CHARACTERIZATION OF THREE MUTATIONS IN A CONSERVED
DOMAIN OF SUBUNIT III OF CYTOCHROME C OXIDASE FROM
RHODOBACTER SPHAEROIDES.

A dissertation submitted in partial fulfillment of the
requirements for the degree of
Master of Science

by

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I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Rachel F. Omolewu ENTITLED Characterization of Three Mutations in a Conserved Domain of Subunit III of Cytochrome c Oxidase from Rhodobacter sphaeroides BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT


Cytochrome *c* oxidase (COX) is the final electron acceptor in mitochondrial respiratory chain and in many bacterial species including *Rhodobacter sphaeroides*. Electron transfer is coupled with the pumping of protons across the membrane. Previous work has shown that reaction of beef COX with dicyclohexylcarbodiimide (DCCD) resulted in an inhibition of proton translocation by covalently binding to the conserved amino acid residue E90 located in a nonpolar region of subunit III (SIII). E90 is involved in a bonding pair with another conserved residue H212, possibly connected by a salt bridge or a hydrogen bond in the three dimensional structure of SIII. Our goal was to test whether the retention of the E90-H212 linkage and the spatial arrangements of these amino acid residues were critical for electron transfer and proton pumping activities of the enzyme.

This work analyzes the functional role of these amino acids through the creation of three mutants in SIII—H212E, E90H, and E90H/H212E. SDS-PAGE verified all mutants contained SI-III. The first mutant, H212E, bacteria cultures grew significantly faster as compared to wild-type; while the other two mutant culture grew at comparable rates to wild-type. Additionally, the visible absorbance spectrum of H212E mutation in bacterial membranes showed little or no heme aa₃ oxidase expression while the other two...
mutants exhibited properties similar to wild-type. Conversely, the spectrum of isolated and purified COX-SIII E90H mutant protein displayed a red shift while the double mutation COX-III E90H/H212E resembled that of wild-type. Electron transfer activity of two purified mutant proteins E90H and E90H/H212E revealed decreases in steady-state activities approximately 40% and 15% respectively. Lastly, the two mutants displayed a slight alkaline shift in pKa value for electron transfer activity. In summary, these results imply that the absolute positions of E90 and H212 are essential to COX activity. The single mutations resulting in two like charges caused changes in COX activity whereas the double mutation that retained the native salt bridge did not. Additionally, the increase in pKa may suggest the environment around the active site is perturbed, which is reflected by the decrease in electron transfer activity.
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I. INTRODUCTION

MITOCHONDRIA AND FUNCTION

The Mitochondrion

Aerobic metabolism is a key process in eukaryotic and prokaryotic organisms alike. It involves a precise set of biochemical reactions within the cell. The driving force for these reactions lies in one cellular organelle--the mitochondrion. The mitochondrion is known as the powerhouse of the cell, providing the primary source of energy by which the cell converts it into ATP.

It is believed that the mitochondria evolved from protobacteria. According to embosymbiotic theory, the mitochondria were separate free organisms, in which they were later taken into the cell by endocytosis (Gray, 1999; Wallace & Fan, 2010). Because of this, mitochondria contain their own DNA apart from the cell nuclear genome (Gnad et al., 2010; Gray, 1993). Furthermore, mitochondrial structure is rather unique compared to other cellular organelles. In addition to having its own ATP generating mechanism, it is enclosed by a double membrane (Figure 1). This double membrane allows for the creation of two separate compartments within the mitochondrion, each with its own special function. The outer membrane consists of a semi-permeable phospholipid bilayer, embedded with various proteins, lipids, and other macromolecules
Figure 1. Mitochondrion structural components. The mitochondrion membrane consists of two parts—inner and outer membrane. Tight folds of the inner membrane make up the cristae. The interior of the organelle, an aqueous mixture of molecules, is known as the matrix. (Frey & Mannella, 2000)
that allow for the transport of ions, nutrients, and other micromolecules in and out of the cell. Its main function is to maintain the shape of the mitochondrion (Tate & Stevens, 2010).

The inner membrane, permeable only to oxygen, water, and carbon dioxide molecules, is more complex and packed with the vital protein complexes involved in energy production of the cell. These proteins make up energy generating metabolic processes oxidative phosphorylation and the electron transport chain. These complexes create an electrochemical gradient which allows charged molecules to easily cross the membrane into the cellular matrix. The inner membrane ‘folds’ itself into tight compartments within the cell, known as cristae (Figure 1)—this serves to increase the surface area of the inner membrane to allow for all the key energy production complexes to exist in multiple copies within the cell (Zick, Rabl, & Reichert, 2009). The volume between the two membranes is known as the intermembrane space (Frey & Mannella, 2000). The space inside the mitochondrion is known as the matrix; it contains ribosomes, mitochondrial DNA, enzymes, and a multitude of other molecules vital to the functioning of the cell (Mannella, 2006; Mannella, 2008). The matrix is also the site for the citric acid cyclic process in the cell—an enzymatic-catalyzed reaction for aerobic metabolism (Srere & Sumegi, 1986).

**Electron Transport Chain**

The electron transport chain occurs in the inner membrane of mitochondria in eukaryotes (Rich & Marechal, 2010) and for prokaryotes, it occurs in the plasma membrane (Srere, Sumegi, & Sherry, 1987). The electron transport chain consists of five main complexes, which collectively, function through a series of biochemical redox
Figure 2. The Mitochondrial Electron Transport Chain. Main complexes of the electron transport chain. Reductants NADH and succinate produced from the citric acid cycle are oxidized to provide protons which drive ATP synthase to produce ATP. The transfer of electrons from one complex to another is coupled with the translocation of protons across the membrane, maintaining an electrochemical gradient. Described in detail in the text. (Hosler, Ferguson-Miller, & Mills, 2006)
reactions to produce ATP (Figure 2). Complex I, also known as NADH dehydrogenase, is the initial ion acceptor. It receives NADH and a free proton H+ generated from the citric acid cycle in which two electrons are transferred to coenzyme Q (“Q” in Figure 2) and four protons pumped are across the membrane. Additionally, Complex II (succinate dehydrogenase), oxidizes FADH₂ to FAD, donating its electrons to coenzyme Q as well. Also called ubiquinone, Coenzyme Q makes up a “Q cycle” with Complexes I and II, using the donated electrons from both complexes to transfer a net of two electrons to cytochrome c via Complex III in a two-step redox reaction. The Q cycle also translocates four protons across the membrane into the intermembrane space (Liu, 2010).

Complex III, cytochrome bc₁ complex receives two electrons from the Q cycle (ubiquinone) and transfers them to cytochrome c. At the same time, two protons are transferred across the membrane, maintaining the electron gradient. Cytochrome c then transfers its electrons to the fourth complex, cytochrome c oxidase. Here, the two electrons are used to reduce oxygen to water and while at the same time pumping protons across the membrane. These pumped protons are transferred to the final complex, Complex V (ATP synthase), the site for ATP synthesis (Hosler et al., 2006). Thus, cytochrome c oxidase serves two roles—electron transfer and as a proton pump.

**CYTOCHROME C OXIDASE STRUCTURE AND FUNCTION**

*Structural Characteristics*

Cytochrome c oxidase (COX) is a vital enzyme in aerobic metabolism. Making up complex IV of the electron transport chain, it is the final electron acceptor catalyzing the
reduction of oxygen to water. Like the other enzymes in the electron transport chain, this
multisubunit protein spans the entire membrane. It is a complex, multifunctional integral
membrane protein that serves as a transporter of electrons and also translocates protons in
energy transduction (Belevich et al., 2010). Each component in its structure serves its
own purpose, making the enzyme a unique and dynamic structure.

COX has been studied in both mammalian and bacteria models. A significant
amount of the work done in mammalian oxidase is done in the bovine heart
mitochondrial form. The crystal structure was originally determined by Yoshikawa et. al
at a resolution of 2.8 Å (Yoshikawa, Tsukihara, & Shinzawa-Itoh, 1996). It has since
been resolved at higher definition of 1.8Å (Tsukihara et al., 2003). (Figure 3).

The bovine mitochondrial form of the enzyme is a dimer, containing thirteen
different polypeptide subunits (Kadenbach, Jarausch, Hartmann, & Merle, 1983). In
addition, the enzyme also contains a multitude of metallic molecules that make COX
redox reactions possible. Within the enzyme, there are Mg^+, Zn^{2+}, and Cu^{2+} metal centers
and iron hemes (Steffens, Biewald, & Buse, 1987; Steffens & Buse, 1988; Tsukihara et
al., 1995). These vital components are all located in the largest subunits—Subunits I-III.
Additionally, contributing to their unique properties, these three subunits are derived
from mitochondrial DNA and synthesized on mitochondrial ribosomes (Hare, Ching, &
Attardi, 1980); the remainder subunits are encoded by nuclear DNA and are made in the
cytosol.

Subunits I-III have been dubbed the “catalytic core”, as they contain the four
different sites on the enzyme that involve the exchange of electrons—heme a, heme a₃,
**Figure 3. Crystal Structure of Bovine Cytochrome c Oxidase.** Crystal structure obtained at a resolution of 1.8 Å. COX is composed a two monomers, forming a dimeric structure. Each monomer contains 13 chains. Subunits I-III, the three largest, are encoded for by mitochondrial genome; while the remaining others, are encoded for by the nuclear genome (gray). Key Subunits are: Subunit I green, Subunit II cyan, Subunit III yellow. Subunit IV (blue). Remainder V-XIII are various colored. (Yoshikawa et al., 1996).
Cuₐ, and Cuₐ. These four redox sites are all located in the first two subunits—subunit I (green) contains heme a, and the heme a₃-Cuₐ active site; while subunit II (cyan) contains Cuₐ and the site for cytochrome c binding (Hosler, 2004; Iwata et al., 1998; Tsukihara et al., 2003). Subunit III (green) contains no redox molecules or active centers however it does contain many phospholipids that have been proposed to play an indirect role in oxygen reduction (Iwata, Ostermeier, Ludwig, & Michel, 1995; Yoshikawa, 1997).

The nuclear encoded subunits VIa and VIb, perform structural roles in that they stabilize the protein in the dimer state (Yoshikawa, 1997). Previous studies to date have not been able to establish a structural or catalytic function for the remaining subunits in the beef form of the enzyme. Most deletion mutation studies on the nuclear encoded subunits yield unassembled oxidase.

Despite having the same metabolic function, the bacteria form of oxidase, has only four subunits. The crystal structure of COX has been identified in two bacterial species—soil bacterium *Paracoccus denitrificans* (Iwata et al., 1995) and photosynthetic bacterium *Rhodobacter sphaeroides* (Svensson-Ek et al., 2002). Figure 4 shows the crystal structure of *R. sphaeroides* resolved at 2.35 Å. In the bacteria model, the first three subunits—the largest subunits—are analogous to that of bovine oxidase subunits I-III (Cao, Shapleigh, Gennis, Revzin, & Ferguson-Miller, 1991). In addition, these three large subunits are highly conserved across species (Hosler et al., 1992; Svensson-Ek et al., 2002). The last subunit of either bacteria form, SIV, is not analogous to bovine COX, nor does it known structural or catalytic properties. SIV, however is homologous between the two bacterial forms (Cao, Hosler, Shapleigh, Revzin, & Ferguson-Miller, 1992). To show the immense homology of subunits I-III, the structural alignment of
Figure 4. Crystal Structure of *Rhodobacter sphaeroides* Cytochrome c Oxidase.
Crystal structure resolved to 2.35 Å. Subunit I yellow, Subunit II green, Subunit III blue, Subunit IV magenta. Blue spheres are Cu metal centers. Red sphere is Fe center of heme aa₃ at the binuclear active site. Adapted from (Svensson-Ek et al., 2002).
the three enzymatic forms is shown in Figure 5. These enzymes exhibit similar energy transduction mechanisms. Due to its ability to grow efficiently, the bacterial \textit{R. sphaeroides} form of the enzyme is highly studied and hence is the model used in this study.

\textit{Functional Characteristics}

Subunit I of COX (Figure 4, yellow) is the largest of the three subunits, containing 12 transmembrane helices (Svensson-Ek et al., 2002). It has been studied intensely and its functions have been characterized. Subunit I contains the key components that are involved in the enzyme’s energy transduction properties—a six coordinate heme \textit{a} (red), heme \textit{a}_3 (aqua), and metal center Cu_{B} (blue). The latter two components make up the binuclear active site.

These molecules function in a linear sequence of events to transfer electrons to the active site. As electrons are being transferred through the enzyme, subunit I also allows for the transfer of protons. Figure 6 shows the three pathways have been proposed for this mechanism. Two of these pathways, the K-pathway and D-pathway are conserved. They are named for the conserved residues at the entrance to the pathway—K362 and D132 respectively (Brzezinski & Johansson, 2010; Ganesan & Gennis, 2010). The third and final pathway, known as the H-pathway is named for the hydrogen bond network and water molecules that outline the channel (Lee et al., 2000; Tsukihara et al., 1996). It spans the entire membrane, extending from the negative side surface to the exterior positive side space of the membrane. Pathways K- and D- connect the binuclear
Figure 5. Multiple Structural Alignment of COX Catalytic Core Subunits I-III. X-ray crystal structures of three oxidase forms of Subunits I-III. Superimposed are: bovine heart (PDB 1OCC blue), *P. denitrificans* (PDB 1QLE green), *R. sphaeroides* (PDB 1M56 red). Adapted from (Geyer, 2007).
Figure 6. Schematic mechanism of the proposed proton pathways of COX R. sphaeroides. K-pathway located in SI, marked by conserved residue K362. D-pathway also in SI, beginning with conserved residue D132. SIII is bound to the D-pathway at residue D132. Both channels lead into the heme $a_3$-Cu$_B$ binuclear active site. Protons are pumped to E286 of SI where they are either shuttled to the active site or across the membrane to the P-side (intermembrane space). Pathway of protons and electrons highlighted by orange arrows. Oxygen binding highlighted by red arrow. Red spheres indicate water molecules that assist with the movement of protons. H-pathway has been omitted as it is not conserved across species. Adapted from (Ganesan & Gennis, 2010).
P side (intermembrane space)

D-pathway
N side (matrix)

K-pathway
center with the negative side space of the membrane (inner space) (Figure 6) (Shimokata et al., 2007). Initially discovered in bovine COX, the H-channel is also present in prokaryotic COX but has been shown to not have any functional importance (Lee et al., 2000).

Subunit II (Figure 4, green) consists of two transmembrane helices and a globular domain that lies on ‘top’ of the enzyme, extending out into the intermembrane space—the positive side of the membrane. This globular domain contains the binding site for cytochrome c, in which electrons are transferred to catalyze the energy transduction process (Ferguson-Miller, Brautigan, & Margoliash, 1976; Papa, Capitanio, & Villani, 1998). Although SII does not have any iron centers, it does contain a bimetallic metal center—CuA-CuA (Figure 4, blue spheres), which serves as a medium for the transfer of electrons from cytochrome c to the heme a3--CuB active site.

SIII consists of seven transmembrane helices which form a V-shape cleft on the side of the enzyme (Figure 4). Its purpose in the enzyme’s function has been the focus of many studies, has it does not contain any metal centers but appears to be indirectly involved with enzymatic activity. Removal of this subunit from the enzyme results in a loss of activity and inhibition of proton pumping (Prochaska & Reynolds, 1986; Puettner, Carafoli, & Malatesta, 1985). SIII also prevents COX from undergoing suicide inactivation, in which the oxygen reduction cycle activity is dramatically reduced to less than 1% (Hosler, 2004; Mills & Hosler, 2005; Varanasi et al., 2006). SIII interacts with CuB at the active site during oxygen reduction—when it is removed, these interactions no longer exist, altering the environment around the active site (M. R. Bratton, Pressler, &
Hence SIII functions to stabilize the integrity of the binuclear active site. As a result of these events, SIII has been established as vital for the COX function.

Subunit III is highly conserved in different species (Abramson, Svensson-Ek, Byrne, & Iwata, 2001). Several studies on SIII have been conducted to analyze its function, including chemical modification and site-directed mutagenesis. When reacted with organic coupling reagent $N, N'$-dicyclo-hexylcarbodiimide (DCCD), the reagent blocks proton translocation in the enzyme by binding to one of its conserved amino acid residues, E90 (Casey, Thelen, & Azzi, 1980; Prochaska, Bisson, Capaldi, Steffens, & Buse, 1981). In addition, when conserved residues in subunits I and III are altered decreases in enzyme function are observed.

Several studies have verified that DCCD inhibits proton translocation in COX. DCCD is a known compound that affects the synthesis and utilization of ATP. It binds to a small polypeptide in the form of a proteolipid complex and forms a covalent bond derivative with ATP synthetase (Beechey, Robertson, Holloway, & Knight, 1967; Cattell, Lindop, Knight, & Beechey, 1971). This derivative significantly inhibits the proton pumping ability of the enzyme. DCCD binds very specifically to the ATP synthetase, forming bonds only with Asp and Glu residues in the $F_0$ unit of ATPase. Formation of this tightly bound complex causes an inhibition of the partial reactions of oxidative phosphorylation by 95-100% (Cattell et al., 1971). Because DCCD binds so tightly, only a small amount is needed to cause a 95% inhibition of ATP synthesis. Consequently, proton translocation of ATP synthetase is inhibited.

Moreover, DCCD binds to COX in a similar way, binding to a particular section of the enzyme, a nonpolar, conserved region in COX (Casey et al., 1980). Incorporation
of DCCD into three subunits of COX—II, III, and IV—yielded significant effects. It was determined that DCCD binds to subunit III with greater specificity of one order magnitude greater than subunits II and IV (Prochaska et al., 1981). Ninety percent of the bound DCCD covalently binds to conserved residue E90, forming a bulky urea derivative. X-ray crystal structure analysis shows that this bulky DCCD molecule faces the interior of the protein, breaking the connecting bond between E90 and conserved residue H207 (bovine heart oxidase numbering; located on an adjacent helix). Additionally, E90 is located within a possible oxygen transfer pathway in subunit III. The bulky DCCD molecule causes two fatty acyl chains imbedded in subunit III to move in such a way that they cover this oxygen transfer channel near E90. This causes the channel in SIII that leads to the oxygen reduction site in SI to be blocked. Hence, the binding of DCCD indirectly leads to decrease in electron transfer of COX (Shinzawa-Itoh et al., 2007).

In addition, when DCCD is bound to the enzyme, there is a substantial decrease in proton pumping of the COX (Shinzawa-Itoh et al., 2007). Because ninety percent of DCCD was solely bound to subunit III, it suggested that DCCD prevents proton release via subunit III (Prochaska et al., 1981). The bound DCCD bulky molecule faces the interior of the protein, causing the helices of subunit III to move in such a way that they affect the interaction with subunit I at the entrance to the D-pathway (Ogunjumi, 2006). This conformation change could account for the observed decrease in proton translocation. Furthermore, the group of amino acids DCCD binds to is similar to the DCCD-binding site of ATP synthetase, which as stated earlier, is an inhibitor of proton translocation. DCCD binding increases with increased pH concentration (Casey et al., 2007).
1980). The inhibition of proton translocation can be attributed to the binding of DCCD at the hydrophobic region of Glu90 and not due to an increase in membrane proton permeability.

A mutagenesis study in *R. sphaeroides* involved altering conserved lipid binding sites in SIII. Helix 3 of SIII binds to helix 6 of SI near the entrance to the D-channel (Hosler et al., 1996). SIII contains two lipid binding sites in a V-shape cleft deep within the enzyme (Cvetkov, 2010). When these sites were mutated, it was found to result in a disruption of the SI and SIII interactions, leading to an increase in the rate of suicide inactivation due to a decrease in the stability of the enzyme (Cvetkov, 2010; Varanasi et al., 2006). Thus, it is known that SIII may play a role in the prevention of suicide inactivation of COX (Haltia, Saraste, & Wikstrom, 1991).

Suicide inactivation, or turnover induced inactivation, is when the enzyme deactivates itself during oxygen reduction; this is caused by structural changes at the binuclear active site (M. R. Bratton et al., 1999). This has been known to occur when SIII is removed from *P. denitrificans* COX, leaving only SI and SII (Mills & Hosler, 2005). The same results are exhibited when this is repeated in *R. sphaeroides* form (M. R. Bratton et al., 1999) of the enzyme. Similar results were found in our laboratory using the beef heart enzyme (Shroyer and Prochaska, unpublished results).

In addition to these results, it has been found that SIII is needed for the uptake of protons from the N-side (matrix side) of the membrane to the active site (Gilderson et al., 2003; Hosler, 2004); without it, the rate of proton uptake is significantly lowered (Smith, Gray, Mitchell, Antholine, & Hosler, 2005). Because SIII interacts with SI at the entrance to the D-channel (Iwata et al., 1995; Tsukihara et al., 1996; Yoshikawa,
Shinzawa-Itoh, & Tsukihara, 1998), it functions to stabilize the active site to enable proton translocation (M. R. Bratton et al., 1999; M. R. Bratton, Hiser, Antholine, Hoganson, & Hosler, 2000). Lastly, SIII may be involved in the proton uptake into the a3-CuB active site (Mills, Tan, Ferguson-Miller, & Hosler, 2003).

**Electron Transfer Mechanism**

The electron transfer process is coupled with the translocation of protons across the inner mitochondrial membrane (Hosler et al., 2006). Each transfer of electron is linked to substrate proton uptake to the active site. The reaction for this multistep process is:

\[
4 \text{cytochrome } c^{2+} + 8H^+ (N \text{ side}) \rightarrow 2 \text{H}_2\text{O} + 4 \text{cytochrome } c^{3+} + 4H^+ \text{pumped (P side)}. 
\]

Electron transfer is initiated with the binding of cytochrome c at SII, causing an electrostatic reaction in which its electrons (2) are transferred to CuA-CuA. From here, the electrons move to heme a and then to the binuclear active site heme a3-CuB. At the active site, the electrons shuttled one more time to molecular oxygen, in which it is reduced to water. (Figure 7) (Antalik, Jancura, Palmer, & Fabian, 2005; Jancura, Antalik, Berka, Palmer, & Fabian, 2006; Yoshikawa et al., 1996; Yoshikawa et al., 1998). The redox center reagents, heme a, heme a3, and CuB are located at about the same distance inward from the membrane of the protein (Tsukihara et al., 1996). As the final electron acceptor, molecular oxygen undergoes a cyclic series of complex steps resulting in the production of several intermediates before it is finally reduced to water (Bloch et al., 2004; Hosler et al., 2006). Figure 8 shows the six intermediates that are produced during this catalytic reduction cycle. The conformation at the active site changes with protonation as the
Figure 7. Schematic mechanism of the Electron Transfer Process of COX. Cytochrome c binds enzyme on the P side (intermembrane space) of the membrane, transferring its electrons to CuA. From here the electrons are transferred to heme a and then finally to the heme a3-CuB active site. Here, the electrons reduce oxygen to water while a proton is simultaneously pumped across the membrane. For each step of electron transfer, one proton is taken up from the N-side (matrix) of the membrane and travels up to the binuclear active site. Adapted from (Garrett & Grisham, 2007).
Figure 8. Oxygen Reduction Catalytic Cycle of COX. Intermediate steps described in detail in text. Superscript indicates number of electrons that are being transferred in the steps. Red arrows refer to protons pumped across the membrane. Green arrows refer to protons/electrons transferred to active site. Substrates in blue text are either produced or used during oxygen redox activity. Adapted from (Cvetkov, 2010).
cycle progresses. The cycle begins at oxidized heme a₃—CuB (Fe³⁺ₐ₃ – Cu²⁺ₕ) active site, O⁴⁰ in the figure. (The superscript refers to the number of electrons being transferred. O is denoted ‘4’ and ‘0’ as it is the start and end of the cycle.) Initially, two electrons are transferred to the active site, the first yielding E¹ and the second R². The active site is now Fe²⁻a₃ – Cu⁺ₕ. At the same time, one proton at each step is taken up from the matrix (N-side), this is to maintain “electrostatic neutrality” because the active site lies in a hydrophobic environment (Artzatbanov, Konstantinov, & Skulachev, 1978; Rich, 1996). These protons either come from the K-pathway (2) or one from K- and D-pathway each. Next, O₂ binds heme a₃ of the active site, yielding A² intermediate. Oxygen will only bind to heme a₃ after Cuₕ and heme a₃ are reduced. The O-O bond is broken when Y288, a residue located in the active site, donates an electron, yielding a tyrosine radical (Proshlyakov et al., 2000). This results in conformational changes near the active site (Iwaki & Rich, 2007). The enzyme is now in the P state. From here, an electron from the P side (intermembrane space) is transferred to the tyrosine radical, resulting in the F³ state. At the same time, another proton is taken up through the D-pathway which occurs at a rate of 100µs (Salomonsson, Faxen, Adelroth, & Brzezinski, 2005). Lastly, an electron is transferred to the active site, along with another proton pumped through the D-pathway at a rate of 1ms, resulting in the O⁴ state. Proton release at the P intermediate leading to the formation of F³ and O⁴ is the rate-limiting step (Adelroth & Hosler, 2006). The enzyme is now in the fully oxidized state, as four electrons have been transferred to the active site. Protons move through the K-pathway only during the reduction phase of the cycle, not during oxidation. Mutagenesis studies have shown that when this channel is blocked, one proton is still able to travel to the active site, allowing for a partial reduction
of heme a₃ (Ganesan & Gennis, 2010). Conversely, mutagenesis of the D-pathway inhibits proton pumping (Fetter et al., 1995; Zhu, Han, Pawate, & Gennis, 2010). Protons move through the D-pathway during both redox phases of the catalytic cycle. Proton release at the P intermediate is rate-limiting. Polar amino acid residues have been known to affect the activity and structure of the active (Hosler et al., 1996; Hosler et al., 2006). Because the protons are taken up from the N side of the membrane, the pH inside the membrane is a factor in the electron transfer between the hemes. Electron transfer between the hemes is sensitive at alkaline pH greater than 9.5 and acidic pH values (Faxen & Brzezinski, 2007).

Although COX has been studied in both mammalian and eukaryotic models, the focus of the work involves the bacterial form of the enzyme, using R. sphaeroides as a model. This work involves analysis of the R. sphaeroides form of the enzyme; more in depth description of its characteristics will be discussed.

Because electron transfer is coupled to proton translocation, SIII may play an indirect role in reduction of oxygen at the active site. As stated earlier, removal of SIII results in decreases in proton pumping (Adelroth & Hosler, 2006; Mills et al., 2003). Another proposed role of SIII involves stability of the enzyme. Previous research has shown that SIII stabilizes water molecules of the D-pathway (Figure 6). When these molecules are stable, it allows for quicker, more efficient proton uptake through the D-pathway (Hosler, 2004). Hence, SIII enables rapid proton uptake via the D-pathway. Within SIII lies a highly conserved triad of amino acids, consisting of E90, H212, and Y246 (Figure 9). These residues are 98% conserved across species, located within a hydrophobic pocket, deep within the core of SIII (Geyer, 2007). E90 lies on helix 6,
Figure 9. Spatial arrangements of conserved triad in hydrophobic pocket of SIII. All three residues are within hydrogen bonding distance of each other. Distance shown in the figure. E90 and H212 may be connected via a hydrogen bond or a salt bridge. Y246 and H212 are located on Helix 6, E90 located on helix 3. Prepared with Accelrys DS Viewer Pro (PDB 1M56). Adapted from (Geyer, 2007).
SII

3.19 Å

3.18 Å

SIII

E90

Y246

H212E

19 Å

3.18 Å
while H212 and Y246 are retained on helix 3. These three amino acid residues are within hydrogen bonding distance. Residue E90 is of particular interest due to the DCCD binding effects on the enzyme is actively leading to inhibition of proton translocation (Prochaska et al., 1981). Additionally, the residue has been mutated to an alanine, in which the enzyme resulted in 25% subunit III loss during purification (Geyer, 2007). It also exhibited a decrease in proton pumping ability followed by inactivation of electron transfer. These changes were due to a loss in SIII.

Furthermore, H212 has also been mutated previously. An alanine and a phenylalanine were inserted—both these mutations retained SIII but resulted in similar decreases in function as E90A. H212F and H212A both had reduced proton pumping abilities and revealed inactivation during enzymatic turnover (Geyer, unpublished results).

Lastly, Y246 has been mutated to a phenylalanine. This mutant form of oxidase exhibited functional activities comparable to wild-type (Geyer, 2007). Hence, it is suggested that Y246 is not directly related to activity of enzyme nor is it involved in interhelical bond interactions with either E90 or H212.

It is hypothesized that E90 and H212 are vital to COX function because the enzyme undergoes decreases in catalytic activity and proton pumping when these residues are mutated. It is unclear as to whether these residues are connected via a salt bridge or hydrogen bond. This study aims to understand if whether the relative positions of these residues are important in enzyme function or if it is the connecting bond that is the driving force of COX’s function. This hypothesis will be specifically analyzed by
creating three different mutants via site-directed mutagenesis: 1) E90 to H90 (E90H), 2) H212 to E212 (H212E), and 3) E90/H212 to H90/E212 (E90H/H212E). The goal behind the creation of these mutants is to disturb the interaction between conserved E90 and its partner H212. The modeled structure of the mutants E90H and E90H/H212E revealed that the side chains of these bonding partners lie within hydrogen bonding distance of each other, 3.35Å and 3.61Å respectively. Modeling of the single mutant H212E showed the two residues to lie slightly farther from each other; the hydrogen-nitrogen pairs of the side chains were 4.77Å and 4.99Å (Figure 10). These mutants will be characterized in the bacterium *R. sphaeroides* via structure-function analysis.

The specific aims of this thesis are:

1) *To determine the effect of the mutations on the growth of Rhodobacter sphaeroides*. After verifying mutagenesis by DNA sequencing, the mutants were grown aerobically in *Rhodobacter sphaeroides* bacteria cultures to an OD of 1.0-1.2. Here, the growth rates of the mutants were compared to that of wild-type, specifically taking note of the expression of the heme centers located in Subunit I (SI), as monitored by visible spectroscopy difference. This will give insight as to how much mutant oxidase is being expressed during growth.

2) *To characterize the mutants’ subunit and redox center composition by use of visible absorbance spectroscopy and SDS-PAGE*. Mutant cells were lysed and harvested via gas homogenizer. Protein was be separated through ultracentrifugation and underwent purification by way of Ni²⁺-NTA ion exchange chromatography. SDS gel electrophoresis was conducted to analyze the purified protein for subunit content. Maximum absorbance peaks were quantified and compared to both isolated wild-type enzyme and cells in vivo. These biochemical methods will provide knowledge of the integrity of subunit composition and may suggest conformational changes, if any.

3) *To determine the effect of mutations on SIII on the functional activities of isolated enzyme via steady-state kinetic assays*. The mutants were analyzed polarographically to test the electron transfer rates in comparison to wild-type. The pH dependence of electron transfer activity was studied in the mutants as
Figure 10. Model structure of *R. sphaeroides* COX-SIII mutation E90H, H212E, and E90H/H212E. Shown is the wild-type COX. Enlarged figures show the three mutants and the proposed distances apart from bonding partner. **A.** Wild-type for of COX. E90 and H212 are shown at a distance of 3.19 Å and 2.97 Å apart. **B.** E90H mutant. Bonding distance modeled is 3.58 Å. **C.** H212E mutant. Bonding distance modeled is 4.2 Å. **D.** E90H/H212E mutant. Distance apart is 4.13 Å and 3.61 Å. The two glutamates of H212E, E90 and E212, lie within hydrogen bonding distance of each other. The two negative side chains of the glutamate residue may cause perturbations in SIII which may ultimately affect SI and the active site. Figure prepared with Accelrys DS Viewer Pro (PDB 1M56).
B.
D.
compared to wild-type oxidase. This will provide insight to the efficiency of the function of the mutant enzymes; and additional information as to whether the active site or its surrounding environment is perturbed.

Understanding the role of conserved E90, may it be involved in a key interaction with H212 or have a vital spatial orientation, will better assist us in understanding the function of SIII in COX as a whole. SIII as stated earlier is critical for the function of the enzyme, removing it results in enzymatic inactivation. Cytochrome c oxidase is a key enzyme in the mitochondria, the powerhouse of muscle and heart cells. Defects in COX and other mitochondria enzymes lead to the onset several muscular diseases, such as seizures, hypertrophic cardiomyopathy, and MELAS--Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (Tam et al., 2008). Further understanding of SIII involvement to COX function will allow for better understanding of the enzyme in relation to the electron transport chain; which can aid in developing better targets for the treatments of mitochondrial diseases.
II. MATERIALS AND METHODS

Mutagenesis of R. sphaeroides COX III

Two residues of the conserved triad in SIII were genetically altered. Mutagenesis of E90 and H212 yielded three different forms of the enzyme. To create the mutants, primers were manually designed. Site-directed mutagenesis was carried out with Stratagene Quik-change Mutagenesis kit and was used to incorporate the mutants into predesigned 4.3kb chloramphenicol resistant pMB301 plasmid (M. Bratton, Mills, Castleden, Hosler, & Meunier, 2003). Figure 11 shows the schematic representation of the plasmids involved in mutagenesis. Samples were sent to Davis Sequencing to verify and test for successful mutation of the desired sequence. Restriction enzyme Xmal was used to cut out the desired mutated subunit III gene sequence (0.9kb). A second enzyme Xho1 (not shown in figure) was used to ensure proper orientation of the SIII sequence. Blunt end ligation was used to insert the sequence into a second ampicillin resistant plasmid, Pmb307 (7.9kb), which contains COX accessory and assembly genes cox10 and cox11. This plasmid also contains Subunit I with a poly-histidine tag—which was used to aid in the purification process (M. Bratton et al., 2003; Hosler et al., 1993). See Figure 11. The plasmid was grown in donor bacterium E. coli. Restriction enzymes EcoR1 and HindIII were used to cut the mutated insert along with the vital COX assembly genes and these were placed into a third and final plasmid, PRK415 (10.5kb). A broad-host range
vector, PRK415 is 10.5 kb in size, tetracycline resistant, and contains a multiple cloning site (Zhen et al., 1998).

After correct insertion of mutated sequence, the vectors were then ligated into the recipient E. coli bacteria strain via New England BioLab Quick Ligation Kits, using supercompetent cells (Stratagene). Bacteria were plated onto LB Agar plates and incubated overnight at 30°C. Colonies are then screened for proper insertion using restriction digests. Davis Sequencing was used to verify mutagenesis.

Triparental conjugation was conducted to insert the vector from the E. coli bacteria strain into the R. sphaeroides bacteria strain (M. Bratton et al., 2003; Hosler et al., 1993). YZ200 R. sphaeroides cells were grown in 1X Sistrom’s (Sis) media which contains a mixture of components using succinic acid as the carbon source. A 10X stock solution of Sis media was made with these reagents in the following order: 200mM KH₂PO₄, 37.8mM (NH₄)₂SO₄, 340mM succinate, 6.7mM glutamate, 2.5mM aspartate, 85mM NaCl, 10mM nitritriacetic acid, 12mM MgSO₄7H₂O, 2.3mM CaCl₂7H₂O, 0.07mM FeSO₄7H₂O, and 1.6µM (NH₄)₆Mo₇O₂₄.4H₂O (Sistrom, 1962). In addition vitamin solution containing thiamine HCl, biotin, and nicotinic acid were added (Sistrom, 1960). A mineral solution consisting of trace elements of the following in 100mL solution: ZnSO₄.7H₂O, 1.095 g.; ethylenediamine tetraacetic acid, 250 mg.; FeSO₄.7H₂O 500 mg.; MnSO₄H₂O, 154 mg.; CuSO₄. 5H₂O, 39-2 mg.; and CO(No)and washed with sterile 1X KPO₄ pH 7.0 twice. To achieve the 1X KPO₄ buffer, a 20X stock solution was made consisting of 40% monobasic KH₂PO₄ and 60% dibasic K₂HPO₄. The pH was adjusted to 7.0 with KH₂PO₄ and diluted to 1X (Sistrom, 1964; Sistrom, 1962).
Figure 11. Schematic Mechanism of the Molecular Biology Behind Constructing Site-directed Mutants of *R. sphaeroides* COX Subunit III. Site-directed mutagenesis involved three different plasmids. A) pMB301 was used as the template DNA for COX SIII. SIII fragment was excised from pMB301 and inserted into pMB307. pMB307 contains COX, histidine tagged-COX-SI, COX-SII, and COX accessory genes cox10 and cox11. B) pMB307 + SIII was ligated into pRK415, creating a vector of size 10.5kb. C) Correct insertion of SIII into pRK415 should yield a vector of size 16.7kb. This will be conjugated into YZ200 (*R. sphaeroides* strain lacking oxidase expression). Arrows show transcription direction (Cvetkov, 2010).
A. Step 1

B. Step 2

C. Step 3
The YZ200 cells were grown to early log phase—reaching an OD$_{660}$ 1.0-1.2. Two milliliters of these cells were harvested in a tabletop centrifuge. Additionally, 1mL each of plasmids JM109-PRK415 (Figure 9) and HB101 (helper plasmid) were grown in *E. coli* cells for 12-16 hours; they were harvested and washed in same manner. The resulting pellets were pooled together with the addition of 100µL 1X KPO$_4$ pH 7.0. This concentrated cellular mixture was placed onto an antibiotic free Peptone-Yeast Extract (PYE) agar plate (Qian and Tabita FR, 1996) and incubated upside down overnight at 30°C. The next day, the mixture was restreaked onto a sterile 1X Sis agar (1X Sistrom’s media with 15g/L agar) plate with 50µg spectromycin, 50µg streptomycin, and 1µg tetracycline. They were placed upside down in light-restricted incubator at 30°C. Colonies began to form in 2-4 days, in which they were screened for proper expression of the desired mutant using restriction map analysis. Once correct mutant strain was identified, large quantities of the bacteria were grown as described below.

**Growth of Bacteria**

The bacteria strains and plasmids used for this thesis project are listed in Table 1; both wild-type and mutant strains of *R. sphaeroides* were grown under the same conditions (Zhen et al., 1998). After determining successful uptake of the plasmid by screening the colonies, *R. sphaeroides* cultures were inoculated by adding 125µL of growth culture to Sis agar plates. Agar plates were placed in incubator 30°C until colonies formed. Colonies were gently removed; each colony was added to 3mL Sis media in 14mL sterile round bottom Falcon tubes (Beckton/Dickinson). These cultures were shaken at 300rpm at 30°C in a light deprived table top incubator shaker until the cultures
**Table 1.** Bacteria Strains and Plasmids used in Site-directed mutagenesis of Subunit III of Cytochrome c oxidase. (Zhen et al., 1998)

<table>
<thead>
<tr>
<th>Bacteria Strain</th>
<th>Plasmid</th>
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<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>pMB301, pMB307, pRK415</td>
</tr>
<tr>
<td>HMB101</td>
<td></td>
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reached an OD660 of 0.9-1.2. From here, the cultures were then placed into a 250mL baffled flask containing 100mL 1X Sis media. The cultures were diluted down to a starting OD660 of 0.1 and grown again under the same conditions until they reached and OD660 of 1.0-1.2. Finally, 10mL of this media was transferred to 250mL Sis media growth cultures in 1L baffled flasks. The cultures were allowed to grow until they reached early log phase, signified by an OD of 0.9-1.2. This typically took a time period of 22-24 hours for wild-type, 16-20 hours for the mutants. Here, they were harvested by centrifugation at 11,300 x g for 10 minutes; then washed with 40mM KPhos, 100mM EDTA solution to remove remaining Tetracycline. The growth period for these *R. sphaeroides* cultures varied from growth to growth, but typically, it was discovered that the mutants E90H and E90H/H212E grew on average at comparable rates to wild-type. The other single mutant, H212E, on the other hand, grew significantly faster than wild type. It reached OD 0.9-1.2 within 15-16 hours, almost 25% faster. The wet weight cellular yield for the wild-type cells was on average 4.4g/L growth culture. The average cell yields for the mutants H212E, E90H, and E90H/H212E were 2.7g/L, 4.9g/L and 3.62g/L respectively.

**Preparation of Rhodobacter sphaeroides crude membranes**

Cells were thawed overnight in -20°C. The lysing process began by placing the cells on ice and adding 1mL Bionebulizer Buffer g/cell weight (30mM Tris, 10mM EDTA, 10mM MgCl$_2$, 20% glycerol, pH 8.0). Small amounts of DNase (Sigma) and lysozyme (Sigma) were added at concentrations of 50µg/mL and 25mg/mL respectively. PMSF in absolute ethanol was added to a final concentration of 100µM (B. van Gelder &
Slater, 1962; B. F. van Gelder, 1966). The sample was homogenized and run through C3 Avestin® Homogenizer two to three times until visual analysis revealed membranes were fully broken. Lysed cells were collected by centrifugation at 24,000 x g for 20 minutes at 4°C.

The supernatant was decanted, and PMSF was added once again to a final concentration of 100µM, and the sample was centrifuged in an ultracentrifuge (Beckman Optima LE-80K, Rotor Ti50.2) for 90 minutes at 150,000 x g. The pellet was extracted, resuspended in 10mL 50mM KHPO4, 1mM EDTA, pH 7.2. PMSF was added to a final concentration of 0.2mM and the extract was spun for a final 90 minutes at 150,000 x g. The supernatant was decanted and the resulting pellet was stored in -80°C (Zhen et al., 1998).

Protein Purification

Cells were purified by histidine-affinity chromatography—there is a histidine-tag on subunit I of the enzyme. The purified cytoplasmic membrane pellets were thawed overnight in -20°C. Cells were then resuspended and homogenized in approximately three pellet volumes of 40mM KCl, 10mM Tris-KOH, pH 8.0 resuspension buffer. The cell membrane mixture was placed on ice to stir as dodecyl maltoside was added to the mixture to a final concentration of 2%. The mixture was then centrifuged at 37,000 x g for 20 minutes at 4°C. The resulting supernatant was added to a 50mL polycarbonate tube. Imidazole was added to a 10mM final concentration along with 0.8mL of Ni²⁺-NTA agarose (Quiagen) per milligram of oxidase in the sample and allowed to rock on a platform at 4°C at moderate speed for 1 hour. The slurry was then placed into a Bio-Rad
glass econo-column (inner diameter=0.7cm, length=30-50cm) with the flow stopped to allow the mixture to settle. After 10 minutes, the column was allowed to pack at a rate of 1 drop every 3-5 seconds. Following the packing, 5-10 column volumes of 10mM Tris, 40mM KCL, 10mM imidazole, 0.1% DM pH 8.0 running buffer was used to wash the column. When the running buffer reached the top of the bed, 2-3 column volumes of elution buffer (10mM Tris-KOH, 40mM KCL, 0.1% DM, 100mM histidine, pH 8.0) were added and the flow rate was slowed to 1 drop every 7-8 seconds to allow the protein to concentrate during elution. The elution buffer ran through the column until all the protein (green) was visibly off the column. Elutants were pooled together, placed in Microcon Centrifugal filter devices (Millipore) with a 100K molecular weight cut off, and washed with 10mM Tris, 40mM KCL, pH 8.0 to remove excess histidine and concentrate the samples. The amount of protein retrieved was calculated using 

$$\Delta_{606(\text{reduced-oxidized})} = 24,000 \text{ M}^{-1}$$ (B. F. van Gelder & Muijsers, 1966).

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

Cytochrome c oxidase from Ni$^{2+}$-NTA column was tested for purity via SDS-PAGE. Three to four micrograms of oxidase was incubated with 2% SDS for 30 minutes at 37 °C. Samples were then loaded onto a 1mm thick SDS gel (Bio-Rad Ready Gel Tris-HCl, pH 8.8, Mini-PROTEAN) consisting of 6% acrylamide-6M urea stacking layer (pH 6.8) and a running layer of 16% acrylamide- 6M urea (pH 8.8). Electrophoresis was carried out at 60V for 20 minutes followed by 120V for two and a half hours (Fuller, Darley-Usmar, & Capaldi, 1981).


**Electron Transfer Activity**

The maximum activity of the purified oxidase was tested polarographically using a Clark oxygen electrode assay (Yellow Springs Instrument Company Model 17372). The purified protein was diluted to 2µM heme aa₃ in 50mM KHPO₄ pH 6.5, 0.1% DM buffer and allowed to incubate on ice for 15 minutes. To measure the auto-oxidation rate of the reductants—20-50µM cytochrome c (Sigma Type III), 0.6mM TMPD, and 18mM ascorbate—the assay was ran without oxidase in 50mM KHPO₄/0.1% DM pH 6.5 buffer for 2.5 minutes. Subsequently, to attain the maximum rate of COX activity, 5-10 picomoles of oxidase were added and the assay was allowed to run for an additional three to four minutes.

**pH Dependence of Wild Type and Mutant Activity**

The pH dependence of the activity of the enzyme was measured also with Clark oxygen electrode (Yellow Springs Instrument Company Model 17372) at pH values at 0.5 pH increments from pH 6.0 to pH 10.0. Purified enzyme was diluted with 0.1% DM, 25mM HEPES pH 7.5 buffer and allowed to incubate on ice for 15 minutes. Three different buffers—25mM MES pH 6.0-6.5, 25mM HEPES pH 7.0-8.5, 25mM CHES pH 9.0-10.0. KCl was added to each buffer as needed to allow for optimal constant ionic strength at each pH. Buffer ionic composition was calculated via online buffer calculator (http://www.liv.ac.uk/buffers/buffercalc.html).

The autooxidation rate was measured without enzyme for 2.5 minutes in 25mM MES, HEPES, or CHES buffer with additional reagents 20-50µM cytochrome c (Sigma Type III), 0.6mM TMPD, and 18mM ascorbate. Subsequently, for rate analysis, 5-10
pmoles of oxidase were added and assay was allowed to run for additional 3-4 minutes. As pH of the buffers increased, a greater amount of oxidase was needed to measure the rate.
III. RESULTS

Previous alterations in the conserved hydrophobic pocket of SIII have yielded decreases in enzymatic activity; either by slowing the proton pumping mechanism, electron transfer activity rate, or both. This study in particular explores the assumption that the connection between helix 3 and 6 of the hydrophobic pocket is vital to SIII and COX function as a whole.

Mutation of conserved amino acid residues E90 and H212 of Subunit III

To accomplish the goal of this study, three mutations were created. E90 was mutated to histidine (E90H) and H212 was mutated to a glutamate (H212E). Third, in the primary amino acid sequence of SIII, the positions of these two residues was exchanged, yielding a double mutation (H212E/E90H). These mutations are different from previous single E90 point mutations in that the inserted residue is not neutral, it contains a charged side chain. This charge may cause additional interactions not just at the hydrophobic region it is inserted, but also affect conformations of the other subunits. After performing DNA sequencing to verify mutation at the correct location in the sequence, large quantities of bacteria *R. sphaeroides* containing each mutation and also the wild type were grown in Sis media. It was found that E90H/H212E and E90H grew at relatively comparable rates to wild-type. In contrast, H212E grew significantly much
faster, almost 1.5x the rate of wild-type (data not shown). Faster growth of the bacterium indicates that oxidase may exhibit abnormal expression (Zhen et al., 1998).

Lysing of each mutated cell strain revealed that some oxidase was present. Spectrophotometric analysis of heme a and aa₃ of the mutants and control membranes were conducted using a Hewlett Packard 8453 UV/Visible diode array spectrophotometer to measure absorbance from 200nm to 800nm. Three enzymatic conditions were analyzed—resting state, oxidized (with FeCN), and reduced (with sodium dithionite). Then the difference spectrum of the reduced state minus the oxidized state (R-O) was taken. Figure 12a shows the fully reduced visible absorption spectra analysis of the mutant strains isolated in *R. sphaeroides* in membranes. A sharp absorption peak at 420nm and 550nm show cytochrome c expression in the membranes. Figure 12b shows reduced-minus- oxidized (R-O) visible absorption spectra of the membrane mutant strains. It has been established that the α-peak of heme *a* (Subunit I), also known as *aa₃*, of oxidase is located at 605-606nm (Shapleigh et al., 1992). The α-peak is present in the wild type and the mutations (although sharply decreased in H212E), showing that heme *aa₃* is present. All of the mutants, with the exception of H212E, exhibited a 1-2nm red shift in the α-peak when compared to wild-type, which may suggest that the environment around heme *a* in SI of these mutations is perturbed. H212E does not display an α-peak at 605nm, suggesting that there is minimal heme *aa₃* expression in the protein bound membrane. This lack of expression may be due to the two negative charges of glutamate causing charge repulsion resulting in distortion of the COX quaternary structure.
Figure 12. Visible absorbance spectra of mutant and wild-type *R. sphaeroides* membrane. **A.** Fully dithionite reduced visible spectrum. The peak at 605-606nm refers to the aa₃-type oxidase. The peak at 420nm refers to cytochrome c. **B.** Dithionite-fully reduced minus ferricyanide-oxidized visible spectra of cytoplasmic membranes of WT, E90H/H212E, E90H, and H212E. Samples were diluted in 50mM potassium phosphate, 1mM EDTA, pH 7.2.
Purification of COX from their cytoplasmic membranes was performed by Ni\textsuperscript{2+}-NTA chromatography using a histidine-tag adhered onto the C-terminus of SI (Mitchell & Gennis, 1995). Before purifying on the Ni\textsuperscript{2+}-NTA column, the mutant oxidase strains were solubilized in 3% DM solution (Zhen et al., 1998). Ni\textsuperscript{2+}-NTA chromatography yielded similar amounts of H212E/E90H and E90H per gram of cellular wet weight as compared to wild-type. However, H212E did not yield significant oxidase during purification. Any H212E protein expressed failed to bind to the nickel column during chromatography, resulting in little or low yield. The chromatography was repeated with two different membrane preps achieving similar results.

**Integrity and Subunit Composition Analysis of purified mutants**

The purified mutants were analyzed via SDS-PAGE to identify subunit composition and determine the quality of purification of the enzyme (Figure 13). It has been well known that mutations involving conserved residues of COX-SIII can result in significant distortion of the protein structure, sometimes leading to the partial loss of one of the subunits (Geyer, 2007). SDS-PAGE gel was used to address this concern. Figure 13 shows the SDS-PAGE gel with the two purified mutants flanked by purified wild-type. All of the subunits in the complex are present in the mutants. Equivalent amounts of heme aa\textsubscript{3} were loaded on each lane. The intensity of the bands is comparable to wild-type, indicating that there is no partial loss of a subunit. Subunit II of both E90H and H212E/E90H both display a ‘doublet’ band, as characteristic of wild-type, which is thought to be due to incomplete processing of SII.
Figure 14 shows the R-O difference visible absorbance spectra of the purified showing heme $a$ absorbance bands. In the difference spectrum, E90 displayed a 1nm red shift (Fig. 4B). The ratio of Soret band (442nm-444nm) to the $\alpha$-band (605nm) is used to verify the presence of both hemes $a$ and $a_3$ (B. F. van Gelder & Muijsers, 1966). The wild-type ratio presented here is 5.45, consistent with previous results published (Hosler et al., 1992). The ratio of E90H is 4.42, while E90H/H212E is 7.1.

CHARACTERIZATION OF THE MUTANT COX ENZYMES

Maximal Steady-state activity

Mutant COX-III enzymes electron transfer rates were analyzed. Ability of the mutated enzymes to reduce oxygen effectively is important as it gives further insight as to whether mutagenesis caused this COX function to be impaired (Zhen et al., 1998). To determine whether the mutation affected the binuclear active site, maximal activity experiments were carried out polarographically in an oxygen electrode in non-denaturing detergent solution. The rate of oxygen reduction for wild-type was consistent with that of published results (Cvetkov, 2010; Geyer, 2007; Gilderson et al., 2003). Both mutations exhibited decreases in steady-state electron transfer activity. The single mutant, E90H, portrayed a significant decrease in activity. The activities of the two different enzymatic preparations were determined with multiple activity assays each. The results are shown in
Figure 13. SDS-polyacrylamide gel electrophoresis of wild-type and mutant *R. sphaeroides* cytochrome *c* oxidase. Comparison and analysis of Subunit content in mutated and WT COX isolated by histidine-tagged Ni$^{2+}$-NTA column chromatography. Three µg enzyme were incubated with 3% SDS in Laemmli buffer for 30 minutes at 37°C. Denatured samples were loaded onto a 16% acrylamide gel containing with 6 M urea and 0.1% SDS, pH 8.8. Voltage was run at 60V for 20minutes, 120V for 2.5 hours. Gel was washed with deionized water three times then stained with Bio-Rad Coomassie G-250 buffer. Picture taken on a Fuji analyzer. Lane 1 and 4, Wild-type; Lane 2, E90H, Lane 3, E90H/H212E. Subunits are labeled. Subunits are present at similar stoichiometries to wild-type. Subunit II displays a doublet, which is characteristic of wild-type and also exhibited in both mutant strains. (Fuller et al., 1981).
Figure 14. Visible absorbance spectra of purified mutants and wild-type cytochrome c oxidase. **A.** Fully reduced dithionite visible spectrum. **B.** Dithionite-reduced minus ferricyanide-oxidized visible spectra of purified oxidase strains of WT, E90H/H212E, and E90H. Soret peak (442nm) exhibits one sharp peak, emphasizing the purity of the protein. Ratio of Soret band to \( \alpha \) peak for is WT 5.45, similar to previous studies (Hosler et al., 1992), and mutants E90H and E90H/H212E exhibit 4.42 and 7.1 respectively.
A.
B.

![Graph showing absorbance at 605nm](image)

- Red line: E90H/H212E
- Green line: E90H
- Black line: WT

Absorbance (AU) vs. Wavelength (nm) with a peak at 605nm.
Table 2. E90H demonstrated an average decrease in activity of 39%. This decrease in activity could be due to perturbation of the SI active site as SIII lies in close proximity to SI (Hosler et al., 1994; Hosler et al., 2006). The single mutation could have caused a conformational change in SI, thus impairing the pathway for electron transfer.

E90H/H212E displayed activity characteristics more closely to that of wild-type, losing only 13% activity where E90H lost nearly 40% activity. This gives positive affirmation that the connecting bond is vital to enzyme activity.

**pH Dependence of Electron Transfer**

Heme $a$ is an important cofactor in the electron transport chain of COX. It allows for the transfer of electrons to the active site (Hosler et al., 2006). In addition, it has been shown that COX activity decreases with increasing pH (Cvetkov, 2010; Gilderson et al., 2003; Riegler et al., 2005; Zaslavsky & Gennis, 1998). Subunit III is necessary for efficient steady state activity of oxidase (Gilderson et al., 2003). Previous work has shown that wild type oxidase steady state activity has a pKa of 8.5 (Gilderson et al., 2003). Here, the pH dependencies of the steady state activities of wild type and mutants are shown (Figure 15). Assays were performed at nine different pH’s, in 0.5 pH increments, starting at 6.0 and concluding at 10.0. Constant ionic strength conditions were maintained. The data were fitted with a sigmoidal curve assuming a single functional pKa group to calculate pKa. Wild type pH titration curve calculations yielded a pKa of 8.5, in agreement with previous work (Cvetkov, 2010; Gilderson et al., 2003). The rate-limiting step in COX steady-state turnover is the internal electron transfer from
Table 2. Maximal Cytochrome c Oxidase Activity. Maximal Oxidase activity was measured polarographically by oxygen electrode. Oxygen reduction activity was analyzed in a reaction containing 50mM KPhos, 0.1% DM at pH 6.5, 18mM ascorbate, 0.6mM TMPD, 30µM horse heart cytochrome c (Sigma), and 1pmol of oxidase.
Figure 15. pH dependence of Cytochrome c Oxidase Electron Transfer Maximal Activity. These assays were performed at varying pH ranging from 6.0 to 10.0. Buffers used were 25mM MES (pH 6.0-6.5), 25mM HEPES (pH 7.0-8.0), 25mM CHES (pH 8.5-10.0). The results are displayed as a fraction of maximum activity with standard error values displayed. A sigmoidal curve equation was used to fit the data. Four to six trials of each form of oxidase was conducted. The pKa value of WT (black curve) was calculated at 8.5, equivalent to published literature. The pKa values of E90H/H212E (red curve) was statistically equivalent at a value of 8.9 (p=2.7), while that of E90H (green curve), 9.0, was not (p=2.2). Conformational equilibrium calculations revealed that the slopes not equal to 1; hence suggesting that more than one proton is interacting with COX at the pKa value (data not shown in figure).
heme $a$ and Cu$_A$ to oxygen at the Cu$_B$-a$_3$ binuclear center and thus, this pKa reflects that electron transfer step (Wilson et al., 1981). The pKa values for E90H and double mutant E9H/H212E were calculated (by sigmoidal fit equation) to be 9.0 and 8.9 respectively; where the E90H/H212E mutant was statistically equivalent. The mutants exhibit a slight alkaline shift in pKa. This change implies that there may be structural changes in SI as a result of the mutation in SI. Additionally, based on conformational equilibrium calculations, there may be more than one proton associating with the enzyme’s active site at this calculated pKa value. This further supports the claim that the proton channel pathways may be perturbed.
IV. DISCUSSION

Summary of results

Subunit III of cytochrome c oxidase is highly conserved—occupying nearly 90% conservation across species. Several theories have been proposed for its function, but its exact role in COX is unknown. Although it does not contain any of the metals or heme proteins involved in the reduction of oxygen, it does however indirectly affect the catalytic activity of the enzyme. When this alphahelical subunit is biochemically removed from the enzyme, there is a significant decrease in proton pumping and electron transfer (Nalecz, Bolli, Ludwig, & Azzi, 1985; Prochaska & Reynolds, 1986). In addition, when SIII is genetically removed from the enzyme, similar results are obtained (Cvetkov, 2010; Haltia et al., 1989). This therefore suggests that SIII is vital to the enzyme and its ability to catalyze the reduction of oxygen in the electron transport chain.

The conserved hydrophobic pocket in SIII contains hydrophilic amino acid residues that are nearly 90% conserved across species. In the native conformation, a cluster of three amino acids lie in close juxtaposition forming a triad. These three residues—E90, H212, and Y246—each residue within hydrogen bonding distance of each other (Figure 9). E90 is located on helix 3 while Y246 and H212 reside on helix 6. Because E90 and H212 are located on adjacent helices, it is assumed they are connected via a salt bridge or hydrogen bond. Ogunjimi et. al determined that these residues are indirectly involved in the electron transfer and proton pumping mechanism of bovine
COX. The organic compound DCCD reacted with COX to covalently bind to E90, leading to a disruption in the E90:H212 interaction. This caused the enzyme to exhibit a reduction in proton pumping and activity (Ogunjimi, Pokalsky, Shroyer, & Prochaska, 2000). In addition, mutation of Y246 to phenylalanine resulted in normal COX activity; hence the Y246 connecting bond must not be vital to COX function (Geyer, 2007).

The motivation of this current work is to focus on and to analyze in-depth the roles of conserved residues E90 and H212 in oxidase functioning. In this investigation they are examined to determine whether the critical nature of the spatial arrangements or the salt bridge/hydrogen bond is required for COX functional activity. It is anticipated that the connecting bond—either a salt bridge or hydrogen bond—is critical for optimal oxidase function. This is modeled when DCCD binds to E90 of COX SIII, disrupting this connecting bond with its partner H212, ultimately leading to decrease in COX functioning.

In the current study, conserved residues E90 and H212 of SIII were successfully mutated to yield E90H, H212E, and double mutant E90H/H212E in the bacterium *R. sphaeroides*. Two of these mutants, E90H and E90H/H212E exhibited growth comparable to wild-type. They reached optical densities of 1.0-1.2 within the 20-24 hour time frame exhibited by wild type protein. Mutant H212E, on the other hand, grew extremely rapid, nearly 50 % faster. This increase in growth rate may be due to the lack of oxidase expression. H212E did not yield significant heme aa₃ with the same amount of growth media; hence, larger quantities of H212E had to be grown to yield significant enzyme for purification. It has been well established in our lab that mutant *R. sphaeroides* cultures that reach early log phase quickly, fail to express sufficient heme aa₃ (Cvetkov,
unpublished results). With the two glutamates E90 and E212 residing in close proximity to each other, there may be some charge repulsion from the negative side chains (See Figure 10). This charge repulsion may cause a conformational change in SIII which in turn affects the proton channel pathways of SI, as SIII is connected to SI at the opening of the D channel (Echabe, Haltia, Freire, Goni, & Arrondo, 1995). The conformation of the environment around the active site may be affected as well, leading to the diminished heme $a$ expression. This decrease in heme $a$ expression was shown in the R-O spectrum of the purified mutant protein; the 605nm $\alpha$-peak displayed a 1nm red shift.

The visible absorbance spectroscopy gave further insight into the quality of the heme $aa_3$ expression in the mutants. In the solubilized membrane enzyme, dithionite reduced wild-type enzyme displays peaks at 420nm and 605-606nm, which corresponds to cytochrome $c$ and heme $aa_3$ respectively. The presence of a peak at 420nm in all three mutations verifies that the cytochrome $c$ binding site of SII is intact and cytochrome $c$ is able to bind to the enzyme. Subunit II is needed during COX formation to ensure proper insertion of CuB at the active site (M. R. Bratton et al., 2000). CuB is the site where oxygen binds; without CuB, the enzyme may lack the ability to reduce oxygen. Cytochrome $c$ binding to COX is imperative because it is the initial electron donor in the electron transfer mechanism of COX, the catalyst for oxygen reduction. These spectra show cytochrome $c$ binds to the enzyme, thus indicating that the mutant enzymes should display some activity.

Furthermore, R-O purified wild-type heme $aa_3$ results in a peak in the visible spectrum at 606nm. This $\alpha$-peak was present in comparable levels to wild-type based on grams per cell weight in both mutants E90H and E90H/H212E. The mutation consisting
of two glutamates—H212E—failed to yield significant aa₃ expression suggesting the environment around the heme a₃–Cu₉ active site is perturbed. Although SIII does not contain any of the metal centers involved in electron transfer, it does however indirectly affect the environment around the active site. Several studies have demonstrated that SIII participates in long range interactions involving charged residues and phospholipids with the active site (Ogunjimi et al., 2000; Varanasi et al., 2006). Hence, in regards to H212E, the mutation may cause conformational changes in SIII that lead to interruptions of the interhelical interactions with the active site at SI. It has previously been shown that during the catalytic reduction cycle, the active site changes conformation (Ganesan & Gennis, 2010). Heme a is the last reductant to transfer electrons to the active site. The lack of oxidase expression seen here in H212E may also be due to the mutant not fully receiving the electrons from heme a, due to possible environmental changes at or near the active site.

Purification

An attempt to purify all three mutants in addition to wild-type was made on Ni²⁺ affinity column chromatography. Purification was successful for three of the four enzymatic forms. H212E failed to bind to the Ni²⁺-NTA column during the initial steps in purification; roughly 75% of the enzyme was lost in the packing step (verified by visible absorbance spectrum analysis of the α peak). The remainder of the enzyme failed to purify; it appeared to become denatured as less than 0.01mg was extracted from the column, whereas 0.5mg of wild-type is expected for each gram of cell purified. Possibly,
the mutant was denatured during the purification process or it may have undergone improper enzyme assembly. In a study conducted by Shapleigh et. al where conserved residues of SI were altered, it was discovered that two histidine residue mutations could not be purified efficiently due to instability of the mutation (Shapleigh et al., 1992). Possibly mutating the H212 residue to a glutamate causes the enzyme to become unstable from charge repulsion within SIII. Furthermore, the two glutamates may cause a conformational change in the environment upstream from the mutation, causing perturbation of subunit III along with subunit I. Further experimentation of total isolated membranes, needs to be conducted in order to verify integrity of the subunits I and III in this particular mutation.

The other two mutants, E90H and H212E/E90H were successfully purified and yielded protein amounts comparable to wild-type per gram of cell weight. Gel electrophoresis revealed that all the subunits were present (Figure 13). SII even displayed a doublet as exhibited in wild-type (corresponding to the two oxidase fractions of differing lengths in SII), hence the subunit composition of the mutants appears to be retained. Visible absorbance spectra analysis of these mutations gave further insight into the nature of the environment around the heme aa3 active site. In the purified native enzyme, heme aa3 is observed at 606nm in the dithionite reduced minus oxidized visible spectrum. E90H/H212E displayed a peak consistent with wild-type, while E90H showed a red shift of 1nm in the purified difference spectra. This shift could imply that the environment around the heme centers is more hydrophobic. Modeling shows the two histidine residues to reside within hydrogen bonding distance of each other; hence the
longer wavelength displayed could be from the formation of a hydrogen bond between H90 and H212, causing a conformational change.

Activity and proton translocation

The DCCD binding to COX results in conformational changes in SIII. Here, it appears that the E90 mutation also altered the conformation of SIII, as the steady-state activity was decreased by 40% in this mutant form of COX. Hence, it is assumed that there is a disruption in the active site, consistent with Ogunjimi et al findings.

The first two steps of electron transfer to heme a and CuA occur rather rapidly (Mills et al., 2008). A recent study by Jancura et al determined that the rate-limiting step of the transfer of electrons to the active site is the initial electron transfer from CuA to the binuclear center (Jancura et al., 2006). The catalytic activity decrease of E90H further suggests that the environment at or around the active site is perturbed, affecting the transfer of electrons through to CuH–heme $aa_3$ active site. In the E90H mutation, the E90:H212 interaction is disrupted, suggesting it is important for COX enzymatic function.

The double mutant E90H/H212E, on the other hand, did not display a similar decrease in catalytic activity as E90H; the activity was reduced by only 13%. This however further supports the claim that the E90-H212 connecting bond is required for optimal enzymatic function.

The pKa values for both mutations did exhibited an alkaline shift; with the E90H mutation revealing a larger change in pKa. The alkaline shift implies that the mutant COX has a higher affinity for protons. The mutation may have caused a conformational
change in SIII that disrupted binding around D132 of SI, the entrance to the D-pathway. There are series of water molecules along the D-channel that assist with proton movement; these molecules may have been altered during SIII conformational change, creating a more hydrophobic environment. SIII may function to stabilize SI and the D-channel. In experiments where SIII is removed from the enzyme, pKa decreases to 7.2 and there is a 50% reduction in steady-state activity (Cvetkov, 2010). This acidic shift in pKa implies there is a decrease in the proton uptake path, suggesting SIII is needed for proper proton translocation. Moreover, proton uptake through the D-channel is coupled with electron transfer to the active site. This further supports the claim that the proton pathway may be perturbed because there is a decrease in electron transfer. Because one electron is transferred for each proton pumped, slow proton uptake may negatively affect electron transfer rate.

In conclusion, this work describes successful mutation of two conserved amino acid residues in the hydrophobic pocket of COX-SIII. Results conclude that it appears that the connecting bond between E90 and H212 of SIII is important to the transfer of electrons of *R. sphaeroides* COX. Both mutations that yielded two like charges opposite each other (E90H and H212E) exhibited changes inconsistent with wild-type COX functioning. The H212E mutation failed to express oxidase and steady-state activity was nearly 50% reduced in E90H. Conversely, E90H/H212E retained the connecting bond and displayed higher activity values than E90H, more comparable to wild-type. Thus, it can be ascertained that the integrity of the active site is altered due to conformational changes in SIII. There is an alkaline pKa shift in both mutants, suggesting that the proton channels of SI are may be disrupted. To further analyze the change in SI and SIII due to
conformational changes, further experiments such as proton pumping analysis should be conducted to further verify this. As of now, it is suggested that it is not the spatial arrangements of conserved residues E90 and H212 that are vital to COX functioning, but the salt-bridge and/or hydrogen bond that connects the two residues in the native state.
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>$A_{560}$</td>
<td>Absorbance measured at 560 nm</td>
</tr>
<tr>
<td>$A_{605}$</td>
<td>Absorbance measured at 605 nm</td>
</tr>
<tr>
<td>$aa_3$</td>
<td>Hemes $a$ and $a_3$ of cytochrome $c$ oxidase</td>
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<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
</tr>
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<td>CHES</td>
<td>N-cyclohexyl-2-aminoethanesulfonic acid</td>
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<td>COX</td>
<td>Cytochrome $c$ oxidase</td>
</tr>
<tr>
<td>Cu+</td>
<td>Copper metal center</td>
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<tr>
<td>DCCD</td>
<td>N, N’-dicyclohexylcarbodiimide</td>
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<tr>
<td>DM</td>
<td>Dodecyl-$\beta$-D-maltoside</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
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<td>EDTA</td>
<td>Ethlenediaminetetraacetic acid</td>
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<td>F State</td>
<td>An oxyferryl intermediate of cytochrome $c$ oxidase</td>
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<td>FAD$^+$</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FADH$_2$</td>
<td>Reduced flavin adenine dinucleotide</td>
</tr>
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<td>H$^+/e^-$</td>
<td>Ratio of protons translocated to electrons transferred</td>
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<td>H-bond</td>
<td>Hydrogen bond</td>
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<td>HEPES</td>
<td>N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid</td>
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<tr>
<td>kb</td>
<td>Kilobase</td>
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<td>KPhos</td>
<td>KPO$_4$</td>
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<td>Mg$^{2+}$</td>
<td>Magnesium metal center</td>
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<td>NADH</td>
<td>Reduced nicotinamide adenine dinucleotide</td>
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<td>O State</td>
<td>The oxidized state of cytochrome c oxidase</td>
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<td>OD$_{660}$</td>
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<td>P$_M$ State</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
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<td>R State</td>
<td>The reduced state of cytochrome c oxidase</td>
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<td>R-O</td>
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<td>R. sphaeroides</td>
<td><em>Rhodobacter sphaeroides</em></td>
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<tr>
<td>SDS</td>
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<td>SI</td>
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</tr>
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<td>Zn$^{2+}$</td>
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