phenol red at 557 nm using a stopped-flow spectrophotometer. An absorbance increase indicates alkalinization, which is observed in the presence of the protonophore CCCP. In the presence of valinomycin, without CCCP, the change of the phenol red absorbance is downward, indicating acidification due to proton pumping for both (A) the wild type oxidase (B) K227M mutant oxidase, and (C) D229N mutant oxidase.
Figure 5.5 RR spectra of oxidized forms of wild type (A), K227M (B), and D229N (C) proteins of CcO upon laser irradiation at 413.1 nm. D and E are calculated spectra made by subtracting A from B and C, respectively, for which the spectral subtractions were done so as to cancel the RR intensity of formyl $\nu_{\text{C=O}}$ mode from heme $a_3$ at 1673 cm$^{-1}$. The RR frequencies and the heme ($a/\ a_3$) and mode assignments in the left panel are those given for the wild type protein. The term 5 chs and 6 chs in the right panel denote five-coordinate high-spin and six-coordinate low-spin, respectively.
Figure 5.6 RR spectra of the reduced forms of wild type (A), K227M (B), D229N (C) oxidases upon laser irradiation at 413.1 nm. D is a calculated spectrum made by subtracting A from B so as to cancel the RR intensity of formyl $\nu_{\text{C-O}}$ mode from heme $a_3$ at 1664 cm$^{-1}$. 
Figure 5.7 RR spectra of the reduced-CO forms of wild type (A), K227M (B), D229N (C) oxidases upon laser irradiation at 413.1 nm. D and E are calculated spectra made by subtracting A from B and C, respectively, for which the spectral subtractions were done so as to cancel the RR intensity of formyl $\nu_{C=O}$ mode from heme $a_3$ at 1665 cm$^{-1}$. 
Figure 5.8 Low frequency RR spectra of the reduced-CO form of wild type (A), K227M (B), D229N (C) oxidases upon laser irradiation at 413.1 nm. The $v_{\text{Fe-CO}}$ RR bands were assigned as indicated, where $\alpha$ and $\beta$ denote the $\alpha$ and $\beta$ conformers of the CO form of wild type CcO.
Table 5.1 Comparison of activity before and after reconstitution, respiratory control ratio, and proton pumping efficiency of wild type and mutant oxidases from *R. sphaeroides*.

Activity, $e^-s^{-1}aa_3^{-1}$

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<th>Uncontrolled</th>
<th>RCR</th>
<th>$H^+/e^-$ (%)</th>
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<tr>
<td>K227M</td>
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<td>335</td>
<td>6</td>
<td>0.14 (28)</td>
</tr>
<tr>
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<td>267 (20)</td>
<td>19</td>
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5.6 References


CHAPTER 6: CONCLUSIONS

Cytochrome c oxidase is an integral membrane protein and a member of the heme-copper oxidase superfamily. CeO catalyzes the oxygen reduction chemistry as the last electron acceptor in the respiratory chain, which produces the majority of the ATP produced in aerobic organisms. Oxygen is converted to water by taking electrons from P-side and protons from the N-side of membrane. This highly exergonic reaction is coupled to proton pumping across the membrane, which forms the proton motive force across the membrane.

The electrons are donated by the reduced cytochrome c from the P-side and sequentially passed to Cu, heme a, and finally to catalytic site consist of heme a and CuB. Protons are taken by K pathway and D pathway from the N-side. Two pathways have been resolved in X-ray crystal structures. The K pathway is known to take the first substrate proton and the rest of the substrate and pumped protons are taken by D pathway. D pathway starts from D132 and extends to E286, which is suggested to be the branch point for the substrate and pumped protons. In the D pathway around 10 water molecules are aligned and facilitate proton translocation. The oxygen reduction chemistry steps, which take place in the catalytic site, are well studied but proton pumping mechanism at the molecular level or the proton exit pathway are not understood yet.

In chapter 2, the functional characteristics of the R481 mutant oxidases were described. R481 is a highly conserved residue located just above the two hemes and hydrogen bonded to heme propionates, which draws attention to this residue as a possible proton loading site. Much study has been done on R481 to investigate its role related to proton pump mechanism. Apparently, R481 itself is not a critical residue for proton pumping since there is no uncoupling
mutant that selectively impairs proton pumping found. The R481 mutations of the *E. coli* oxidase indicates that although a positively charged side chain in the R481 position is not absolutely required for proton pumping, stabilization by hydrogen bonding of the deprotonated carboxylate of the D-propionate of heme $o_2$ is critical to pumping (1). The R481Q mutation is 70% active and pumps protons, whereas R481L (about 40% active) did not pump protons. In current study the equivalent mutants were introduced in *Rhodobacter sphaeroides*. Interestingly, the mutants introduced in *R. sphaeroide* behaved differently from the *E. coli* system by showing R481L (about 5% active) pump protons whereas R481Q (about 5% active) did not pump protons. In addition, the activities of the mutant oxidases were dramatically decreased to around 5% of that of wild type oxidase. Despite R481L being slowed in all steps of oxidation kinetics, slow rate of binding by O$_2$ (R$\rightarrow$A), slow electron transfer (A$\rightarrow$P$_R$), slow proton transfer (P$_R$$\rightarrow$F) and slow coupled electron/proton transfer (F$\rightarrow$O), this mutant managed to pump protons. Therefore, it is difficult to imagine that the dynamics of the R481/D-propionate ion pair plays a critical part of the mechanism of proton pumping. In the R481L case, the midpoint potential was shifted, which supports its slowed oxidation kinetics and dramatic decrease in steady-state activity.

All of the R481 mutants showed shifted UV-Vis spectra. These results provided the motivation for the investigation of the heme environment of R481 mutants by applying Resonance Raman spectroscopy. The changes in heme environment of R481 mutants are described in chapter 3. The assignments of well-established vibrational modes, including the totally symmetric porphyrin skeletal vibrational modes (2, 3) and the formyl stretching modes ($n_{C=O}$) (4) implied that the R481 mutants have perturbed conformation of the formyl group. The spectra also showed induced partial conversion of the high-spin heme $a_3$ to a low spin species, which suggests that the induced movement of the heme $a$ and $a_3$ is associated with the rearrangement of the catalytic
center. The disturbed catalytic site could support the functional changes in the mutant oxidases. In mammalian CcO, the hydrogen bonding interaction between the formyl group of heme $a$ and R38 (equivalent to R52 in RsCcO) is suggested to be essential for proton pumping (5, 6). But in R481L mutant of RsCcO still pump protons although the H-bonding interaction between R52 and the formyl group of heme $a$ in this mutants is disrupted implying that the proton pumping model proposed by Yoshikawa and coworkers is not operative in RsCcO. Finally, Resonance Raman marker lines of four propionate groups of hemes, which is known to play critical roles in both the electron transfer and proton translocation events (7-9), are established.

In chapter 4, we tried to understand the proton-pumping machinery of CcO, which requires an understanding of the detailed interactions between amino-acid residues and water molecules within the D pathway. A region that is of particular interest in this respect is a water cluster, near two serine residues, Ser200 (S200) and Ser201 (S201). This cluster has been proposed to define a “proton trap” region (10-12) ~7 Å from E286. Early studies of the Ser201Ala (S200A) mutant CcO showed that it displays 50 % activity compared to wild-type CcO and that the proton-pumping stoichiometry was essentially unaffected (13). To further investigate the role of the water cluster, we introduced the single and double mutations Ser200Ile (S200I), Tyr33Phe (Y33F), Ser200Val/Ser201Val (S200V/S201V) and Ser200Val/Ser201Tyr (S200V/S201Y). Furthermore, to investigate only internal proton transfer in these mutant CcOs, they were combined with the Asp132Asn (D132N) mutation, which blocks proton uptake from solution near the N-side surface of the protein. The S200I, Y33F, S200V/S201V and S200V/S201Y structural modifications resulted in lower catalytic activities, but only in the Ser200Val/Ser201Tyr mutant CcO proton pumping was impaired. In both the S200V/S201V and S200V/S201Y mutant CcO the slowed activities could be explained by dramatically slowed
proton transfer through the D pathway during O₂ reduction. Furthermore, the apparent E286 pKₐ was elevated from 9.4 in the wild-type CcO to >12 in the S200V/S201V mutant CcO. Clearly, the serine residues are not critical for proton pumping ability. The structural details of the hydrogen-bonded water is important for maintaining rapid proton transfer rates, however, the water structure within the D pathway is not critical for proton pumping. The proton pumping mutant, S200V/S201V confirmed that the change in pKₐ of E286 and proton pumping are not directly correlated.

In the last chapter the functional characterization and spectroscopic analysis using Resonance Raman spectra of mutants introduced in putative exit pathway. The proton exit pathway is not resolved in X-ray crystal structure. A theoretical exit pathway was predicted based on electrostatic calculations considering the energy profile for the proton translocation. The most favorable pathways have been suggested. One includes highly conserved K227, and the other pathways with T337. In this study, T337, K227, Q228, D229, and Q127 were included for the characterization. These residues form a ring shaped channel and Q127 is located at the end of the pathway facing the P-side of the membrane with numerous waters around it. The goal of the mutation was to change the charge status of the residue in order to disturb the proton translocation or the water molecules to affect the proton pumping ability. The results showed that none of the mutants were uncoupling mutants, which implies these residues are not directly linked to the proton pumping mechanism. But by the fact that these residues are located at the interface of subunit I and II the mutant oxidases possibly caused the structural disturbance in the area, which was observed in the heme environment changes. K227M and D229N mutant oxidases showed changes in formyl vibration modes as well as the spin state of heme a₃. The mutations introduced in this region influenced the optical function of the mutants located 15-20
Away from the catalytic site but was not able to selectively stop proton pumping, which implies the complexity of the area above two hemes containing a significant amount of ordered water.

References


APPENDIX A: DNA AND PROTEIN SEQUENCES OF $\alpha A_3$-TYPE

CYTOCHROME C OXIDASE

Subunit I DNA sequences:

1  ATGGCCGACG  CAGCCATCCA  TGGCCACGAG  CACGACCGGA  GGGGTTCTT  CACCCGCTGG
61  TTCATGTCGA  CGAACCACAA  GGACATCGGC  GTTCTCTATC  TCTTCACCAG  GGGCCTCGTC
121  GGGCTGATCT  CGGCTGCTCG  GCCTGCTCGC  ATGCTACTTG  ATGAGCTTAC  GCCGACATGG
181  CAGTTCATGT  GCACCAGGAA  CAGGCGCCATC  ATTCATCAGG  GGGCCGCTGT  TGGTCTCGTG
241  GGCCACGGGA  TCCTGATGAT  GGTCTTCGTG  GTCATTCCCG  CGCTCTTCGG  CGGCTTCGGC
301  AACTATTTCA  TGCCGCTGCA  CATCGGCGCG  CCGGACATGG  CCTTCCCGG  GATGAACAC
361  CTCTCCTACT  GGCTCCTATG  CGCCGCCACC  TCGCTGCGG  TGGATCGCTG  GTCGCGGCGG
421  ACCATGCACA  AGGTACGCTG  CTTCACGCTC  GGGCGCTGC  GGGCTGCTCG  CGGCGCTATT
481  ACCACCTCTT  TCAGCCCTTC  GGGCCGGGCG  GACCCGGTGC  GCTACAGCCA  CATCTGCGG
541  TCTCTCGGCC  ACCCGGAGGT  CTACATCATC  GTGCTGCCGG  CCTTCGGCAT  CGTCAGCCAC
601  GTCATCGCGA  CCTTCCGCCA  AAGGCCGATC  TTTCGCTATT  TGCCGATGGT  CTATGCGATG
661  GGGCTGCTGG  CGGCACCATC  TTACCCGCGT  GGCATCGAGC  AGTATGACG  GCCACCCGCG
721  ACCAGCGCG  CGGACGCTG  ATCTCTGGG  CGCTCGGGTT  CCTCTTCCTC  TTCACCGTGG  CGGGCGTCAC
781  ACCAGGCGCA  AGCAGAGCTA  CTTTATGATG  GCGACCATGG  TGATCGCAGT  GCCCACCGGC
841  TTCGTGGGCG  CGAACCTCAC  CTTCTTCCCG  CAGCACTTCC  TCGGCCGCCA  GGGCATGCCG
901  GTCATCGCGA  CCTTCCGCCA  AAGGCCGATC  TTTCGCTATT  TGCCGATGGT  CTATGCGATG
961  GTGCGGATCG  CGGTCTGGTC  TTGGGCCACC  ACATGTATAC  AGCCGGGCTG  AGCAGGCGCA
1021  AGGCTCCCCA  AGGACCAGCTA  CTTTATGATG  GCGACCAGATG  TGATCGCAGT  GCCCACCCGC
1081  ATCAAGATCT  TCTCTGGGAT  CGCCGCCATC  TGAGGCCTGGT  CGATCGAGCT  CAAGACGCCG
1141  ATGCTCTGGG  CGCTCCTGTC  TTCCACCGTG  GGGCCGCTAC  GCGCATCTG  CTAAGGACCG
1201  CTAGACCGAG  CGGACCTGCG  CGCTATTAT  CACGACACCT  ACTATGCTTG  GCCGACACCT
1261  CATTATGTGA  TGAGGCCCGT  CGGGGTCTTG  GCAGTATGCC  CGGGGATCTC  CTCTTGCGATC
1321  GGCAAGATGT  CGGGCGCGCA  ATATCCGGAAG  TGGGCCGCGA  AGCTGCGATT  CTGGATGATG
1381  TTTCGCTGGC  CGCAACCTCAC  CTTCTTCGCAG  CAGCACTTCC  TCGGCGCGCA  GGCGATCGGC

144
Subunit I protein sequences:

MADAAIHGHEHDRGFFTRWFMSNTHKDIGVYLFTGGLVGLISVAFTVYMELMAPGVQFMCAEHLLESGLVKGF
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ASLFAPGGNQLGSGIGWLYPLPLSTESYSGTDLAIFAVHLSGASSILGAINMITTFNLMPAGTMHKVLF
IFVTAWLILLALPVLAGAITMLLDDRNGFQTPFSGDPVLYQLWHFFGPEYIVVLPAGIVSHVIAFTAKK
PIFGYLPMVAMVAIGVLFVVWAWHMYTAGLSLTQSYFMMATMVIAPGTGIFIKIFSMAWGISSLKLF
GFLFLFTVGGTVGTIVSLQASVDRYHDYTYYVAHFHYVMSLGAVGFGASTSSIGKMSGRQPEWAGKLF
GANLTFPPQHFLQGMRPRYIDYPFEATNWNVSSLGAFLSFASLFILGVIFVSGARVTANNYNEHADTLE
TLTSEPPEHTFQLPKREDWERAPAH

Subunit II DNA sequences:

1  ATGACATCTG TCCTTGTACCC AGATCAAATG ATGACGCTTC GAGCCATCGT TTTCCCTTGC
61  GTGCACAAAA CAATATGCTAT CTAATCGGGTT CAGGAACAC GGGAAATCTG CCCCCTTTCC
121  GGCACGCCGAA CGAGACGGCAT GATGCGCCGC CGAAGCCAAAC GGGATCTGAA CATGAGACAT
181  TCCACCCCTTC TGACCACATG GCCACCGGGG GCGCAGGGCC TTCTGGCGGC CACGGCCGGG
241  GCCCGCCGAC CGAGACGGCT CGAGATCATC GGGCGGCCGC AGCCGGGGGG CACGGGCTTC
301  CAGCCCTCGG CGAGCCCGTCG ATCCATGATT TCGACGGGTG CATCTCGTC
361  ATCATGCCTG CCATCCACCAT CCCTCGTCAG CTCCCTATCC TCTATGCGGT CTGGCGCTTC
421  CATGAGAAGC GCAACAGAGT GCCCGGCCGGC TCCACCCACA ATCCCGCGCT CGAGATCGGC
481  TGGACGATCG TCCCGATCGT CATCCTCGTG GCCATCGGGG CCCTCCTCGT GCCGGTGCTG
541  TTCAACCAGC AGGAAATCCC CGAGGCGGAC GTGACGGTGA AGGTCACGGG CTACCAGTGG
601  TACGTGGGCT AGCAGATATCC CGACGAGGAA ATCTCGTGTC AGAGCTACAT GATCGGCTCG
661  CCCGCACCGG GTGCGACCAA CGTATGTCG CCCAGGTCTG AGCAGCAGCT GATCGGGCCG
721 GGCTACAGCC GCGACGAGTT CCGTCTGCGC ACCGACACCG CCATGGTCTG GCCGGTGAAC
781 AAGACGCTGC TGGTGCGAGT GCCACGCTGCC GACGTGATCC ACTCCTGGAC GTGCCCCGCC
841 TCTCCGGCTGA AGCAGGATGC GTGCGCGGCG CGGCTCGCGC AGCTCTGGTT CCGGGCGGAG
901 CGCGAGGCGA TTCTCTTCCG CCAGTGCTTG GAGCTCTGCC GCATCTGCGA GCCTCAGCAT
961 CCATCGAGG TCAAGGGCTG TCGAGGAAAG GCTTATGCAG CCGGCTCGGA ACAGTGA

Subunit II protein sequences:
MTTVLDSDQMMLRRIVFLVVAKTWQSQVEOREICPVSGRTDGMRSQRLNMRHSTTLTPCATGAAGLLAATAAAQQQTEIIIRGQPQPGTFQPSASPAMVATQIHWLDGFLVIIAATIFVTLILYAVWRPEKRNNKVPARFTHNSPLEIAWTVIPVILVAIGASFLPVFNLNQEIEPEADVTVKVTGYQNYGWEYPDDEISFESYMSPATGGDNRSFPEVEQQLIEAGYSDFEFLATMDMVVPNVTQVTGADVIIHSVTVPAFGVKQDAVPGLALWAFRAEREGIFGQCSELCGISHYMPITVKVSEEAAYAAWLEG

Subunit III DNA sequences:
1 ATGGCCCACG CCAAGAACCA CGACTACCAC ATCTCTGCGC CCTCGATCTG GCCCTTCATG
61 GCCTCGTTCG GACCTCTCGT CATGCTGAC GGGCCTCGGC TCTGGATGCA CGGCTCGGGG
121 CCCTGGATGG GCCTGATCGG GCTCGTCGTG GTGCTCTACA CGATGTTCGG CTGGTGGTCC
181 GACGTGTTGA CGGAAACGCT CGAGGGCAGC CACACGCCGG TGGTGCGTCT GGGCTCGCC
241 TGGGGCTTCA TCTCTCCTCAT CATGCTCCAG GTATCTTCTC TCTGGCGCTG GTTCTGGAGC
301 TTCTTCAAGC ACGCGGCTTA TCCGATGGGG CCCGAGAGCC CGATCATCGA CGGGATCTTT
361 CGGCCGAGG GGATCATTAC CTTCGATCCG TGCTCATGAG CGCTCATCAA CACGGCTGATC
421 CTGCCTCTCTG CGGGCTCGGC GCCACCTTGG GCCACCATATG CGCTGGTCGA TGAAGAACAT
481 CGGCCGCGACG TGCCCTGGGG GCTGGCGGTC GCCATCGGC CCGGCCTGGCT CTTCACCGTG
541 TTCCAGGGCT ACAGATTACG CCACCGGCGG TTCCGCGTGG CGGGCAACAT CTATGGCGGC
601 AAACCTCTCA TGCCGACGGG ATTCGAGCGG TTCGCAATGCA TGTGGGGCAG GATCTTCTTG
661 CTCACTCTGG TGATCCGGGT GCAGCGCGGC CACTTCACCC CCGAGAAGCA TGTCGGCTTC
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781 ATCTCATCTG GGGCCAGTA A
Subunit III protein sequences:
MAHAKNHDYHILPPSIWPFFMASVGAFVMLNGAVLWMHGSGFWMGLICLVKLVTMFGWSDVVTESELEGDHTPVVRGLRWFILFIMSEVIFFSAWFWSFFKHALYPMGPESPIIDGFIPPEGIITFDPLWHLPLLTLILLCSGCAATWAHHLVHENRRDVAWGLALAIALGALFTVFQAYEYSHAAGFAGNIYGANFFMATGFGHVIVGTTYFELVCLIRVQRGHTFPEKHVGFEEAMWYWHFVDVVWLFLFASIYIWGQ

Subunit IV DNA sequences:
1 ATGGCAGATC ACTCGCATCC CGCTCAGGG CATGTGCCAG GCTCGATGGA CATCACCCAG 61 CAGGAGAAGA CCTTCGCGGG CTTCGTCCGG ATGGTGACAT GGGCCGCCGT GGTCATCGTC 121 GCGGCCCTGA TCTTCTCGC GCTGGCCAC GCCTGA

Subunit IV protein sequences:
MADHSHPAIGHVAGSMSMDITQOEKTFAGFVRMVTAAVVIVAALIFLALANA
APPENDIX B: PRIMERS

Subunit I mutants:
R481H: 5'-GGCCGCCAGGGCATGCCGCAA CGCTACATCGAC-3' (A SaeII site is introduced)
R481L: 5'-GGCCGCCAGGGCATGCCGCTGCGCTACATCGAC-3'
R481Q: 5'-GGCCGCCAGGGCATGCCGCAGCGCTACATCGAC-3'
R481N: 5'-GGCCGCCAGGGCATGCCGAAACGCTACATCGAC-3'
S200I: 5'-CACCTGTCGGGCGCCTCACCATCTCCTCGGCACGATC-3'
S200V/S201V: 5'-GTGCACCTGTCCGGGCGCCGA GTGGTAGCTCTCGGCACGATC-3'
S200V/S201Y: 5'-CACGTGCACCTGTCCGGGCGCCGA GTGTATGCCTCGGCACGATC-3'
Y33F: 5'-GACATCGGCGTTCTCTTCCTCTCTTCACCCTGCCG-3'
D132N: 5'-CACATCGGCGCCCGCAACATGGCCTTCGCGG-3'
T337K: 5'-CACATCGGCGCCCGCAAGATGGGCGGCTGAGCACC-3'
T337R: 5'-CACATCGGCGCCCGCAAGATGGGCGGCTGCATCC-3'

Subunit II mutants:
K227M: 5'-CCCGCCTTTCGGCGTATCGGATGCCGGTG-3' (A HpyCH4V site is introduced)
Q228K: 5'-GCCTTTCGGCGTGAAGAAAGATGCCGGTGTCGCGGC-3'
D229N: 5'-GGCGTGGAAGCAGATGCCGGTGTCGCGGC-3'
Q127K: 5'-GTGCTGTTCACCCAGAAGAAATCCCGAGGC-3'
## APPENDIX C: STRAINS AND PLASMID

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APPENDIX D: PROTOCOLS

CELL PREPARATION

1. Grow cells to 60 KU (early to mid log phase, ~2 hrs from overnight)
2. Spin down at 4 ºC, 4000 rpm in GSA rotor/ 5000 rpm in SS34, for 15 mins.
3. Resuspend in one volume of cold 1 mM HEPES (1mM CaCl$_2$).
4. Repeat centrifugation.
5. Resuspend in $\frac{1}{2}$ volume 1 mM HEPES (water works well).
6. Repeat centrifugation.
7. Resuspend in 1/50 original volume of cold 10 % glycerol.
   (e.g.: If started with 100 ml cells = 2 mL)
8. Repeat centrifugation.
9. Resuspend in 1/500 original volume of cold 10 % glycerol.
10. Aliquot final suspension in Eppendorf tubes, 40 µL each.
11. Freeze in dry ice/ethanol bath or use right away.
CONJUGATION

Day 1:
1. Start a 5 mL culture of JS100/YZ200 cells in Sistrom’s media with 50 µg/mL streptomycin and spectinomycin at 30 °C at least 24 hours in advance of desired conjugation date.

Day 2:
1. Start a fresh 2-3 mL culture of S-17-1 strain with the desired mutant pRK plasmid in it, with 15 µg/mL tetracycline in the morning of the desired conjugation.
2. When the S-17-1 reaches early log phase (3-4hrs), take 100 µL of culture and 1mL of 24 hr JS100/YZ200 culture and spin down (3000 rpm, 5 min) separately.
3. Pour off the supernatants and resuspend each pellet in 500 µL LB to wash out any remaining antibiotics. Combine the two suspensions (JS100/YZ200 and S-17-1) and spin down slowly. The pellet will contain a mixture of the two cell types.
4. Resuspend the pellet with 1 mL and spin down the cell, discard 900 µL and resuspend the cell with remaining 100 µL. Deposit the suspension on a Millipore 0.45 µm filter placed on an LB plate. Allow the liquid to dry by diffusing into the agar (15-30 mins) and incubate the plate overnight at 30 °C.

Day 3:
1. The filter should now look like a fried egg. Place it in 1 mL of Sistrom’s media (no antibiotics) with sterile tong and allow the cells to resuspend in the media. Vortex briefly if needed.
2. Remove 50 µL of the suspension and dilute it with 1 mL of Sistrom’s media.
3. Plate 100 µL of the diluted suspension on a Sistrom’s plate with 50 µg/mL streptomycin, spectinomycin, and 1 µg/mL tetracycline. Spread with sterile glass rod.
4. Incubate at 30 °C for 4-5 days. Colonies will be visible after two days.

5. Streak out some isolated colonies on fresh Sistrom’s plates to allow them to grow more.

6. After 2-3 days the cells will be abundant enough to start a 5 mL liquid culture.

7. Grow for 1-2 days and make a low temperature stock (15 % glycerol, 85 % culture; mix and store at -70 °C).
**SISTROM’S MEDIUM**


- **Preparation of 1 L of 10X medium**

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<th>Component</th>
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<th>Add this to make 20 L of 12X (g)</th>
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<td>5.0</td>
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</tr>
<tr>
<td>(NH&lt;sub&gt;4&lt;/sub&gt;)&lt;sub&gt;6&lt;/sub&gt;Mo&lt;sub&gt;7&lt;/sub&gt;O&lt;sub&gt;24&lt;/sub&gt;4H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>0.002 g (160 µL) 0.2 mL (1 % soln.)</td>
<td>1.6 µM</td>
<td>0.048</td>
<td>2.4 mL (2 % soln.)</td>
</tr>
<tr>
<td>Trace elements soln.</td>
<td>1.0 mL</td>
<td></td>
<td>24 mL</td>
<td></td>
</tr>
</tbody>
</table>

- pH will be around 4.5-4.9

- Freeze at -80 ºC. To use, dilute to 1X; adjust with KOH (approx 4g/L)

- To make solid medium, add agar to 1.5 %
Trace elements solution stock: 1000X for 10X

<table>
<thead>
<tr>
<th>Component</th>
<th>MW</th>
<th>g/100 mL</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA (disodium salt)</td>
<td>372.24</td>
<td>1.765</td>
<td></td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>278.02</td>
<td>5</td>
<td>0.18 M</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>287.54</td>
<td>10.95</td>
<td>0.38 M</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>169.01</td>
<td>1.54</td>
<td>0.09 M</td>
</tr>
<tr>
<td>CuSO₄·H₂O</td>
<td>249.69</td>
<td>0.392</td>
<td>0.016 M</td>
</tr>
<tr>
<td>Co(NO₃)₂·6H₂O</td>
<td>291</td>
<td>0.248</td>
<td>8.5 mM</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>61.83</td>
<td>0.114</td>
<td>0.018 mM</td>
</tr>
</tbody>
</table>

- Add H₂SO₄ drop wise until solution is clear (usually not needed, solution is bright green at first like NiSO₄ soln.)
- Store at 4 °C.
- Wrap in Aluminum foil.
### ADDITION OF BIO-BEADS

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Bio-Beads mg/mL</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>-20</td>
<td>0</td>
<td>RT</td>
</tr>
<tr>
<td>0</td>
<td>33.2</td>
<td>4</td>
</tr>
<tr>
<td>30</td>
<td>33.2</td>
<td>4</td>
</tr>
<tr>
<td>60</td>
<td>33.2</td>
<td>4</td>
</tr>
<tr>
<td>90</td>
<td>33.2</td>
<td>4</td>
</tr>
<tr>
<td>120</td>
<td>66.5</td>
<td>4</td>
</tr>
<tr>
<td>180</td>
<td>66.5</td>
<td>4</td>
</tr>
<tr>
<td>240*</td>
<td>133.0</td>
<td>RT</td>
</tr>
<tr>
<td>270</td>
<td>133.0</td>
<td>RT</td>
</tr>
<tr>
<td>300</td>
<td>266.0</td>
<td>RT</td>
</tr>
<tr>
<td>360</td>
<td>266.0</td>
<td>RT</td>
</tr>
<tr>
<td>420</td>
<td>-</td>
<td>RT</td>
</tr>
</tbody>
</table>

* 0.5 mL of 100 mM HEPES-KOH pH 7.4 is added per mL mixture.