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Investigating the Undefined Role of Subunit III in Cytochrome c Oxidase Functioning Using Dicyclohexylcarbodiimide Chemical Modification; Insight Into Enzyme Structure and Molecular Mechanism

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I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Kelli N. Fisher ENTITLED Investigating the Undefined Role of Subunit III in Cytochrome c Oxidase Functioning Using Dicyclohexylcarbodiimide Chemical Modification; Insight Into Enzyme Structure and Molecular Mechanism BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Abstract

Fisher, Kelli N. M. S., Department of Biochemistry and Molecular Biology, Wright State University, 2014. Investigating the Undefined Role of Subunit III in Cytochrome c Oxidase Functioning Using Dicyclohexylcarbodiimide Chemical Modification; Insight Into Enzyme Structure and Molecular Mechanism.

In the cell metabolic cycle, cytochrome c oxidase (COX) is the final electron acceptor of the respiratory chain which reduces molecular oxygen into water. It is bound in the inner mitochondrial membrane and on the plasma membrane of bacterial species. Energy produced through electron transfer is coupled to the pumping of protons against the electrochemical gradient in order to fuel the proton motive force for the synthesis of most of the ATP in the cell. The three mitochondrial encoded subunits of COX, I, II, and III, are conserved across species and the complete function of subunit III remains unknown. Dicyclohexylcarbodiimide (DCCD) is an inhibitor of function which binds specifically to the conserved Glu-90 residue of subunit III. DCCD modification of COX has been shown to induce a conformational change in subunit III, which inhibits the proton pumping and electron transfer mechanisms occurring in subunit I of the enzyme.

This work analyzes the catalytic mechanism and environment of bovine heart and R. sphaeroides COX upon DCCD modification in order to gain insight into the significance of subunit III in the functioning of the enzyme as well as to compare the effects of the modification on catalytic activity. The effect of DCCD modification was also analyzed in both physiological and alkaline environments due to data which showed that bovine heart COX exhibits less inhibition of electron transfer activity at pH values 9.5 and 10.0, while R. sphaeroides COX shows less inhibition at pH values 6.5 and 7.0.
Both COX enzymes exhibited a steady biphasic pH dependence for electron transfer activity, suggesting that there are two proton binding sites critical in electron transfer activity. Bovine heart COX displayed an alkaline shift from 8.8±0.2 to 9.3±0.1 at site 2, while *R. sphaeroides* COX displayed an acidic shift from 7.8±0.4 to 7.3±0.4 at site 1.

To examine the effects of DCCD modification on the environment of hemes $a$ and $a_3$, the Soret region of the CD spectrum was analyzed. DCCD induced a red shift from 427.7 ± 0.3 nm in control to 428.2 ± 0.1 nm at pH 7.0 in bovine heart COX and from 429.2 ± 0.2 nm to 429.6 ± 0.1 nm at pH 10. In *R. sphaeroides*, a red shift in the CD spectrum was observed from 429.4 ± 0.1 nm in WT to 430.2 ± 0.1 nm in the DCCD-modified enzyme at pH 7.0 and from 431.5 ± 0.5 nm in WT to 431.9 ± 0.4 nm in DCCD-modified enzyme at pH 10.0. The heme $a$ and $a_3$ environment was also monitored using heme $a$ reduction during steady state electron transfer. Heme $a$ was found to be 18±1% reduced in control bovine heart COX during electron transfer and 33±2% reduced in DCCD-modified COX at pH 7.0 At pH 10.0, control bovine heart COX exhibited a heme $a$ reduction level of 67±7% and DCCD-modified enzyme yielded a reduction level of 93±6%. In0 *R. sphaeroides*, heme $a$ was found to be 41 ± 2% reduced and DCCD-modified enzyme was 36 ± 2% reduced at pH 7.0. At pH 10.0, WT *R. sphaeroides* exhibited a heme $a$ reduction level of 33 ± 4% and DCCD-modified enzyme yielded a reduction level of 47 ± 5%. In summary, our results indicate that the modification at Glu-90 in subunit III causes a perturbation to the catalytic cycle and its environment in subunit I. DCCD, while binding at a similar site in subunit III, leads to differential effects on the conformation and activity of the enzyme in bovine heart and *R. sphaeroides* COX. DCCD modification to subunit III of bovine heart COX may cause blockage of the
putative O$_2$ transfer pathway, while modification of subunit III in *R. sphaeroides* COX may induce a slowed proton uptake. Both of these effects will cause a decreased efficiency of electron transfer activity, and the difference in mechanism of inhibition between the two enzymes could be explained by variation in subunit structural homology between the two COX forms. Overall, it is clear that subunit III of cytochrome c oxidase, which lacks metal redox active centers, continues to reveal its significance to the catalytic mechanism and maintaining structural integrity of the enzyme.
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I. Introduction

Mitochondrion

Nearly all eukaryotic cells possess mitochondria, including plants, animals, fungi, and most protists (Reece et al., 2011). A mitochondrion is cylindrical in shape and is generally observed as 1 to 2 um long and 0.5 to 1 um wide, which is comparable to the size of an *Escherichia coli* bacterial cell (Tzagoloff 1982). These organelles are often referred to as “powerhouses” of the cell due to the vital metabolic processes it supports. The tricarboxylic acid cycle and the generation of ATP yield all life forms an energy currency and thus maintain viability (Willey et al., 2011). For this reason, the number of mitochondria within a cell correlates with its cellular metabolic activity and required energy expenditure. Some cells contain only one mitochondrion, while more commonly other cells contain hundreds or thousands of these organelles (Reece et al., 2011).

A mitochondrion is bound and compartmentalized by two phospholipid bilayers, an outer mitochondrial membrane and an inner mitochondrial membrane, which are separated by a 6 to 8 nm intermembrane space. Each compartment is unique in its enzymatic and chemical composition (Willey et al., 2011). The smooth outer mitochondrial membrane contains many enzymes which are involved in fatty acid and amino acid metabolism (Benz 1986). Another feature of the outer mitochondrial membrane is porins, known as voltage-dependent anion-selective channels (VDAC). As the most abundant protein of the outer membrane, porins are predicted to exist as a β barrel structure similar to its bacterial counterparts. They function by forming a pore that allows the passage of small molecules across the membrane, allowing the outer membrane to be more permeable (Krimmer et al., 2001).
The inner membrane of the mitochondrion is able to obtain a large surface area due to its dense infoldings called cristae. This bilayer is embedded with enzymes and electron carriers which aid in electron transport and oxidative phosphorylation. The inner membrane also varies in its lipid arrangement as compared to the outer membrane. Phosphatidylcholine (40%), phosphatidylethanolamine (35%), and cardiolipin (15%) are the major elements which compose the unique lipid content of the inner membrane. The inner bilayer is also highly enriched in protein (70%), with bovine heart mitochondria using close to 50% of these proteins for catalyzing the oxidative phosphorylation system. It is the abundance of protein as well as the addition of transport proteins which add control and create a much lower permeability of the inner membrane. (Hatefi 1985).

The innermost region of the mitochondria is the matrix, and the enzymes located in this area are involved in the catabolism of fatty acids as well as the tricarboxylic acid cycle. These pathways create the reduced cofactors NADH and FADH$_2$ which are shuttled to and used as a major electron source for oxidative phosphorylation (Willey et al., 2011). Here these reduced substrates will donate electrons through a respiratory chain composed of metalloprotein complexes that are embedded in either a mitochondrial or bacterial membrane (Hosler et al., 2006).

*The Respiratory Chain and its Role in Oxidative Phosphorylation*

The respiratory chain of eukaryotic organisms is embedded within the inner membrane of the mitochondria, while the prokaryotes respiratory chain is located in the cytoplasmic membrane (Hatefi 1985). Also known as the electron transport chain, this pathway has been shown to exhibit three properties which are fundamental to metabolic
Figure 1. The Respiratory Chain. Located in the inner mitochondrial membrane of eukaryotes and the plasma membrane of prokaryotes, the respiratory chain and its components create a stepwise process responsible for creating ATP. Reduced cofactors (NADH and FADH$_2$) created through cellular respiration transfer electrons to the chain, where they are passed from one complex to the next in order to simultaneously produce a proton gradient. (Rich and Merechal, 2010)
and regulatory functions of a cell; (1) maintaining essential oxidized levels of NAD within an aerobic cell through the transfer of protons and electrons from substrates to oxygen; (2) acting as a system of three of more energy conservation steps which promotes ADP to be converted to ATP, making a common medium available for energy expenditure throughout the cell; (3) regulating metabolism according to levels of control substances and rates of efficiency of energy conservation in the cell (Chance and Williams, 1956).

Bovine heart mitochondria exhibit an electron transfer chain separated into four distinct functional enzyme complexes, which are rich in phospholipid (Green and Tzagoloff, 1966). These complexes are NADH-ubiquinone oxidoreductase (complex I), succinate-ubiquinone oxidoreductase (complex II), coenzyme Q-ferrocytochrome c oxidoreductase (complex III; bc1 complex), and cytochrome c oxidoreductase (complex IV), and function within the chain using the electron carriers coenzyme Q and cytochrome c (Hatefi, 1985). To complete the chain, ATP synthase (complex V) produces ATP from ADP and phosphate using chemiosmosis (Wittig and Schägger, 2009). The commonly cited ratio for the oxidative phosphorylation complexes I, II, III, IV, and V is 1:2:3:6-7:3-5. This was based on the measured content of FMN, covalently bound FAD, cytochrome c1, cytochrome a-a3, and F1, which are markers for complexes 1, 2, 3, 4, and 5 (Schägger, 2001). Bacterial respiratory chains are known to be similar in many aspects to those mitochondrial chains found in higher organisms. They are both bound by a membrane, composed of similar types of respiratory carriers, and capable of conserving energy which can be used for ATP synthesis or for other energy-dependent processes.
Electrons are passed stepwise to carriers with increasingly more positive reduction potentials, which aids in the completion of their transport throughout the entire respiratory chain. Oxidative phosphorylation, therefore, begins when electrons from NADH are passed to the flavin mononucleotide (FMN) and then to multiple iron-sulfur centers in complex I. Eventually two electrons and two protons reach ubiquinone (coenzyme Q), a small hydrophobic molecule which is mobile within the membrane, and four protons will be pumped through complex I. FADH$_2$ is another source for the electron transport chain, and it donates electrons to complex II, which are then passed to coenzyme Q. NADH and FADH$_2$ each contribute equivalent amounts of electrons to oxygen reduction, however, FADH$_2$ provides one-third less the energy for ATP production (Reece et al., 2011) The $bc_1$ complex moves protons from the negative to the positive side of the membrane using coenzyme Q in a redox loop mechanism called the Q-cycle. In this pathway, a fully reduced quinol carrying two electrons and two protons docks at the ubiquinone binding site near the positive surface of the enzyme. One electron is passed to a high potential Fe-S center, which then reduces cytochrome c. The other electron is sent to heme $b_L$ while two protons are released to the outer surface and the now oxidized quinol remains idle at a second quinone-binding site. After the binding and catalysis of a second reduced quinol, this oxidized quinol is reduced with accompanying uptake of two protons from the matrix. The overall pathway yields two reduced cytochrome c carriers and four pumped protons from the matrix into the intermembrane space or periplasm for bacteria (Hosler et al., 2006). The reduced cytochrome c carriers transfer electrons one at a time to complex IV, where oxygen acts as the final electron acceptor and is reduced to two water molecules with the use of four
chemical protons. Additionally, four more pumped protons are taken up from the matrix and expelled across the membrane (Willey et al., 2011).

Vectorial proton movement occurs at the complexes in which the chemical transfer of electrons conserves enough energy for proton uptake. The result of this proton expulsion creates a pH gradient of chemical potential energy as well as an electrochemical gradient of electrical potential energy (Willey et al., 2011). This produces a more alkaline and negative environment in the matrix or cytoplasm of bacteria. The combined potential differences produce the proton motive force that allows protons to flow down the concentration and charge gradients through exergonic means in order to fuel ATP synthase in phosphorylating ADP (Willey et al., 2011); however, a portion of protons pumped by the respiratory chain will return to the matrix through leaks in the inner membrane rather than driving ATP synthesis and transport (Kelso et al., 2001).

**Cytochrome c Oxidase**

Since its discovery, cytochrome c oxidase has been studied intensively due to its physiological importance and remarkable catalytic reaction (Tsukihara et al., 1996). It is a firmly membrane-bound enzyme which is undoubtedly a component of the cristae in mitochondria or the cell membrane in bacteria (Svensson-Ek et al., 2002). As the terminal catalyst in the respiratory chain, it is a two heme-a, three-copper metalloenzyme which functions as the final electron donor to molecular oxygen. The redox reactions occurring within this enzyme allow energy to be released and transduced into an electrochemical gradient by pumping protons vectorially across the mitochondrial inner
membrane (Ogunjimi et al., 2000). It is the uptake of protons to form water, accompanied by the reduction of O2 which plays a central role in maintaining the proton pump mechanism (Wikstrom 1998).

During each turnover of the enzyme, eight protons are taken from the matrix side of the membrane, with four chemical protons used to reduce oxygen and four pumped protons used to create the proton motive force. Crystal structures of cytochrome c oxidase indicate two proton input channels which lead from the inner surface of cytochrome c oxidase toward the buried binuclear center where O2 binds to heme a3 (Hosler 2006). The K channel is named for its highly conserved lysine residue K362 (*Rhodobacter sphaeroides* numbering is used if not indicated otherwise) and is responsible for delivering two of the four chemical protons to the heme-copper active site for O2 reduction. The D channel, named for its conserved aspartate residue D132 (D124 of bovine CcO), provides all four pumped protons as well as two of the four chemical protons used in reduction of O2 at the binuclear center. (Zhu et. al, 2010; Tomson et al., 2003). The proton uptake is strongly enhanced for the D-pathway due to proton-collecting “antenna” groups which are located around its entrance (Wikstrom and Verkhovsky, 2007; Alnajjar et. al, 2014).

Additionally, a network of hydrogen-bonded water molecules link the aspartate residue to another highly conserved glutamate reside (E286), which is proposed as the branching point for both chemical and pumped protons (Zhu et al., 2010; Adelroth et al., 1997). The H channel in bovine cytochrome c oxidase is composed of a hydrogen-bond
Figure 2. Schematic Diagram of the Reaction Catalyzed by Cytochrome c Oxidase.

A reduced cytochrome c passes electrons to the Cu₂ site in subunit II. Electrons are then transferred to subunit I to heme a and the heme a₃-Cu₄ binuclear center, where oxygen is reduced to water by chemical protons. Pumped protons are also taken up through the D channel and expelled to contribute to the proton motive force. Adapted from (Gennis and Ferguson-Miller, 1996).
network which is connected to the intermembrane side of the enzyme and a water channel which opens into the matrix side. The hydrogen bond network forms two hydrogen bonds with a fixed water molecules from the network to heme a through its propionate and formyl groups. Asp-51 is a key residue in this pathway and is suggested to function as a proton pumping site in bovine CcO (Yosikawa et al., 2011). There is no analogous amino acid residues for the H channel in the R. sphaeroides enzyme.

Electron transfer reaction

Cytochrome oxidase contains four metal redox centers, two of which are copper atoms, and two heme a groups. These centers are engaged in the electron transferring as well as active in the dioxygen-binding site (Saraste 1990). The net equation for the complete reaction of cytochrome c oxidase is: 4 Cyt. $c^{2+}$ + O$_2$ + 8 H$^+$ (mitochondrial matrix) $\rightarrow$ 2 H$_2$O + 4 Cyt. $c^{3+}$ + 4 H$^+$ (intermembrane space). The catalytic cycle begins when a soluble cytochrome c binds to subunit II to donate one electron at a time to Cu$_A$. After a cytochrome c molecule delivers an electron to the Cu$_A$ site, the electron is transferred to the six coordinate heme a site in subunit I and then passed to the heme $a_3$-Cu$_B$ binuclear center, also in subunit I (Bratton et al., 1999). At the heme $a_3$-Cu$_B$ O$_2$ reduction site, oxygen intermediates are converted to water by two chemical protons through the K channel. At the same time, two protons are pumped through the D channel. To complete the catalytic cycle of cytochrome c oxidase, two more cytochrome c molecules will bind to subunit II sequentially, leading to the reduction of an oxygen intermediate by two chemical protons through the D channel this time. This yields a total of two water molecules produced. The D channel will also uptake protons to be pumped
and yield a net of four pumped protons/4e\(^-\) altogether. The catalytic cycle therefore uses protons as substrate in the reaction that drives proton pumping and as the ion which is pumped across the membrane (Brzezinski et al., 2013).

As shown in Figure 3, there are two phases of the catalytic cycle in cytochrome \(c\) oxidase. In the R state, heme \(a_3\) and Cu\(_B\) are reduced. Oxygen binds to heme \(a_3\) only after heme \(a_3\) and Cu\(_B\) are reduced, creating the A state (Hosler 2006). Electron transfer from heme \(a\) to the catalytic site then breaks the dioxygen bond to form the \(P_R\) state. The transfer of a proton from the E286 residue to the binuclear center will create the F state. E286 is reproto

\[\text{Electron transfer from heme } a \text{ to the catalytic site then breaks the dioxygen bond to form the } P_R \text{ state. The transfer of a proton from the E286 residue to the binuclear center will create the F state.} \]

\[\text{E286 is reproto} \]

\[\text{and yields the oxidized } O \text{ state of the enzyme (Johansson et al., 2013). The two phases of the catalytic cycle—metal reduction and } O_2 \text{ reduction—merge to some extent; however, the metal reduction phase is fundamentally } O \text{ to } R \text{ and the } O_2 \text{ reduction phase is } A \text{ to } F \]

(Hosler 2006).

\[X\text{-ray Crystal Structure of Bovine Heart Cytochrome } c \text{ Oxidase} \]

Crystals from cytochrome \(c\) oxidase from beef heart muscle have been resolved to 1.8 angstroms (Tsukihara et al., 2003). The x-ray crystallographic images reveal 13 unique subunits, 10 of which are encoded by nuclear genes and 3 highly conserved subunits encoded by mitochondrial genes. The structure also contains five phosphatidyl ethanolamines, three phosphatidyl glycerols and two detergent molecules (cholates), along with the redox components: two hemes a, and three coppers. One magnesium and
Figure 3. The Catalytic Intermediates. During steady-state turnover, the metal reduction phase (O to R) and oxygen reduction phase (A to F) occur within a catalytic cycle, producing a sequence of intermediates. In this figure, Y refers to the Y288 residue. O refers to the oxidized enzyme, with heme a$_3$ and Cu$_B$ are in their oxidized forms. The R state indicates the enzyme is reduced when heme a$_3$ and Cu$_B$ gain electrons and proton addition creates H$_2$O. O$_2$ then binds to form A, the oxy state, and O$_2$ bond scission occurs to form the more stable P oxoferryl state. The F state occurs upon addition of one proton and is considered the protonated form of P, while H$_2$O is produced. The addition of two more electrons then recreates the O state. (Hosler 2006)
one zinc atom are also embedded in the structure (Tsukihara et al., 1996). X-ray images, cross-linking and gel filtration studies suggest that bovine cytochrome c oxidase exists as a dimer in the membrane (Estey et al., 1990; Nyugen et al., 2002; Rubinson et al., 2013). Each monomer has a calculated molecular mass of 204,005 kD, with the molecular mass of each subunit ranging from 56.9-4.9 kDa (as seen in Table I) and contains of 28 transmembrane alpha helices. 21 of these transmembrane alpha helices are found within the mitochondrial encoded subunits, while 7 transmembrane alpha helices are in the nuclear encoded subunits. The monomers face one another with a concave surface, creating a large central opening and limiting contact between them (Tsukihara et al., 1996; 1998). The x-ray crystal structure largely attributes the monomer-monomer contact to subunits VIa and VIb, which are subunits that are lacking in bacterial forms of the enzyme (Cvetkov and Prochaska 2007).

**Mitochondrial Encoded Subunits**

**Subunit I**

Subunit I is the largest subunit of cytochrome c oxidase and has mainly a transmembrane domain composed of 12 membrane helices which are arranged in three semi-circular bundles of four helices each. Two of these bundles hold heme \( a \) and heme \( a_3 \). The starting and ending points of each helix are essentially located at the membrane surface, creating a flat top and bottom to the enzyme and a cylindrical shape. The enzyme has an orientation perpendicular to the membrane surface, and holds Cu\(_B\) near the molecular exterior, facing the space between the monomers (Tsukihara et al., 1996).
The low spin heme (heme $a$) iron, with paired electrons filling the lower energy orbitals, is bound to six nitrogens in both of its oxidation states. In subunit I, it is coordinated to His 61 (bovine) of helix II and His 378 (bovine) of helix X, and also functions to transfer electrons from Cu$_A$ to the binuclear center. When reduced, the charges associated with Fe$^{2+}$ are stabilized by utilizing the resonance of the porphyrin ring. While oxidized, the Fe$^{3+}$ has one positive charge which is not neutralized, and the relocation of this charge within the porphyrin electron system can promote deprotonation of heme-propionic acid side chains (Tsukihara et al., 1995; Tsukihara et al., 2003). According to studies done by Tsukihara et al. (2003), improved x-ray structures at 1.8/1.9 Å resolution in the fully oxidized and reduced states of bovine heart cytochrome $c$ oxidase hypothesized that this net positive charge drives the active proton transport from the interior of the mitochondria to the Asp-51 (a key residue of the H channel in bovine CcO) across the enzyme through a water channel and a hydrogen-bond network. Thorough analysis of x-ray structures of both bovine and bacterial cytochrome $c$ oxidase suggest heme $a$ as the driving element of proton pumping, although there is no homologous amino acid to the bovine heart Asp-51 found in the H channel structure of the bacterial enzymes (Yoskikawa et al., 2011).

The binuclear center of cytochrome $c$ oxidase is the site of O$_2$ reduction. It includes a high-spin heme a$_3$ in which higher energy orbitals are filled with unpaired electrons and a type-II Cu$_B$ ion, which are located 4.5 angstroms apart. Heme a$_3$ is a five coordinate heme, bound to His 376 (bovine) in helix X of subunit I. Cu$_B$ is a ligand to three histidine residues: His 240 (bovine) of helix VI and His 290 and 291 (bovine) in non-helical fragments between helices VII and VIII (Tsukihara et al., 1995). A unique
Figure 4. Crystal Structure of Oxidized Beef Mitochondrial Cytochrome c Oxidase.

(a) A transmembrane view of beef mitochondrial cytochrome c oxidase. (b) An overhead view of the molecule, clearly displaying its dimeric property. Mitochondrial encoded subunits: Subunit I (cyan) is composed of 12 transmembrane helices and contains heme $\alpha$ (yellow) as well as the binuclear center, heme $\alpha_3$-$\text{Cu}_B$ (yellow heme, green sphere $\text{Cu}_B$). Subunit II (orange) is composed of two transmembrane helices, the cytochrome c binding site located above the membrane, and also contains the bivalent $\text{Cu}_A$ site (green spheres). Subunit III (pink) is composed of seven transmembrane helices and holds none of the redox centers of the enzyme. Nuclear encoded subunits (gray). Accelrys DS Viewer Pro. was used to prepare this figure. PDB 3aso (Tsukihara et al., 2003).
Table I

Subunits and Molecular Weights of Bovine Heart Cytochrome c Oxidase

<table>
<thead>
<tr>
<th>Subunit&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Molecular Weight (Da)&lt;sup&gt;b&lt;/sup&gt;</th>
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</tr>
<tr>
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<td>5,541</td>
</tr>
<tr>
<td>VIII</td>
<td>4,962</td>
</tr>
</tbody>
</table>

<sup>a</sup> The nomenclature of Kadenbach et al. (1983) is used for subunits, and they appear in order of their migration on SDS-PAGE except for subunit VIII which migrates with VII<sub>b</sub>.

<sup>b</sup> Molecular weights of the subunits were determined using known amino acid sequences (Buse et al., 1983; Capaldi, 1990) or mitochondrial DNA sequence (Anderson et al., 1982).
property of the binuclear center is the covalent link of Tyr-280 to one of the three histidine ligands of CuB. This residue is located at the end of the K channel and is highly conserved among heme-copper oxidases (Gray et al., 1994). Data reported by Pinakoulaki et al. (2002) have shown that the cross-link His-Tyr generates a unique environment around CuB and holds it in a certain distance and position from heme $a_3$, suggestive of structural and functional importance.

Subunit II

Subunit II is composed of two transmembrane helices. It exhibits a large extramembrane domain above the cytosolic surface of subunit I, which holds the binding site for reduced cytochrome c; this is composed of a ten strand $\beta$ barrel structure that holds a diatomic copper (CuA) site 7 angstroms to the nearest surface atom, allowing for electron transfer from the cytochrome c carrier (Tsukihara et al., 1996)

The CuA center is made up of six ligands: Cys-196, Cys-200 (type I Cu), His-161, His-204, Met-207, and a peptide carbonyl of Glu-198. X-ray crystals provide an arrangement which is consistent with a dinuclear copper center, exhibiting two copper atoms bridged by two sulfur atoms from Cys-196 and Cys-200 with a 2.7 angstrom separation. One copper atom is bound with the imidazole nitrogen of His-161, the sulfur of Met-207, and the two cysteine sulfur atoms to form a tetrahedral coordination. The second copper atom also displays tetrahedral coordination, bound to the imidazole nitrogen of His-204, the peptide carbonyl of Glu-198, and the two sulfur atoms of the cysteine residues (Tsukihara et al., 1995)
**Subunit III**

Subunit III is the second largest subunit of COX which lacks active redox centers, and its function is unknown (Nguyen et al., 2002). This subunit contains seven transmembrane helices which are arranged into two bundles that create a V-shaped cleft between them; there is no extensive extramembrane domain. Though no direct contact takes places between subunit II and subunit III as subunit I is located between them, interactions occur between subunit III and subunit I. This contact is made through helices III and IV of subunit I and helix I of subunit III, as well as through helices IV and V of subunit I and helix III of subunit III (Tsukihara et al., 1996). Another unique feature at the junction of subunit I and subunit III is the start of the D channel, with half of the residues which surround D132 coming from subunit III (Hosler 2006). Among these residues located in close proximity to the mouth of the D-channel are three conserved and surface exposed histidines at the N-terminus of subunit III. A triple-histidine mutation to glutamine in *R. sphaeroides* yielded an enzyme which retains only 60% of WT activity, supporting the idea that these histidines function to stabilize interactions of subunit I and subunit III (Alnajjar et al., 2014).

Though subunit III does not contain any metal centers, interactions between subunit I and subunit III are also found in the V-shaped cleft formed by the transmembrane helices in subunit III, where it hold two extensively conserved lipid binding sites. The lipids in these sites in the bovine CcO structure are phosphatidylglycerol and *R. sphaeroides* is shown to contain phosphatidylethanolamines. Subunits I and III provide residues which form a noncovalent network to hold PE1 in place at the back of the cleft, and PE2 is located anteriorly to PE1 with no contact to
subunit I; however, side chains from subunit III extend into the cleft to interact with the fatty acids of both PE1 and PE2 (Varanasi et al., 2006). Experimental mutations to alter the binding sites of these lipids were performed by Varanasi et al. (2006) in which they concluded that the conserved lipid binding sites in subunit III are significant to its ability to stabilize the active site during catalytic turnover, preventing suicide inactivation of the enzyme.

Nuclear Encoded Subunits

In mitochondrial COX, the function of the remaining ten nuclear encoded subunits is unknown; however, one explanation for the complexity of the mammalian respiratory complex is that these extra subunits provide protection and stability in the presence of toxic compounds. Because semiquinone is known to reduce O$_2$ directly, production of reactive O$_2$ species (ROS) like the hydroxyl radical must be counteracted in mitochondria. The mitochondrion contains sophisticated systems which scavenge toxic compounds, and it is possible that the nuclear encoded subunits of COX aid in the defense against their damaging effects (Hosler 2006). Some small molecules have been shown to have an interaction with the CcO nuclear encoded subunits to affect kinetic properties of the enzyme. Subunits IV and VIa have been identified as containing ATP and ADP binding sites, regulating the activity of the enzyme with dependence on the levels of these substrates (Srinivasan and Avadhani, 2012). Under levels of hypoxia, when cells are under bioenergetic crisis, the expression of the isoform subunit IV-i2 will prevent further inhibition of the enzyme by ATP, and CcO containing this isoform have been shown to be catalytically more efficient with a higher affinity for cytochrome $c$ (Fukuda et al., 2007). Allosteric regulation is also seen in thyroid hormone (T2), which
directly binds to subunit Va to forcibly activate CcO activity, and subunit Vb also interacts with Rlα, a subunit in protein kinase, where dissociation of Rlα from subunit Vb induces inhibition of CcO activity (Srinivasan and Avadhani, 2012; Arnold et al., 1998; Yang et al., 1998).

Crystal images of nuclear encoded subunits reveal seven of ten subunits as having transmembrane regions. Subunit IV is shaped like a dumbbell, with two extramembrane domains on each side of the transmembrane helix. It makes contact with helices XI and XII of subunit I, while also contacting subunit VIIb and VIII. Aside from its transmembrane region, subunit VIIb has an extended structure on the cytosolic side of the enzyme. Subunit VIII has a short extended structure on the matrix side, and maintains close contact with helices I and XII of subunit I. Subunit VIIc lies adjacent to subunit VIII on the surface of subunit I. These subunits VIIb, IV, VIII, and VIIc are located side by side from left to right in the enzyme, with subunit III located to their right and the cytosolic side of the membrane upward. Subunit VIIa also has an extramembrane region on the matrix side and cross interacts with subunit III at helices I and II. Another dumbbell shaped subunit with a transmembrane domain, subunit VIc, makes contact with helix I of subunit II. Lastly, subunit VIa interacts with helix IV of subunit III and is composed of ten residues on the NH₂ terminal which are in extended conformation to make contact with helices V and VII of subunit I in the other monomer, likely stabilizing the dimeric structure (Tsukihara et al., 1996).

The remaining three nuclear encoded subunits are classified as the extramembrane subunits. Subunit Va is located on the matrix side of the enzyme below subunit I and is composed of five alpha helices which create a right-handed superhelix. Subunit Vb is
also located on the matrix side and attaches tightly below subunit I and subunit III. It contains a tetrahedral zinc site with four cysteine ligands and a zinc finger motif located in a β barrel structure formed in the COOH-terminal domain involving an extended segment of subunit I. No physiological role of this zinc site has been determined in the enzyme. Subunit VIb is the only nuclear encoded extramembrane subunit located on the cytosolic side of the enzyme. It associates with subunits II and III and contains two disulfide bridges. The connection between Cys-39 and Cys-53 (bovine) is in contact with the corresponding segment in the other monomer, giving it a role in intermonomer contact like subunit VIa. Both the bridging peptides in subunit VIb and subunit VIa are coded by nuclear genes, which explains why the dimer form may not exist in the bacterial enzyme in which these nuclear subunits are absent (Tsukihara et al., 1996).

*Models for Proton Pumping*

Protons are taken up from the matrix for pumping and for reduction at the binuclear center through two different pathways: the K channel and the D channel. All pumped protons follow the D channel while at the most, 2 of the 8 protons follow the K channel (Siegbahn and Blomberg, 2014). The conserved lysine K362 leads the K channel which starts near the Glu-101 residue of subunit II (Tsukihara et al., 1996) After the reduction of heme $a_3$ and CuB through electron transfer from heme $a$, oxygen binds to the iron of heme $a_3$. In the reaction, the first oxygen is reduced by the uptake of two chemical protons via the K channel. A mutation of T359 to alanine in the K channel has shown to slow but does not eliminate the proton transfer or CcO activity. This ability to retain
efficient proton pumping activity supports the conclusion that pumped protons are not taken up through the K channel (Hosler, 2006).

The D channel is located within subunit I, consisting of hydrogen bonded waters which are anchored by D132 on the surface and E286 in the interior juxtaposed between heme $a$ and heme $a_3$ (Hosler 2006). The D channel is used for the uptake of the remaining six protons which is composed of all pumped protons and two chemical protons. A network of hydrogen-bonded water molecules link the aspartate residue to the highly conserved glutamate residue (E286), which is proposed as the branching point for both chemical and pumped protons (Zhu et al., 2010; Adelroth et al., 1997). It likely adopts different positions during turnover, controlling access of protons toward the catalytic site or toward the acceptor site for pumping (Lepp et al., 2008). The proton uptake is strongly enhanced for the D-pathway due to proton-collecting “antenna” groups which are located around its entrance (Wikstrom and Verkhovsky, 2007; Alnajjar et al, 2014). Studies in which the Asn-139 of the D channel was replaced by an Asp reveal that proton pumping is blocked without changing the rate of chemical proton uptake to reduce oxygen. This mutation changed the pKa of the branch point Glu-286 from 9.4 to 11 (Lepp et al., 2008). In another study done by Lepp et al. (2008), Asn-139 was replaced by a neutral threonine residue and an uncoupling of proton pumping from oxygen reduction was seen with an acidic pKa shift from 9.4 to 7.6 in Glu-286.

Another channel found in bovine cytochrome c oxidase, the H channel, is composed of a hydrogen-bond network which is connected to the intermembrane side of the enzyme and a water channel which opens into the matrix side. The hydrogen bond network forms two hydrogen bonds with a fixed water molecules from the network to
heme $a$ through its propionate and formyl groups. Asp-51 is a key residue in this pathway and is suggested to function as a proton pumping site in bovine CcO (Yoskikawa et al., 2011). Upon reduction of the enzyme, a proton is ejected from the aspartate to the mitochondrial exterior. Additionally, a peptide bond within the hydrogen-bond network seems to critically inhibit reverse proton backflow through the network. Bacterial models of the enzyme do not contain an analogous residue to Asp-51; however, they do contain a pathway analogous to the H channel in which the Asp-51 (bovine) is replaced with glycine and a water molecule (Tsukihara et al., 2003). Because proton pumping is a chemically simple reaction, various amino acids can facilitate proton pumping in different ways. Precise sequence comparisons done by Pereira et al. (2001) show that completely different sets of amino acids can have an identical physiological function. The evidence that the Asp-51 residue is not conserved in bacterial models is not conclusive against the possibility that Asp-51 may play a critical role in proton pumping of animal CcO.

**Bacterial Cytochrome c Oxidases**

Iwata et al. (1995) obtained x-ray crystallographic images of cytochrome c oxidase isolated from the cytoplasmic membrane of *Paracoccus denitrificans*, a soil-dwelling bacteria, at a resolution of 2.8 angstroms. This enzyme contains four subunits composed of 22 membrane-spanning alpha helices. Subunit I contains 12 transmembrane alpha helices as well as heme $a$, heme $a_3$, and Cu$_B$, important components of the redox catalysis. Subunit II, which is projected to be involved in transfer of electrons to heme $a$, contains two transmembrane alpha helices and the Cu$_A$ site. Subunit III is unknown in
function, however, it contains 7 transmembrane helices which are separated into two bundles which create a V-shaped cleft. Subunit IV also has unknown function and contains one single transmembrane alpha helix which is in contact with all other subunits. It may be used to stabilize the *Paracoccus* cytochrome c oxidase (Iwata et al., 1995).

*Rhodobacter sphaeroides* is another bacterial model used in the study of cytochrome c oxidase. Capable of many modes of growth, this bacteria dwells in soil, mud, and organic-rich waters. X-ray crystallography was employed to determine crystal structures of this enzyme at 2.3/2.8 angstroms using molecular replacement of subunits I-III of the *P. denitrificans* model. *Rhodobacter sphaeroides* is a four subunit enzyme with a molecular weight of approximately 130 kDa (Cvetkov and Prochaska 2007). Its crystal structure reveals a gap between subunit I and subunit II which may allow for water molecules to form a network of hydrogen bonds that connect the propionate groups of heme *a* and heme *a*₃ to the exterior of the molecule. Propionate groups are suggested to be involved in proton pumping, which indicates that this hydrogen bonded network could be a possible proton exit pathway for the enzyme (Svensson-Ek et al., 2002). *R. sphaeroides* shares a high sequence identity with *P. detnitrificans*, and the overall structure and components of the subunits I, II, III, and IV are very similar; however, subunit IV does not directly interact with any of the other subunits. In addition, Subunits I, II, and III are also homologous to the corresponding subunits of the bovine heart mitochondrial encoded form of the enzyme (Svensson-Ek et al., 2002).

The mitochondrial encoded subunits of bovine heart COX (subunits I, II, and III) compose the major catalytic core and have been evolutionarily conserved from bacterial forms of oxidase. The corresponding three subunits of *R. sphaeroides* show 70%
Figure 5. *Rhodobacter sphaeroides* Crystal Structure. Subunit I is shown in green, containing heme $a$ (light blue), the binuclear center (heme $a_3$: red; Cu$_B$: dark blue), magnesium (light green) and calcium (pink). Subunit II (light gray) contains the diatomic Cu$_A$ site (dark blue) and serves as the cytochrome c binding site. Subunit III is shown in dark gray and the non-conserved subunit IV is shown in magenta. Lipids (orange) and water molecules (red spheres) are also shown throughout the enzyme. (a) A side view of the bacterial enzyme structure shows all subunits, cofactors, water molecules and lipids which are bound to the enzyme. (b) Helix arrangement is clearly seen in the top view of *Rhodobacter sphaeroides*. This figure was prepared using Bobscript and RAS-TER 3D programs (Svensson-Ek et al., 2002).
Table II

Subunits and Molecular Weights of *Rhodobacter sphaeroides*

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Molecular Weight (Da)$^a$</th>
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<td>II</td>
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<td>III</td>
<td>30,022</td>
</tr>
<tr>
<td>IV</td>
<td>5,273</td>
</tr>
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</table>

$^a$ Molecular weights were determined using MALDI-TOF mass spectrometry (Distler et al., 2004)
homology to the mammalian form of the enzyme (Hosler et al., 1993; Shapleigh and Gennis, 1992). In terms of catalytic components, the redox active metal centers (binuclear center, Cu_A, heme a) of *R. sphaeroides* as well as the non-redox active centers (Mg^{2+} and Ca^{2+}) are surrounded by environments which share close structural similarities to those of *P. denitrificans* and bovine heart oxidases (Svensson-Ek et al., 2002). Mg^{2+} is located near the surface between subunit I and subunit II and coordinated to the residues E254, D412, and H411, while Ca^{2+} is located in subunit I and coordinated to six oxygen molecules supplied by Q61, G59, A57, an immobilized water molecule, and two bonds to E54 (Schmidt et al., 2003; Lee et al., 2002). These similarities in the structural environments allow for the strong resemblance found in kinetic and spectral characteristics, as well as a similar corresponding Raman spectrum between *R. sphaeroides* and mammalian bovine COX (Hosler et al., 1992; Shapleigh et al., 1992).

The intricate structure and gene organization involved in mitochondrial cytochrome c oxidase has not easily allowed for its thorough analysis through molecular genetic techniques. Direct mutations are difficult to complete in the mammalian cells which can contain up to 800 mitochondria. Every DNA molecule and hence every enzyme in each mitochondria must contain the mutation in order to obtain interpretable results. The homologous *R. sphaeroides* and *P. denitrificans* bacterial models are those which allow for simplistic and efficient site-directed mutagenesis experiments of COX, yielding more insight into the relationship between structure and molecular mechanism of the enzyme (Cvetkov and Prochaska 2007).
The Role of Subunit III in the Functional Aspects of COX

Subunit III is a large, hydrophobic component of cytochrome c oxidase, binding to the transmembrane region of subunit I. (Bratton et al., 1999; Iwata et al., 1995). Without any metal centers, subunit III is not suspected to play a direct role in electron transfer but has been clearly identified as a member of the catalytic core through its gene retention in the mitochondrial genome as well as its highly conserved primary structure (71%) between *R. sphaeroides* and humans (Bratton et al., 1999; Cao et al, 1992). Furthermore, its primary sequence is conserved (45%) between *R. sphaeroides* and humans (Cao et al., 1992) and its unique V-shaped cleft is maintained between the bacterial and mitochondrial forms as well (Gilderson et al., 2003; Svensson-Ek et al., 2002; Yoshikawa et al., 1998).

A major function of subunit III in cytochrome c oxidase is the ability to prevent the enzyme from undergoing spontaneous inactivation, known as suicide inactivation during catalytic turnover (Mills et al., 2005; Haltia et al., 1994). Subunit III can be removed from the enzyme using polyoxyethylene detergents, such as TX-100, which indicates weak binding to subunit I (Bratton et al, 1999; Solioz et al., 1982). Subunit III depleted preparations promote irreversible structural changes at the heme $a_2$-$Cu_B$ active site which results in a complete loss of $Cu_B$ and dramatically decreases the catalytic life span of the enzyme. The I-II oxidase of *R. sphaeroides* halts O$_2$ reduction and inactivates with a catalytic lifetime which is 0.5% of that of wildtype enzyme with subunit III present, while also exhibiting a decrease in proton pumping abilities (Gilderson et al., 2003; Bratton et al., 1999). Thus, the function of subunit III is likely physiologically important due to its drastic decrease in catalytic turnovers and is further supported
through a suicide inactivation found in the mitochondrial form of the enzyme lacking subunit III as well (Mills et al., 2005). Specifically, studies have led to the indication that one of the key functions of subunit III in wildtype oxidase is to preserve the integrity of the Cu₄ center during the catalytic cycle of the enzyme (Gilderson et al., 2003).

Subunit III plays a role in proton pumping efficiency, proton channeling, oxidase assembly, and O₂ channeling; however, the molecular mechanisms for these proposals remain unknown (Bratton et al., 1999; Wilson and Prochaska, 1990; Haltia et al., 1989; Riistama et al., 1996). It has been experimentally shown that the removal of subunit III in COX induces a decrease in the proton pumping efficiency of the enzyme (Prochaska and Fink, 1987). Studies done with COX reconstituted into phospholipid vesicles (COV) have also shown that subunit III depleted COV (COV-III) compared to WT COV exhibited a 40% decrease in proton pumping stoichiometry (H⁺/e⁻) (Nguyen et al., 2002; Prochaska and Fink, 1987).

_Dicyclohexylcarbodiimide_

Carbodiimides are molecules that are most often considered a symmetrical anhydride of urea, however some asymmetrical forms exist. They are highly reactive with many organic functional groups, which does not allow for accreditation as a specific reagent; however, a certain specificity can be induced by conditions of the reaction. It also explains its use in a wide variety of procedures. The specific carbodiimide of interest to our application is known as N, N'-dicyclocxyl-carbodiimide (DCCD). This hydrophobic, non-cyclic carbodiimide is stable over long periods of time and does not tend to polymerize (Azzi et al., 1984). Each proton-translocating enzyme complex in the
respiratory chain of the inner mitochondrial membrane reacts with DCCD, in addition to an outer membrane ion channel (Hassinen and Vuokila, 1993; Prochaska et al., 1981). Furthermore, the proton translocation activity in ATP synthase is known to be inhibited by a covalent modification of DCCD (Fillingame, 1975).

DCCD reacts with amino, phenolic, hydroxyl, sulfhydryl and carboxyl groups, but the major reactive groups are carboxyls and and sulfhydryls in aqueous solutions of acidic or neutral conditions. (Carraway and Koshland, 1972). In a reaction with DCCD and a carboxyl group, a highly reactive dicyclohexyl-O-acyl-isourea intermediate is formed. A hydrophilic environment will support hydrolysis of the intermediate, generating dicyclohexylurea and restoring the carboxyl group of the protein (Prochaska et al., 1981; Khorana, 1953). There are two fates for dicyclohexyl-O-acyl-isourea located in a hydrophobic environment, such as the one provided by cytochrome c oxidase. Rearrangement of the intermediate can occur to form a covalent adduct, and a reaction with a close nucleophilic center can form an amide bond to promote cross-linking within the protein (Prochaska et al., 1981; Hoare and Koshland, 1967).

Prochaska et al. (1981) used radioactive DCCD to monitor and confirm the covalent modification of cytochrome c oxidase. There is evidence of DCCD binding to subunit II of cytochrome c oxidase both at hydrophilic stretches of amino acids at the C terminal and also in the more hydrophobic binding domain from cytochrome c. Subunit III of the enzyme is strongly suggested to bind DCCD at a single site. Studies have shown that essentially all bound DCCD is incorporated at a highly conserved glutamic acid 90 in beef oxidase. Fragmentation studies have also shown DCCD interactions with subunit IV, exclusively within a domain containing several glutamic acid residues.
Figure 6. Schematic Diagram of Dicyclohexylcarbodiimide (DCCD) Reaction in Cytochrome c Oxidase. Upon reaction of DCCD with a carboxyl group, the highly reactive intermediate dicyclohexyl-O-acylisourea is formed. In a hydrophobic environment such as the one provided by cytochrome c oxidase, this intermediate has two fates. In the presence of a nucleophile, a new peptide bond is formed in a nucleophilic attack along with dicyclohexylurea, leaving the amino group unmodified. Shown in the diagram is the rearrangement which occurs in the reaction with cytochrome c oxidase, resulting in a covalent adduct dicyclohexyl-N-acylurea (Prochaska et al., 1981; Hassinen and Vuokila, 1993; Azzi et al., 1984). Schematic adapted from Azzi et al., 1984.
Dicyclohexylcarbodiimide (DCCD)

\[ \text{R-substituted dicyclohexyl-}O\text{-acylisourea} \]

Rearrangement (hydrophobic region)

\[ \text{modified R-substituted dicyclohexyl-N-acylurea} \]
Figure 7. Subunit III of Bovine Heart Cytochrome c Oxidase and the Site of Covalent DCCD Modification. This is a three-dimensional crystal structure of bovine heart COX in which transmembrane helices of subunit III are shown in light pink. DCCD (pink) is shown bound to the Glu-90 residue of subunit III, which remains conserved throughout the bovine heart and *R. sphaeroides* models. In WT COX, PGV609 (light green) and PGV1267 (light blue) are present in the conformation as shown; however, upon reaction with DCCD, PGV1266 (dark green) and PGV307 (dark blue) adopt a new conformation in the enzyme. Modeling was done by Chris Pokalsky.
(Prochaska et al., 1981). Because subunit IV protrudes into the matrix of the mitochondria, it does not have a particularly hydrophobic amino acid composition. This environment does not favor stable binding of DCCD and explains why no labelling is seen in cytochrome c oxidase in mitochondria. In lipid free cytochrome c oxidase preparations, subunit IV can be manipulated into a more hydrophobic environment, which can allow for more favorable DCCD binding (Casey et al., 1980; Steffans and Buse, 1976; Ludwig et al., 1979). Due to the multiple binding sites for DCCD in subunit II, it is suggested that the single major site in subunit III will have a specificity for the inhibitor which is at least one order of magnitude greater than that of other sites in subunit II or IV. Furthermore, the observed inhibition of the enzyme caused by DCCD modification can be attributed to the alteration at subunit III by itself (Prochaksa et al., 1981).

The Functional Effect of Dicyclohexylcarbodiimide on Cytochrome c Oxidase

When dicyclohexyl-O-acyl-isourea rearranges to produce a covalent adduct or reacts with a close nucleophile to form crosslinking in the protein, there is potential for inhibition of enzymatic activity (Prochaska et al., 1981). In subunit III of cytochrome c oxidase, the glutamic acid residue (Glu-90) has the ability to carry protons due to its negative charge and maintains integrity in the enzyme with its high evolutionary conservation. Prochaska et al. (1981) have shown that Glu-90 is the site for covalent modification by DCCD, and cytochrome c oxidase is partially inhibited in its electron transfer activity and in proton pumping capabilities when this modification occurs (Shinzawa-Itoh et al., 2007; Casey et al., 1980; Prochaska et al., 1981). There is
approximately a 1:1 stoichiometric relationship between the proportion of cytochrome c oxidase bound by DCCD and the proportion of inhibition of proton-pumping. (Ogunjimi et al., 2000; Casey et al., 1980). When the protein is reacted at levels higher than the one half saturation, DCCD is incorporated into sites in subunit II, which could affect proton pumping through the inhibition of electron transfer or denaturation of the entire enzyme. (Prochaska et al., 1981).

Mutagenesis experiments of the Glu-90 site in P. denitrificans models of COX resulted in wild-type proton translocation activity in the mutant enzyme when assayed in the bacterial membrane (Haltia et al., 1991). These experiments indicated that inhibition of proton pumping induced by DCCD modification at Glu-90 is indirect and not attributed to the blockage of a specific amino acid residue in the proton-conducting channel of the enzyme (Musser et al., 1993). To provide a mechanism for DCCD-induced inhibition, Ogunjimi et al. (2000) indicated that the binding of the bulky substituent DCCD induces a conformational change in subunit III which inhibits the proton pumping and electron transfer mechanisms of the enzyme.

Chymotrypsin digestions reveal that subunit III of COX bound by DCCD was 15-37% more labile to proteolytic cleavage, suggesting that changes in the structural configuration of subunit III are induced by DCCD modification (Ogunjimi et al., 2000). In addition, SADP, a known heterobifunctional cross-linking reagent which extensively creates cross links within COX, was reacted with the DCCD-modified enzyme (Ogunjimi et al., 2000; Estey et al., 1990; Estey and Prochaska, 1993) and showed that compared to WT, DCCD-reacted COX displayed a protection against cross-linking within the enzyme (Ogunjimi et al., 2000). Furthermore, the reactivity of two cysteine residues found in
subunit III of COX (Cys-115 and Cys-218) was increased in DCCD reacted COX when using the sulphhydryl reagent iodoacetamide (Anderson et al., 1982; Malatesta and Capaldi, 1982; DiBiase and Prochaska, 1985; Ogunjimi et al., 2000). When DCCD is reacted to COX displays a significant increase in iodoacetyl-biotin labelling as compared to WT, indicative of more surface exposed cysteine residues and support of an induced conformational change in subunit III due a DCCD modification (Ogunjimi et al., 2000).

Specific Aims of Research

Analyzing the effects of DCCD binding to subunit III may provide insight into the significance of the subunit and how its interactions within the enzyme support its function. I am proposing that in the event when DCCD is bound to subunit III in cytochrome c oxidase, a structural change will occur which perturbs the activity of the enzyme and alters the molecular mechanism through which it functions. A conformational change in subunit III which could indirectly affect components involved in the enzymatic pathway, therefore altering activity, could give more insight into how subunit III specifically maintains integrity of the enzyme during catalytic turnover. By analyzing both bovine heart and R. sphaeroides COX, comparisons and contrasts can be applied to determine whether the DCCD modification in subunit III affects the enzyme and its function through the same mechanism.

The specific aim presented in this thesis is to test the hypothesis that DCCD modification in subunit III will perturb the subunit structure and alter the catalytic mechanism of the enzyme. The hypothesis was tested experimentally as follows:
1. To assess the DCCD modification of subunit III by SDS gel electrophoresis;

2. To analyze the environment of heme regions of the enzyme by CD spectroscopy in both bovine heart and *R. sphaeroides* COX to determine if any structural changes in heme *aa*3 of the enzyme are induced by DCCD binding;

3. To investigate deviations in the steady-state molecular mechanism of electron transfer activity within bovine heart and *R. sphaeroides* COX by DCCD modification through measurements of reduction at heme a;

4. To determine the effect that DCCD has on steady-state electron transfer activity and its dependency on proton concentration in bovine heart and *R. sphaeroides* COX.

5. To assess the effect of DCCD modification on the irreversible inactivation of electron transfer activity by measuring the number of catalytic turnovers of COX before suicide inactivation.
II. Materials and Methods

Preparation of Bovine Heart COX

Bovine heart COX was purified by cholate solubilization and ammonium sulfate precipitation using the procedure of Yonetani (1961). COX was then prepared for assays by incubation in 0.1% DM solution on ice for a period of 1 hour then transferred to buffer solutions specific to the assay being performed. The heme $aa_3$ concentration of the solubilized enzyme was determined by absorbance spectroscopy on a HP-diode array spectrophotometer using the reduced minus oxidized spectrum and an extinction coefficient of 24 mM$^{-1}$ cm$^{-1}$ at 605 nm.

Growth of Rhodobacter sphaeroides and purification of R. sphaeroides COX

100 µL of Rhodobacter sphaeroides YZ-300 WT glycerol stock cells were used to inoculate 3 milliliters of 1X Sistrom’s media in the presence of 50 µg/mL streptomycin, 50 µg/mL spectinomycin, and 1 µg/mL tetracycline. Cells were grown in a shaking incubator at 30-32°C at 250 rpm until they reached an OD$_{660}$ of 0.8-1.2. The 3 mL cultures were then used to reached an OD$_{660}$ of 0.8-1.2, 10-12 mL were used to inoculate 250 mL cultures grown in 1 L baffled flasks. Cultures routinely reached log phase after approximately 20-24 hours and an OD$_{660}$ of 1.0-1.2. Cells were then centrifuged at 11,300 x g and the resulting pellet was washed in 50 mM potassium phosphate, 1 mM EDTA, pH 7.2 and stored at -80°C.

Frozen pellets are thawed and resuspended in 10 mM Tris, 10 mM EDTA, 10 mM MgCl$_2$, 20% glycerol, pH 8.0. DNAse (50 µg/mL), lysozyme (25 mg/mL) and
phenylmethylsulfonylfluoride (PMSF, 1 mM) were added and cells were mechanically homogenized with a tissue grind tube and passed through an EmulsiFlex-C3 apparatus at 15,000-20,000 psi using Nitrogen gas. The lysed cell mixture was centrifuged at 24,000 x g for 20 minutes at 4ºC to remove the unlysed cells. The supernatant was analyzed for heme \( \text{aa}_3 \) concentration using a reduced minus oxidized extinction coefficient of 24 mM-l at 606-630 in a Hewlett Packard 8453 UV/Visible diode array spectrophotometer (van Gelder, 1966). A final concentration of 1 mM PMSF was added to the supernatant and the solution was centrifuged for a 1.5 hours at 4ºC at 153,000 x g in a Beckman Optima LE-80K ultracentrifuge using a Ti50.2 rotor. The resulting pellet was washed with a solution of 50 mM potassium phosphate, 1 mM EDTA, pH 7.2 and 1 mM PMSF. The ultracentrifugation was repeated, the supernatant was decanted, and the resulting pellet was stored at minus 80ºC (Zhen et al., 1998).

In the YZ300 strain of \textit{R. sphaeroides}, COX has a poly-histidine tag on subunit I of the enzyme. Thus, affinity chromatography using Ni-NTA columns can be used for purification. (Hosler et al., 1992; Zhen et al., 1998). The cytoplasmic membrane pellet was resuspended in 10 mM Tris-KOH, 40 mM KCl, pH 8.0 and 2% DM. This mixture was stirred for 15 minutes at 4ºC then centrifuged for 20 minutes at 4ºC at 18,000 rpm. The supernatant was decanted into a polycarbonate tube and imidazole was added to 10 mM. 0.8 mL of Ni\(^{2+}\)-NTA resin was added per mg of COX to allow the interaction between the poly-histidine tag on subunit I and the nickel resin. This mixture was mixed on a rocking platform for 1 hour at 4ºC. The slurry was transferred to a Bio-Rad glass econo-column (0.7 cm inner diameter, 30-50 cm length) and allowed to settle without flow for 10-15 minutes. Gravity flow (2-3 drops/sec) was used to pack the column. 5-10
column volumes of running buffer (10 mM Tris-KOH, 40 mM KCl, 10 mM imidazole, 0.1% DM, pH 8.0) were then used to wash the column, and when the elution became clear, 2-3 column volumes of elutant buffer (10 mM Tris-KOH, 40 mM KCl, 100 mM histidine, 0.1% DM, pH 8.0) were added to the column (1 drop/5-7 sec) to maintain concentrated enzyme. The column was run until all of the green color eluted. To decrease histidine concentrations below 1 mM in the preparation, the green tinted elution fractions were pooled, concentrated, and washed using resuspension buffer (10 mM Tris-KOH, 40 mM KCl, pH 8.0) and 2 mL Millipore YM-100 Centron devices at 4°C.

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

COX enzyme (5-7 µg) was incubated in 2% SDS at 37°C for 30 minutes and then loaded onto a 1 mm thick gel containing a 6% polyacrylamide stacking layer (pH 6.8) and a 16% polyacrylamide resolving gel (pH 8.8) with 6 M urea (Fuller et al., 1981). The gel was run at 60 V for 25 minutes followed by 120 V for 2.5 hours on a BioRad PowerPac 1000 apparatus. Staining was carried out using Coomassie G-250 Bio-Rad directions and washed in a destaining solution (20% methanol, 10% acetic acid). All images were taken on a Fuji LAS-4000 imager in precision mode.

**Reaction of COX with DCCD**

1 µM (αα3) beef heart and R. sphaeroides COX enzymes were reacted with a 0.25 mM and a 0.5 mM concentration of DCCD, respectively in order to maintain approximately 50% inhibition in the enzyme. The N,N’,5,5’-dicyclohexylcarbodiimide was dissolved into methanol to create a 100 mM stock solution. Reactions between
DCCD and COX were conducted at room temperature for a period of 1 hour in 25 mM Hepes buffer pH 7.0, 0.1% DM. 100 mM succinate, pH 8.0 was added to at room temperature for 15 minutes in order to quench the reaction.

**Control and DCCD treated COX for Circular Dichroism Spectroscopy**

For both beef heart and *Rhodobacter sphaeroides* COX, aliquots of control, unreacted COX at pH 7.0 were allowed to incubate at room temperature for a 1 hour period to mimic DCCD reacted aliquots. 100 mM succinate, pH 8.0 was also added to controls. After the DCCD reaction at pH 7.0, bovine heart COX control and 0.25 mM DCCD reacted samples were titrated to a final concentration of 1 uM and a desired pH 7.0 or pH 10.0 using 25 mM HEPES pH 7.0, 0.1% DM or predetermined amounts of 25 mM CHES pH 10.5, 0.1% DM and 0.1 M NaOH. Both control and 0.5 mM DCCD-reacted *R. sphaeroides* samples were concentrated by ultracentrifugation at 4º C for 10 minutes at 7,500 g and then for 2 minutes at 1,000 g in 2 mL Amicon Ultra 100K devices. 25 mM HEPES, pH 7.0, 0.1% DM and 25 mM CHES, pH 10.0, 0.1% DM were used to dilute the centrifuged samples to a final concentration of 1 µM.

**pH Dependence of COX electron transfer activity**

A Clark oxygen electrode (Yellow Springs Instrument Company Model 17372)

The pH dependence of the enzymatic activity for both bovine heart COX and *R. sphaeroides* COX was measured using a Clark oxygen electrode (Yellow Springs
Instrument Company Model 17372). All COX samples were then diluted with 25 mM HEPES pH 7.0, 0.1% DM and incubated on ice for 20 minutes. DCCD reactions and control were carried out as described above. 25 mM MES buffers pH 6.0-6.5, 25 mM HEPES buffers pH 7.0-8.5, and 25 mM CHES buffers pH 9.0-10.0 were prepared and each with the addition of KCl to maintain a constant ionic strength within the reaction. An online buffer calculator was used to determine the constant buffer ionic composition for each buffer (http://www.liv.ac.uk/buffers/buffercalc.html).

The autooxidation rate for cytochrome c was measured for 3 minutes in the corresponding MES, HEPES, or CHES buffer along with additional reagents 20-50 µM cytochrome c (Sigma Type III), 18 mM ascorbate, and 0.6 mM TMPD. For initial rate analysis, 5-10 pmol of cytochrome c oxidase were then added and the assay was run for an additional 3 minutes. Assays determining suicide inactivation were run for 3-20 minutes depending on their activity. As pH value for the assay became more alkaline, an increased amount of oxidase was added in order to efficiently measure enzymatic rates due to decrease enzyme turnover number.

**Circular Dichroism (CD) Spectroscopy**

For each CD experiment, beef heart COX was solubilized and treated with DCCD as previously described. Control or DCCD reacted enzyme was titrated to the corresponding pH value described in the previous section. All CD spectra were measured on a Jasco J-815 CD spectrometer at both pH 7.0 and 10.0 using a COX concentration of
1 µM. The CD spectra were performed using a quartz cuvette with a 1 cm path length and scanned from 370 nm to 470 nm in order to analyze the Soret region of heme $aa_3$.

Samples measured at pH 10.0 were titrated to pH 7.0 after spectral analysis in the same buffer using small volumes of 1 M HCl. After incubation on ice for 1 hour, the spectra were recorded again in order to monitor the enzyme denaturation.

**Steady State Heme $aa_3$ Reduction**

All sample preparations were described in previous sections. 50 mM HEPES buffer pH 7.0, 0.1% DM or 50 mM CHES buffer pH 10.0, 0.1% DM were used to dilute COX to a 0.25 µM final concentration. All spectra were corrected for buffer absorbance prior to measurement in the diode array. The HP-diode array spectrophotometer was blanked with corresponding buffers. Steady state reactions were recorded at wavelengths 445 nm, 460 nm, 605 nm, and 650 nm in 15 second intervals for 600 seconds at room temperature using a stirring 1 cm path length glass optical cell. 100 mM TMPD and 0.6 M ascorbate were added to the reaction after 30 seconds to initiate electron transfer and steady state absorbance measurements recorded (Hiser et al., 2013). After 300 seconds, a small amount of dithionite was added to the cuvette to completely reduce heme $a$.

Reduction levels of heme $a$ were determined using the ratios of the 605 nm absorbance levels of the aerobic steady-state to the dithionite fully reduced absorbance, while subtracting the contribution of the oxidized heme from both and then normalizing data to the baseline. Heme $a$ absorbance is assumed to comprise 85% of the total absorbance at 605 nm (van Gelder, 1966; Alnajjar et al., 2014).
III. Results

Ogunjimi et al. (2000) previously showed that DCCD modification of Glu-90 in bovine heart cytochrome c oxidase induces a conformational change in subunit III of the enzyme. When DCCD is bound, proton pumping and electron transfer functions of the enzyme are inhibited. Compared to the control, DCCD bound to COX induces a 70% inhibition of proton pumping as well as a 40% inhibition of the electron transfer activity. This suggested that subunit III may play a role in the proton pumping efficiency and catalytic mechanism of the enzyme (Prochaska et al., 1981; Casey et al., 1980). Experimental work in which subunit III was removed from COX shows a decrease in proton pumping capabilities (Prochaska and Fink, 1987; Nguyen et al., 2002) and induces suicide inactivation of the enzyme by completely diminishing its catalytic turnover (Mills et al., 2005; Haltia et al., 1994). Subunit III has been shown to maintain the integrity of the binuclear center in subunit I during catalytic turnover, however, its full role in COX functioning is still unknown (Gilderson et al., 2003; Bratton et al., 1999).

The goal of this study is to investigate how DCCD binding to subunit III affects the structure and catalytic activity of COX. These experiments also analyze how DCCD modification perturbs the molecular mechanism by which COX functions and determine if the bovine heart and R. sphaeroides activities are affected by a similar mechanism. Both bovine heart and R. sphaeroides COX were reacted with DCCD and the pH dependence of the catalytic activity of the enzyme was also measured. Changes in the spectral properties of the heme groups were also analyzed in order to determine any structural changes in the environment surrounding the catalytic center. All experiments were performed at pH 7.0 in physiological range as well as at pH 10.0 in order to monitor
how the DCCD modification of subunit III affects the enzyme under different pH conditions and whether different conformations of the DCCD-bound enzyme occur in neutral and alkaline environments.

**Concentration Dependence of DCCD-induced Inhibition of Catalytic Activity in COX**

Bovine heart and *R. sphaeroides* COX were reacted for 1 hour with varying concentrations of DCCD in order to determine a concentration for one half maximal inhibition of activity. Bovine heart COX was reacted with 0.25 mM DCCD and with 0.5 mM DCCD and the samples were assayed for electron transfer activity. *R. sphaeroides* COX was also reacted with 0.5 mM DCCD, 1 mM DCCD, and 1.5 mM DCCD and similarly assayed (Figure 8). The DCCD modification of bovine heart COX inhibited electron transfer activity 55% at a DCCD concentration of 0.25 mM and 74% at a DCCD concentration of 0.5 mM. Alternatively, *R. sphaeroides* COX activity was inhibited by 43%, 80%, and 91% at 0.5 mM, 1.0 mM, and 1.5 mM respectively. In these experiments, the goal was to find a DCCD concentration which inhibited the enzyme’s catalytic function by approximately 50%, giving the ability to measure efficiently the inhibited activity without completely abolishing electron transfer. For bovine heart COX, a 0.25 mM DCCD reaction yielded approximately 50% values while *R. sphaeroides* COX yielded a 0.5 mM DCCD reaction as most optimal for assays. Figure 8 shows that higher stoichiometries of DCCD added per mole of COX are required to obtain equivalent levels of inhibition of electron transfer activity.
Figure 8. Concentration Dependence of DCCD-induced Inhibition of Electron Transfer Activity in Bovine Heart and \textit{Rhodobacter sphaeroides} COX. Electron transfer activity of bovine heart and \textit{R. sphaeroides} COX was measured in 25 mM HEPES buffer, pH 7.0, 100 mM constant ionic strength with use of KCl at varying concentrations of DCCD reactions. Results were plotted as percent inhibition of activity compared to \textit{R. sphaeroides} WT COX or control bovine heart COX versus the ratio of moles of DCCD added per mole of COX to determine an optimal DCCD concentration for each form of the enzyme for subsequent experiments. Bovine heart COX (closed black circles) and \textit{R. sphaeroides} (open circles) are shown. A hyperbolic curve was fitted to the \textit{R. sphaeroides} data using SigmaPlot. The results presented are representative of one trial, however, multiple reactions were carried out.
Confirming DCCD Modification of Subunit III in COX

After reacting bovine heart and *R. sphaeroides* COX with DCCD, the enzymes were run on a 16%, 6 M SDS polyacrylamide gels in order to confirm the covalent modification in subunit III. On an SDS-PAGE gel, the bovine enzyme displays subunits which run at 36 kD (subunit I), 26 kD (subunit II) and 22 kD (subunit III). Subunit I has an actual molecular weight of 54 kD and subunit III has an actual molecular weight of 30 kD (Anderson et al., 1982). The reason these subunits run at a lower molecular weight on SDS-PAGE than their actual molecular weight is due to incomplete denaturation in SDS. CD spectra were used to verify that subunit III is in fact not fully denatured, resulting in a band which displays a lower molecular weight than its actual molecular weight (Prochaska et al., 1994). When reacted with DCCD, subunit III displays a slower migrating species on SDS-PAGE gel, indicative of a higher molecular weight (Figure 9a) (Ogunjimi et al., 2000). This result is likely due to an increased ability of the subunit III to undergo denaturation when the DCCD is covalently bound. DCCD bound to subunit III at Glu-90 was observed in X-ray crystallography (Shinzawa-Itoh et al., 2007).

On SDS-PAGE gel, the WT *R. sphaeroides* enzyme exhibits subunits which run at 49 kD (subunit I), 37 kD (subunit II), and 22 kD (subunit III), however, their actual DNA-derived molecular weights are 62.6, 32.0, and 30.1 kD, respectively (Hosler et al., 1992). The variances in actual molecular weight and SDS-PAGE molecular weight are similar to those seen in bovine heart COX. In subunit II, two bands are seen due to native post-translational proteolytic processing which results in the removal of 25 amino acids at
Figure 9. DCCD Modification of Subunit III in Bovine Heart and R. sphaeroides COX on SDS-PAGE. Bovine heart and R. sphaeroides COX was reacted with DCCD then control and DCCD-modified samples were incubated in 2% SDS. SDS-PAGE was performed on a 16%, 6 M urea polyacrylamide gel and run in order to confirm a covalent DCCD modification of subunit III in the enzyme (Fuller et al., 1981). In part A, 0.25 mM DCCD reacted bovine heart COX SDS-PAGE gel subunit III exhibits a shift toward a higher molecular weight in lane 2, verifying a covalent DCCD modification. Lane 1 exhibits control bovine heart COX. In part B, 0.5 mM DCCD reacted R. sphaeroides COX SDS-PAGE subunit III shows a similar shift toward a higher molecular weight in lane 2, also indicating the covalent binding of DCCD. The control R. sphaeroides COX is shown in lane 1.
(A) Subunits I, II, III, IV, V, VI, Va, Vb, VII, VIII, VIa, VIb, VIc.

(B) Subunits I, II, III.
the N terminal and 15 amino acids at the C terminal, causing a decrease in molecular weight as compared to an unprocessed subunit II (Distler et al., 2004). *R. sphaeroides* also displays a band shift to a higher molecular weight in subunit III when DCCD is bound to the enzyme (Figure 9b), emphasizing that the mechanism of DCCD binding to subunit III of *R. sphaeroides* COX may be similar.

**pH Dependence of Electron Transfer Activity of WT and DCCD-modified COX**

*Bovine Heart COX*

Electron transfer activity of WT and DCCD modified bovine heart and *R. sphaeroides* COX was measured at pH values from 6.0-10.0 in MES, HEPES, and CHES buffers at constant ionic strength as described in the methods. This also allowed for the indirect measurement of a relative proton uptake in the enzyme due to interdependence with electron transfer activity of the enzyme (Verkhovsky et al., 2006). Results from two different determinations of bovine heart COX modeled with a single-fit pKa yielded pKa values of 7.7 and 8.0, while 0.25 mM DCCD-modified COX yielded pKa values of 7.0 and 7.2 respectively, an acid shift approximately 0.7 units. Representative data from bovine heart COX and DCCD treated enzyme are shown in Figure 10. Overall, this shows data results which are inconsistent with a titration curve of one site for proton binding. This suggested further multiple-site analysis in order to more definitively evaluate the effect of DCCD on the enzyme.

The indication of two proton binding sites which are correlated with the activity of the enzyme led to analysis using Michaelis-Menten curves modeled with two sites for
**Figure 10. pH Dependence of Control and 0.25 mM DCCD-modified bovine heart COX.** WT and 0.25 mM DCCD-reacted bovine heart COX were assayed for their pH dependence activity ranging from pH 6.0-10.0 using 25 mM MES, HEPES, and CHES buffers at constant ionic strength. By fitting a sigmoidal single-fit pKa curve to the data using SigmaPlot, WT resulted in a pKa of 7.7, and 0.25 mM DCCD-modified COX yielded a pKa of 7.0. Filled circles represent the WT control bovine heart COX, while open circles represent the 0.25 mM DCCD-modified COX results. This data is representative of multiple trials.
proton binding (Figure 11). Activity was plotted as a function of H+ ion concentration and fitted with a double-site saturation curve in order to determine the pKa of two sites for WT and 0.25 mM DCCD-modified enzyme. For three trials of data, site 1 in WT control enzyme yielded an average pKa of 7.0±0.4, while site 2 yielded an average pKa of 8.7±0.1. DCCD-modified enzyme yielded an average pKa of 7.2±0.1 for site 1 and an average pKa of 8.9±0.4 for site 2.

Because bovine heart COX obeys Michaelis-Menton kinetics for two sites of proton binding, Eadie-Hofstee plots were used to further investigate the effect of DCCD on the pH dependence of COX electron transfer kinetics. These plots also indicate a two-site mechanism while yielding a pKa value for each site and V_{max}. As seen in Table 3, bovine heart control COX yielded an average pKa of 7.6±0.1 for site 1 which results in an average V_{max} of 830±70 sec\(^{-1}\). Site 2 yields an average pKa value of 8.8±0.2 which corresponds to an average V_{max} of 520±110 sec\(^{-1}\). In 0.25 mM DCCD-modified bovine heart COX, site 1 yielded an average pKa of 7.7±0.2 resulting in a V_{max} of 1120±35 sec\(^{-1}\). Site 2 yields a pKa of 9.3±0.1 with a V_{max} of 160±30 sec\(^{-1}\). This suggests that upon the modification of DCCD, an alkaline shift is occurring in the high pH value for activity, while the other remains unaffected. Representative data are shown in Figure 12.

The pH dependence of the inhibition of electron transfer activity in bovine heart COX induced by 0.25 mM DCCD was plotted to determine if the pH affected the amount of inhibition of the enzyme’s catalytic activity when DCCD was bound to subunit III. As shown in Figure 13, the amount of inhibition induced by DCCD modification from pH 6 to pH 9 was 45-59%, values within 10% of the observed 50% inhibition. At pH 9.5, the 0.25 mM DCCD-reacted COX activity was inhibited by only 31% and 32%, while pH
Figure 11. Michaelis-Menten Kinetics as a Function of pH for Two-site Saturation in Bovine Heart COX modified with 0.25 mM DCCD. Control bovine heart COX activity is shown (filled black circles) plotted as a function of H+ ion concentration, reaching a maximum velocity at a H+ ion concentration corresponding to pH 6.5. The data was fit to a double-site saturation curve which yielded a pKa of 7.2 and 8.7 at site 1 and site 2 respectively. 0.25 mM DCCD-modified bovine heart COX activity is also shown (open circles) reaching a maximum velocity at pH 6.5 at an activity approximately 50% inhibited compared to the control enzyme. The pKa values which resulted from the fitted double-site saturation curve in DCCD-modified enzyme yielded pKa values of 7.3 and 9.3 at site 1 and site 2 respectively. Each data point corresponds to 1 experimental trial which is comprised of 3 data sets per trial.
Figure 12. Eadie-Hofstee Analysis of the pH Dependence of Electron Transfer Activity in DCCD-modified Bovine Heart COX. Control bovine heart COX (blue circles) display a two site binding mechanism. Fitting each site with a linear regression (solid blue line) yielded a slope with was used to calculate a pKa of 7.7 for site 1 and a pKa of 8.8 for site 2. 0.25 mM DCCD-modified COX (red triangles) also displayed a two site binding mechanism. The slope from the linear regression fitted to this data (dashed red line) allowed for the calculation of pKa values of 7.7 and 9.3 for site 1 and site 2 respectively. Similar slopes are seen between site 1 of control and DCCD-modified enzyme, while site 2 shows a significant difference upon the modification of the enzyme.
Table III

The Effects of pH on Electron Transfer Activity in Control and DCCD-modified Bovine Heart and *R. sphaeroides* COX.

<table>
<thead>
<tr>
<th></th>
<th>Treatment</th>
<th>Average pKa Site 1</th>
<th>Vmax Site 1 (sec⁻¹)</th>
<th>Average pKa Site 2</th>
<th>Vmax Site 2 (sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine heart COX</td>
<td>Control</td>
<td>7.6 ± 0.1</td>
<td>830 ± 70</td>
<td>8.8 ± 0.2</td>
<td>520 ± 110</td>
</tr>
<tr>
<td></td>
<td>+0.25 mM DCCD</td>
<td>7.7 ± 0.2</td>
<td>1120 ± 35</td>
<td>9.3 ± 0.1</td>
<td>160 ± 30</td>
</tr>
<tr>
<td><em>R. sphaeroides</em></td>
<td>COX Control</td>
<td>7.8 ± 0.4</td>
<td>2800 ± 180</td>
<td>9.4 ± 0.1</td>
<td>825 ± 35</td>
</tr>
<tr>
<td></td>
<td>+0.5 mM DCCD</td>
<td>7.3 ± 0.4</td>
<td>2750 ± 110</td>
<td>9.3 ± 0</td>
<td>425 ± 35</td>
</tr>
</tbody>
</table>

*Average values and standard deviations were calculated from three trials of data for each treatment*
Figure 13. pH Dependence of Inhibition of Electron Transfer Activity in DCCD-modified Bovine Heart COX. Percent inhibition of 0.25 mM DCCD modified to bovine heart COX was plotted as a function of pH from 6.0-10.0. Trial 1 (closed circles) and trial 2 (open circles) are presented with standard deviation bars for the three trials performed at each point.
Inhibition of Electron Transfer Activity (% of control) vs pH
10.0 yielded results which indicated a higher rate of electron transfer rather than an inhibition of activity in the DCCD-modified enzyme. At these pH values the enzyme’s electron transfer activity was actually higher than that of the control COX, suggesting a stimulation of activity versus the inhibition we observed at other pH values. These results stimulated further analysis of the effects of the DCCD reaction with COX enzyme at a physiological pH value 7.0 as well as an alkaline value 10.0 for all subsequent experiments.

Turnover-induced inactivation or suicide inactivation occurs in COX when the enzyme’s activity is destroyed during catalysis (Bratton et al., 1999). It is defined by the parameter \( CC_{50} \) which is the number of catalytic turnovers for oxygen reduction which are required until one half of enzyme electron transfer activity is diminished (Hosler, 2004). By utilizing this calculation, the suicide inactivation of bovine heart COX reacted with 0.25 mM DCCD was converted to \( CC_{50} \) values (Hosler, 2004). This parameter was plotted as a function of pH to determine the catalytic turnover dependence on pH which occurs in the DCCD-reacted COX. Data shown in Figure 14 are the \( CC_{50} \) values from two experiments where the assay pH was valued 6.0 to 10.0 for 0.25 mM DCCD-reacted bovine heart COX. A single-fit pKa curve was also fitted to the data and yielded a pKa values of 7.9.

\textit{R. sphaeroides COX}

WT and 0.5 mM DCCD-modified \textit{R. sphaeroides} COX were assayed for electron transfer activity at pH values from 6.5-10.0 in MES, HEPES, and CHES buffers. Representative results modeled with a single-fit pKa yielded a pKa value of 7.9, while 0.5 mM DCCD-modified COX yielded pKa value of 6.9, resulting in an acid shift
Figure 14. The pH Dependence of Suicide Inactivation Induced by 0.25 mM DCCD Modification of Bovine Heart COX. CC50 values are plotted as a function of pH to determine the effect of pH on the suicide inactivation of the enzyme (Hosler, 2004). Each point is the result of two experiments which each contained three points of data.
Figure 15. pH Dependence of Electron Transfer of Control and 0.5 mM DCCD-modified *R. sphaeroides* COX. pH dependence assays were performed from pH 6.5-10.0 using 25 mM MES, HEPES, and CHES buffers at constant ionic strength for control and 0.5 mM DCCD-reacted *R. sphaeroides* COX. A sigmoidal single-fit pKa curve was created using SigmaPlot, and control COX resulted in a pKa of 7.9. 0.5 mM DCCD-reacted COX yielded a pKa of 6.9. Filled circles represent the WT control *R. sphaeroides* COX, while open circles represent the 0.5 mM DCCD-reacted COX results. This data is representative of multiple trials.
approximately 0.9 units which is similar to the acid shift seen in bovine heart COX (Figure 15). The overall data was also inconsistent with a one-site titration curve which caused the inability of some data trials to be fit to the single-fit pKa curve and suggested that two-site saturation analysis be performed.

Michaelis-Menten plots were also created for double-site saturation analysis as a result of the pH dependence data. A two-site saturation curve was fitted to WT control data and yielded an average pKa value of 7.7±0.5 for site 1 and an average pKa value of 9.4±0.2 for site 2. 0.5 mM DCCD-modified enzyme yielded average pKa values of 6.7±0.1 and 8.7±0.4 for site 1 and site 2 respectively. Representative data are shown in Figure 16. Site 1 exhibited a statistically significant acidic shift in pKa value. These differences in DCCD-modified enzyme also prompted further analysis.

Also seen in Table 3 and Figure 17, analysis of Eadie-Hofstee plots produced an average pKa value of 7.8±0.4 corresponding to an average $V_{\text{max}}$ of 2800±180 sec$^{-1}$ for site 1 of WT *R. sphaeroides* COX and an average pKa value of 9.4±0.1 which corresponds to an average $V_{\text{max}}$ of 825±35 sec$^{-1}$ for site 2. 0.5 mM DCCD-modified COX displayed an average pKa of 7.3±0.4 with an average $V_{\text{max}}$ of 2750±110 sec$^{-1}$ for site 1 and an average pKa of 9.3±0 with an average $V_{\text{max}}$ of 425±35 sec$^{-1}$ for site 2.

The inhibition of catalytic activity induced by DCCD was also calculated and plotted as a function of pH for DCCD-modified *R. sphaeroides* COX in comparison to control WT COX. As shown in Figure 18, inhibition of activity (25-40%) at pH 6.5 and 7.0 are less than the 50% inhibition observed at higher pH values. From pH 7.5-10.0 values, approximately 48-70% inhibition was observed, suggesting that low pH values were less affected by DCCD binding on COX than that at higher pH values.
Figure 16. Michaelis-Menten Kinetics as a Function of pH for Two-site Saturation in *R. sphaeroides* COX Modified with 0.5 mM DCCD. Control *R. sphaeroides* COX activity is shown (filled black circles) plotted as a function of H+ ion concentration. The data was fit to a double-site saturation curve in SigmaPlot which yielded a pKa of 7.9 and 9.3 at site 1 and site 2 respectively. 0.5 mM DCCD-modified bovine heart COX activity is also shown (open circles). The pKa values which resulted from the double-site saturation curve in DCCD-modified enzyme yielded pKa values of 6.8 for site 1 and 8.4 for site 2 which are indicative of an acid shift upon DCCD modification. Each data point corresponds to 1 experimental trial which is comprised of 3 data sets per trial.
Figure 17. Eadie-Hofstee Analysis of the pH Dependence of Electron Transfer Activity in DCCD-modified *R. sphaeroides* COX.

WT control *R. sphaeroides* COX (blue circles) also display a two site binding mechanism as shown by Eadie-Hofstee analysis. Fitting each site with a linear regression (solid blue line) yielded a slope with was used to calculate a pKa of 9.3 for site 1 and a pKa of 7.5 for site 2. 0.5 mM DCCD-modified COX (red triangles) also displayed a two site binding mechanism in which the linear regression (dashed red line) allowed for the calculation of pKa values of 9.3 and 7.0 for site 1 and site 2 respectively. Similar slopes are seen between site 1 of control and DCCD-modified enzyme, while site 2 shows a significant difference upon the modification of the enzyme. These results are dissimilar to bovine heart COX in which site 1 shows differences upon modification with DCCD.
Figure 18. The pH Dependence of Inhibition of Electron Transfer Activity Induced by DCCD Modification in *R. sphaeroides* COX. Percent inhibition of activity for modified *R. sphaeroides* COX as compared to control COX from pH values of 6.5-10.0. Each point is the result of two trials of experiments which contain three data points each.
**Figure 19. pH Dependence of Suicide Inactivation Induced by 0.5 mM DCCD**

**Reaction with *R. sphaeroides* COX.** The amount of catalytic turnover which occurs when *R. sphaeroides* COX is reacted with 0.5 mM DCCD before suicide inactivation is induced is shown in the CC50 values plotted as a function of pH. Trial 1 (closed circles) and trial 2 (open circles) are represented with standard deviation bars displayed for the three trials performed at each point plotted on the graph.
Suicide inactivation of DCCD-modified R. sphaeroides COX was also monitored as a function of pH. As shown in Figure 29, the CC50 values mimic the bovine heart DCCD-modified COX in that as alkalinity increases, the number of catalytic turnovers required for suicide inactivation decreases. This trend mimics the pH dependence of suicide inactivation observed in subunit III-depleted R. sphaeroides COX (Hosler, 2006).

**CD Spectroscopy of the Heme Region in Control and DCCD-modified COX at Physiological and Alkaline pH Values**

Circular dichroism spectral analysis was performed on bovine heart COX and R. sphaeroides COX in order to determine whether the covalent DCCD modification of Glu90 in subunit III induces any perturbations in the environment of heme a and heme a$_3$. Physiological pH 7.0 and alkaline pH 10.0 were used experimentally to assess the molecular mechanism of the differential inhibition between DCCD-modified bovine heart and R. sphaeroides COX previously seen in pH dependence assays.

**Bovine Heart COX**

Figure 20A,B shows the CD spectra of the Soret region of heme a at pH 7.0 and 10.0 for oxidized bovine heart COX. Maximum molar ellipticity values were observed at the wavelengths 427.7±0.3 nm for control COX and 428.2±0.1 nm for oxidized 0.25 mM DCCD-modified COX (Table 4). Previous work on bovine heart COX at physiological pH has shown a CD signal centered at 426 nm in the Soret region (Hill et al., 1988). Average maximum molar ellipticity values for pH 10.0 were determined to be 429.2±0.2 nm for control COX and 429.6.0±0.1 nm for DCCD reacted to COX.
Table IV

Maximum Molar Ellipticity Average Wavelengths for the Soret Region of Bovine Heart and R. sphaeroides COX Upon DCCD Modification

<table>
<thead>
<tr>
<th>Model and Treatment</th>
<th>pH</th>
<th>Average Max. Molar Ellipticity Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Heart COX Control</td>
<td>7.0</td>
<td>427.7 ± 0.3</td>
</tr>
<tr>
<td>Bovine Heart COX +0.25 mM DCCD</td>
<td>7.0</td>
<td>428.2 ± 0.1</td>
</tr>
<tr>
<td>Bovine Heart COX Control</td>
<td>10.0</td>
<td>429.2 ± 0.2</td>
</tr>
<tr>
<td>Bovine Heart COX +0.25 mM DCCD</td>
<td>10.0</td>
<td>429.6 ± 0.1</td>
</tr>
<tr>
<td>R. sphaeroides COX Control</td>
<td>7.0</td>
<td>429.4 ± 0.1</td>
</tr>
<tr>
<td>R. sphaeroides COX +0.5 mM DCCD</td>
<td>7.0</td>
<td>430.2 ± 0.1</td>
</tr>
<tr>
<td>R. sphaeroides COX Control</td>
<td>10.0</td>
<td>431.5 ± 0.5</td>
</tr>
<tr>
<td>R. sphaeroides COX +0.5 mM DCCD</td>
<td>10.0</td>
<td>431.9 ± 0.4</td>
</tr>
</tbody>
</table>

*Averages and standard deviation values are calculated from four different trials of data in which samples for each trial are scanned 5 times to create an average CD spectrum.

*Though a red shift is suggested between control and 0.5 mM DCCD-modified R. sphaeroides, a t-test confirms that there is not a statistically significant difference between the two data sets.
**Figure 20. CD spectra of Control and 0.25 mM DCCD-modified Bovine Heart COX at pH 7.0 and pH 10.0.** In part A, the CD spectrum of the Soret region of heme $a$ for WT control COX at pH 7.0 is shown as a solid black line. The maximum molar ellipticity is determined at the wavelength 428.0 nm. Bovine heart COX reacted with 0.25 mM DCCD is presented as a dashed line and has a maximum molar ellipticity value at 428.8 nm, a red shift as compared to control results. This data is representative of one trial among four total spectral analyses. In part B, the solid black line is the CD spectrum of the Soret region of heme $a$ for WT control COX at pH 10.0. The maximum molar ellipticity is determined at the wavelength 429.8 nm. COX reacted with 0.25 mM DCCD is seen as a dashed line and also displays a red shift as compared to control COX with a maximum molar ellipticity value at 430.8 nm. This data is representative of one trial among four total spectral analyses.
The red shifts in the maximum molar ellipticity wavelength observed from pH 7.0 to pH 10.0 as well as from control COX to DCCD-modified COX is indicative of an electrostatic perturbation in the heme α environment (Riistima et al., 2007). Samples at pH 10.0 were titrated to pH 7.0 in an attempt to shift maximum molar ellipticity values back to values seen at pH 7.0, in order to show reversibility of the structural perturbation of the hemes by the pH 10.0. After titration, a maximum molar ellipticity average wavelength of 429.2±0.2 was calculated for control COX and an average wavelength of 428.7±0.3 was calculated for 0.25 mM DCCD modified COX. As compared to control and DCCD modified values measured at pH 7.0, bovine heart COX does not completely shift its maximum molar ellipticity wavelength from pH 10.0 to pH 7.0 in both the control and DCCD modified enzyme.

*R. sphaeroides* COX

Figure 21A,B shows the CD spectra of the Soret region of heme α for pH 7.0 and 10.0 of *R. sphaeroides* COX. Four trials of *R. sphaeroides* COX samples yielded CD spectra at pH 7.0 with an average maximum molar ellipticity value of 429.4±0.1 nm for control COX and 430.2±0.1 nm for 0.5 mM DCCD modified COX (Table 4). At pH 10.0, maximum molar ellipticity values were determined to be 431.5±0.5 nm for control COX and 431.9±0.4 nm for DCCD modified COX. A red shift is observed in samples from pH 7.0 to pH 10.0 for control COX as well as for control and 0.5 mM DCCD modified COX at pH 7.0. Though a red shift trend seems to be occurring, control and DCCD-modified samples at pH 10.0 do not display a statistical significant shift in wavelength in the bacterial form of the enzyme. When samples at pH 10.0 were titrated back to pH 7.0,
Figure 21. CD spectra of WT and 0.5 mM DCCD-reacted *R. sphaeroides* COX at pH 7.0 and pH 10.0. In part A, the CD spectrum of the Soret region of heme *a* for WT control COX at pH 7.0 is shown as a solid black line and its maximum molar ellipticity is determined at the wavelength 429.3 nm. *R. sphaeroides* COX reacted with 0.5 mM DCCD is displayed as a dashed line and has a maximum molar ellipticity value at 430.2 nm, a red shift as compared to control results. This data is representative of one trial among four total spectral analyses. In part B, the CD spectrum of the Soret region of heme *a* for WT control COX at pH 10.0 is displayed as a solid black line. The maximum molar ellipticity is determined at the wavelength 431.4 nm. *R. sphaeroides* COX reacted with 0.5 mM DCCD is seen as a dashed line with a maximum molar ellipticity value at 432.1 nm. This data is representative of one trial among four total spectral analyses.
maximum molar ellipticity values shifted back to an average wavelength of 429.9±0.4 for control COX and 428.9±0.4 for 0.5 mM DCCD modified COX as compared to control and DCCD modified values measured at pH 7.0. *R. sphaeroides* COX shifts its maximum molar ellipticity wavelength from pH 10.0 to pH 7.0 more efficiently than bovine heart COX samples in both the control and DCCD modified enzyme.

**Heme a reduction of Control and DCCD-modified COX at Physiological and Alkaline pH Values**

Heme a reduction experiments during steady-state electron transfer were performed using control and DCCD-modified bovine heart COX and *R. sphaeroides* COX at pH 7.0 and 10.0 to determine if the electron transfer from heme a to heme a3 is affected by the binding of DCCD to Glu-90 in subunit III. The oxidized spectrum, spectrum during steady-state electron transfer, and reduced spectrum of the Soret region (400-460 nm), which is comprised of 50% heme a and 50% heme a3 (van Gelder et al., 1966), and the alpha peak (550-650 nm), which is comprised of 85% heme a (van Gelder et al., 1966), were analyzed to determine the effect of DCCD upon the enzyme’s electron transfer from heme a to the binuclear center (Alnajjar et al., 2014). Baseline calculations using the alpha peak were performed to determine the percentage of reduction remaining at heme a, which is indicative of the efficiency of catalysis (Alnajjar et al., 2014). Time traces were used to measure changes in absorbance at 605 nm in order to observe the reduction of heme a in the steady state, which correlates to electron transfer to the binuclear center. After a source of electrons are added to the environment containing oxidized enzyme, steady-steady electron transfer kinetics are measured. Dithionite is then
added to fully reduce the enzyme and remove oxygen from the environment. These traces can be analyzed to determine how efficiently electrons are passed from heme $a$ to heme $a_{3}$-Cu$_B$ Bovine Heart COX

As seen in Table 5 and in Figures 22-23 control bovine heart COX yielded an average of 18 ± 1 percentage of reduction at heme $a$ at pH 7.0, whereas DCCD-modified enzyme at pH 7.0 yielded an average of 33 ± 2% heme $a$ reduction during electron transfer. Figure 24 shows the heme $a$ kinetic time traces at 605 nm of control and DCCD-modified bovine heart COX at pH 7.0 and displays a significantly higher reduction level in the DCCD-modified enzyme, which is indicative of less electron transfer to the binuclear center. In Figures 25-26, at pH 10, control enzyme yielded an average reduction of 67 ± 7% and DCCD-modified enzyme yielded an average reduction of 93 ± 6%. As expected, reduction of heme $a$ increases from pH 7.0 to pH 10, and in addition, upon DCCD modification of the enzyme, heme $a$ reduction exhibits a significant increase compared to control, suggesting that the modification at Glu-90 in subunit III affects the efficiency of the electron transfer to the binuclear center. Figure 27 displays the time trace kinetics of heme $a$ at 605 nm for control and DCCD-modified COX at pH 10.0, in which a significantly higher reduction occurs in DCCD-modified enzyme as compared to control. Kinetic reduction levels also indicate less efficient electron transfer to the binuclear center at alkaline pH compared to pH 7.0.

*R. sphaeroides* COX

Similarly heme $a$ reduction levels of *R. sphaeroides* COX undergoing electron transfer is shown in Table 5 and Figures 28-29. The results yielded average reduction levels of 41 ± 2% and 36 ± 2% for WT and 0.5 mM DCCD-modified enzyme at pH 7.0,
respectively. Figure 30 displays the kinetics of heme a reduction at 605 nm of WT and DCCD-modified *R. sphaeroides* COX at pH 7.0. The WT level of reduction is much greater than that of previously published data due to an unknown factor (Alnajjar et al., 2014). The reduction level of heme a is only slightly higher in DCCD-modified enzyme. At pH 10.0, WT COX yielded an average reduction of 33 ± 4% and DCCD-modified enzyme yielded an average reduction of 47 ± 5% (Figures 31-32). Inconsistent with the bovine heart COX model, *R. sphaeroides* displays a significant increase in heme a reduction only at pH 10.0 when modified with DCCD. Figure 33 shows the heme a kinetic time traces of *R. sphaeroides* WT and DCCD-modified COX at pH 10.0. Heme a of DCCD-modified enzyme is significantly more reduced than WT enzyme in these time traces. Overall, data indicate that compared to pH 7.0, far less reduction of the binuclear center occurs in the alkaline environment.
Figure 22. Soret Region and Alpha Peak Analysis of Heme $a$ Reduction for Control Bovine Heart COX at pH 7.0. In part A, the Soret region is shown. The oxidized spectrum is displayed as a thick solid black line, the steady-state spectrum is shown as a thin solid black line, and the reduced spectrum is shown as a dashed line. In part B, the alpha peak is shown. The oxidized spectrum is again shown as a thick solid black line, the steady-state spectrum is shown as a thick solid black line, and the reduced spectrum is shown as a dashed line. Calculations using this peak determined a heme $a$ reduction of 19%. This representative data is one of three trials performed.
Figure 23. Soret Region and Alpha Peak Analysis of Heme a Reduction for 0.25 mM DCCD-modified Bovine Heart COX at pH 7.0. In part A, the Soret region is shown. The oxidized spectrum is displayed as a thick solid black line, the steady-state spectrum is shown as a thin solid black line, and the reduced spectrum is shown as a dashed line. In part B, the alpha peak is shown. The oxidized spectrum is shown as a thick solid black line, the steady-state spectrum is shown as a thick solid black line, and the reduced spectrum is shown as a dashed line. Calculations using this peak determined a heme a reduction of 31%, indicating less efficiency of the catalytic cycle. This representative data is one of two trials performed.
**Figure 24. The Kinetics of Heme a reduction in Control and 0.25 DCCD-modified Bovine Heart COX at pH 7.0.** Absorbance changes at 605 nm are shown for control (solid black line) and 0.25 DCCD-modified (dashed line) enzyme. Time traces for 600 total seconds, with data collection occurring in 15 second intervals, were used to measure the reduction of heme a. After approximately 30 seconds, a source of electrons is added to the sample, which is shown as the short, sharp rise in absorbance, and steady-state electron transfer activity occurs. After 300 seconds, dithionite is added to remove oxygen from the environment and allow full reduction of the enzyme. The increase in absorbance at this point is indicative of the reduction of heme a. These traces indicate a significant increase in heme a reduction and less efficiency in electron transfer to the binuclear center in the enzyme when modified with DCCD. Data are representative of one of five trials.
Figure 25. Soret Region and Alpha Peak Analysis of Heme a Reduction for Control Bovine Heart COX at pH 10.0. In part A, the Soret region is shown. The oxidized spectrum is displayed as a thick solid black line, the steady-state spectrum is shown as a thin solid black line, and the reduced spectrum is shown as a dashed line. In part B, the alpha peak is shown. The oxidized spectrum is shown as a thick solid black line, the steady-state spectrum is shown as a thick solid black line, and the reduced spectrum is shown as a dashed line. Calculations using this peak determined a heme a reduction of 52%, indicating even less efficiency of the catalytic cycle as compared to pH 7.0 values. This representative data is one of five trials performed.
Figure 26. Soret Region and Alpha Peak Analysis of Heme $a$ Reduction for 0.25 mM DCCD-modified Bovine Heart COX at pH 10.0. In part A, the Soret region is shown. The oxidized spectrum is displayed as a thick solid black line, the steady-state spectrum is shown as a thin solid black line, and the reduced spectrum is shown as a dashed line. In part B, the alpha peak is shown. The oxidized spectrum is shown as a thick solid black line, the steady-state spectrum is shown as a thick solid black line, and the reduced spectrum is shown as a dashed line. Calculations using this peak determined a heme $a$ reduction of 73%, indicating less efficiency of the catalytic cycle than the control value at pH 10.0. This representative data is one of three trials performed.
Figure 27. The Kinetics of Heme a reduction in Control and 0.25 DCCD-modified Bovine Heart COX at pH 10.0. Absorbance changes at 605 nm are shown for control (solid black line) and DCCD-modifed (dashed line) enzyme. As previously described, time traces for 600 total seconds were used to measure the kinetics of heme a reduction. Steady-state electron transfer activity occurs, and addition of dithionite removes oxygen from the environment to allow full reduction of the enzyme. The increase in absorbance at this point is indicative of the reduction of heme a, and these traces indicate that heme a reduction levels are increased when enzyme is modified with DCCD. Traces also indicate a significantly slower kinetic transfer of electrons to the binuclear center at alkaline pH than at pH 7.0. Data are representative of one of three trials.
Table V

Analysis of Electron Transfer Efficiency by Reduction at Heme \( a \)

<table>
<thead>
<tr>
<th>Model and Treatment</th>
<th>Percent Reduction at Heme ( a )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7</td>
</tr>
<tr>
<td>Bovine Heart COX Control</td>
<td>18 ( \pm ) 1%</td>
</tr>
<tr>
<td>Bovine Heart COX +0.25 mM DCCD</td>
<td>33 ( \pm ) 2%</td>
</tr>
<tr>
<td>\textit{R. sphaeroides} COX Control</td>
<td>41 ( \pm ) 2%</td>
</tr>
<tr>
<td>\textit{R. sphaeroides} COX +0.5 mM DCCD</td>
<td>36 ( \pm ) 2%</td>
</tr>
</tbody>
</table>

* Averages for bovine heart COX at pH 7.0 for control and DCCD-modified enzyme were calculated from three and two trials respectively. Averages and standard deviations at pH 10.0 for control and DCCD-modified enzyme were calculated from five and three trials respectively.

* Averages for \textit{R. sphaeroides} COX at pH 7.0 for control and DCCD-modified enzyme were calculated from five and three trials respectively. Averages and standard deviations at pH 10.0 for control and DCCD-modified enzyme were calculated from three trials each.
Figure 28. Soret Region and Alpha Peak Analysis of Heme $a$ Reduction for WT $R$. sphaeroides COX at pH 7.0. Part A displays the Soret region of R. sphaeroides COX. The oxidized spectrum is shown as a thick solid black line, the steady-state spectrum as a thin solid black line, and the reduced spectrum is shown as a dashed line. Part B shows the same spectra at the alpha peak. Oxidized (thick solid black line), steady-state (thin solid black line), and reduced (dashed line) spectra were used to calculate a heme $a$ reduction of 40%. This is representative data of five trials of experiments.
Figure 29. Soret Region and Alpha Peak Analysis of Heme $a$ Reduction for 0.5 mM DCCD-modified *R. sphaeroides* COX at pH 7.0. Part A displays the Soret region of *R. sphaeroides* COX. The oxidized spectrum is shown as a thick solid black line, the steady-state spectrum as a thin solid black line, and the reduced spectrum is shown as a dashed line. Part B shows the same spectra at the alpha peak. Oxidized (thick solid black line), steady-state (thin solid black line), and reduced (dashed line) spectra were used to calculate a heme $a$ reduction of 38%. This is representative data of three trials of experiments.
Figure 30. The Kinetics for Heme a reduction in WT and 0.5 DCCD-modified *R. sphaeroides* COX at pH 7.0. Absorbance is shown for WT (solid black line) and DCCD-modified (dotted line) enzyme at pH 7.0. Kinetic time traces for 600 total seconds were used to measure heme a reduction and, therefore, the efficiency of reduction of the binuclear center, The increase in absorbance at the point of dithionite addition is indicative of the reduction of heme a. To a lesser extent than bovine heart COX, these traces show a slight increase in heme a reduction in DCCD-modified enzyme. Data are representative of one of five trials.
Figure 31. Soret Region and Alpha Peak Analysis of Heme a Reduction for WT *R. sphaeroides* COX at pH 10.0. Part A displays the Soret region of *R. sphaeroides* COX. The oxidized spectrum is shown as a thick solid black line, the steady-state spectrum as a thin solid black line, and the reduced spectrum is shown as a dashed line. Part B shows the same spectra at the alpha peak. Oxidized (thick solid black line), steady-state (thin solid black line), and reduced (dashed line) spectra were used to calculate a heme *a* reduction of 36%. This is representative data of three trials of experiments.
Figure 32. Soret Region and Alpha Peak Analysis of Heme a Reduction for 0.5 mM DCCD-modified *R. sphaeroides* COX at pH 10.0. Part A displays the Soret region of *R. sphaeroides* COX. The oxidized spectrum is shown as a thick solid black line, the steady-state spectrum as a thin solid black line, and the reduced spectrum is shown as a dashed line. Part B shows the same spectra at the alpha peak. Oxidized (thick solid black line), steady-state (thin solid black line), and reduced (dashed line) spectra were used to calculate a heme a reduction of 52%, indicating less efficiency of catalysis upon DCCD modification of the enzyme at alkaline pH. This is representative data of three trials of experiments.
(A)

Absorbance

Wavelength (nm)

(B)

Absorbance

Wavelength (nm)
Figure 33. The Kinetics of Heme a reduction in WT and 0.5 DCCD-modified *R. sphaeroides* COX at pH 10.0. Absorbance is shown for WT (solid black line) and DCCD-modified (dashed line) enzyme at pH 10.0. Kinetic time traces for 600 total seconds were used to measure heme a reduction which is linked to the efficiency of electron transfer to the binuclear center. As previously described, the increase in absorbance at the point of dithionite addition is indicative of the reduction of heme a. Though to a much lesser extent than in bovine heart COX, these traces show a slight increase in heme a reduction and, therefore, a decrease in the efficiency of electron transfer to the binuclear center when enzyme is modified with DCCD. Compared to pH 7.0 kinetics, reduction levels of heme a are also significantly higher at alkaline pH. Data are representative of one of three trials.
IV. Discussion

Despite its lack of metal binding sites for redox centers, the gene for subunit III of cytochrome $c$ oxidase is retained in the mitochondrial genome along with the genes for redox active subunits I and II (Bratton et al., 1999). The specific function of subunit III in cytochrome $c$ oxidase catalysis is unknown; however, its removal from the enzyme causes a turnover-induced loss of activity known as suicide inactivation (Bratton et al., 1999). The enzyme rapidly loses O$_2$ reduction activity as well as proton pumping efficiency upon depletion of subunit III (Haltia et al., 1994; Prochaska and Fink, 1987). When WT enzyme is modified with DCCD at the conserved Glu-90 site on subunit III, a 70% decrease in efficiency of proton pumping has been observed (Prochaska et al., 1981). Upon covalent modification, a conformational change is induced in subunit III, suggesting that the structural integrity of subunit III may be significant in maintaining optimal activity of the enzyme (Ogunjimi et al., 2000).

To gain more insight into the role of subunit III in COX activity, mammalian bovine heart COX and bacterial $R. sphaeroides$ COX have been modified with DCCD and compared for similarities in mechanism of inhibition. Electron transfer activity of both the DCCD-modified models as well as the indirect environmental effects which they potentially have on the catalytic cycle have been analyzed. We found that DCCD modification affects the activities of bovine heart COX and $R. sphaeroides$ COX models by significantly different mechanisms. In addition, DCCD modification causes an indirect perturbation of the heme environments in subunit I and affects some electron transfer activity to the binuclear center. A higher concentration of DCCD was required to elicit the effects on the enzyme structure and function in $R. sphaeroides$ COX. This supports
the idea that subunit III plays an indirect role in maintaining the integrity of the environment of the catalytic cycle and, therefore, optimizes activity of the enzyme.

**Summary of Data**

To determine a DCCD concentration which would inhibit electron transfer activity of the enzyme by approximately 50% for optimal measurement, concentration-dependent assays were performed for both bovine heart and *R. sphaeroides* COX (Figure 8). In order to confirm that the DCCD modification at Glu-90 did occur in subunit III, SDS-PAGE gel electrophoresis was performed for all assays and analyzed for the characteristic shift in subunit III toward a higher molecular weight (Figure 9A, B). DCCD-modified bovine heart and *R. sphaeroides* COX were assayed for the pH dependence of their electron transfer activity (Figure 10, 15). Through Michaelis-Menten and Eadie-Hofstee plot analysis of the pH dependence curves, two proton binding sites for catalytic activity were observed (Figure 11, 12, 16, 17). These results suggested one site in which the pKa is affected by DCCD-modification and one site in which the pKa remains unaffected by modification; however, these sites differed between the mammalian and bacterial COX enzymes. The pH dependence of inhibition of electron transfer activity as compared to WT *R. sphaeroides* COX and control bovine heart COX was also determined, with bovine heart COX exhibiting less inhibition by DCCD modification at alkaline pH values (Figure 13) and *R. sphaeroides* COX having less inhibition at physiological pH values (6.5-7.5) (Figure 18). The pH dependence of suicide inactivation induced by DCCD modification was calculated using CC50 values (Hosler, 2004), and both models mimic the suicide inactivation trend seen in subunit III depleted enzyme in *R. sphaeroides* (Bratton et al., 1999) (Figure 14, 19).
To further analyze the inhibition seen in the electron transfer activity of DCCD-modified enzyme, the environment of the hemes in bovine heart COX and *R. sphaeroides* COX were measured using circular dichroism spectroscopy. The differences in the inhibition of electron transfer activity induced by DCCD observed at physiological and alkaline pH values prompted the analysis of the heme environments under both conditions in the modified and unmodified enzymes. DCCD-modified bovine heart COX resulted in a red-shift in maximum molar ellipticity wavelength at both pH 7.0 and 10.0 values (Figure 20). DCCD-modified *R. sphaeroides* COX resulted in a red-shift in the maximum molar ellipticity wavelength at the pH 7.0 value, and a red-shift is suggested at pH 10.0; however, statistically it is not significant (Figure 21). Interestingly, DCCD induced similar effects of the CD spectrum of both enzymes.

Heme *a* reduction experiments were performed at pH 7.0 and 10.0 values to determine if electron transfer activity to the binuclear center was indirectly affected by DCCD modification of subunit III and if pH had any effect on the extent of heme perturbation. Bovine heart COX showed that DCCD-modified enzyme exhibited a higher percentage of reduction at heme *a* during steady-state electron transfer, which corresponds to slower transfer to the binuclear center and lower catalytic efficiency, at both pH 7.0 and 10.0 values (Figure 22-24, 25-27, Table 5). DCCD reaction with *R. sphaeroides* COX induced different properties than bovine heart COX in that DCCD-modified enzyme yielded a higher heme *a* reduction percentage during steady-state electron transfer activity only at pH 10.0, again indicating that the two enzymes are affected by DCCD through different mechanisms (Figure 28-30, 31-33, Table 5).
The Conformational Change Induced by DCCD Modification in Subunit III of COX

When carbodiimides react with Glu-90 in subunit III of cytochrome c oxidase, a significant conformation change occurs and perturbs helical interactions within the subunit as well as affects how subunit III interacts with other subunits of COX (Ogunjimi et al., 2000; Musser et al., 1993). Both bovine heart and R. sphaeroides COX display and confirm this conformational change specifically attributed to DCCD through SDS-PAGE gel electrophoresis (Figure 9A, B). As routinely used in these experiments, a shift in subunit III toward a higher molecular weight for both enzymes is indicative that DCCD has covalently been bound to Glu-90.

DCCD Modification in Bovine Heart Cytochrome c Oxidase

The conserved Glu-90 residue of subunit III in bovine heart COX is located within the wall of the putative O2 transfer pathway, whose function has been studied through mutational analysis in subunit I (Riistima et al., 1996; Tsukihara et al., 1996). This pathway passes through fatty acid tails of PG1 and PG2 located on the negative side of the membrane. In WT bovine heart COX, two oxygen atoms in the carboxyl group of Glu-90 hydrogen bond with the imidazole group of His207 and phenol group of Tyr241. Terminals of two palmitates of PG1 and PG2 are positioned about 5 angstroms from the carboxyl group of Glu-90 (Shinzawa-Itoh et al., 2007). The modification of Glu-90 does not have a major influence on the protein structure; however, the hydrogen bond with His-207 is broken, and a newly introduced water molecule forms a hydrogen bond between the imidazole of His-207 and the hexylamine of DCCD. The hydrogen bond
with Tyr-280 remains. The DCCD molecule itself does not directly contribute to the blockage of the O₂ transfer pathway, but indirectly, the two cyclohexyl groups induce the movement of the two palmitate tail ends of PG1 and PG2 from the wall of the channel to completely occupy the channel space.

Figure 34A shows DCCD bound to Glu-90 in bovine heart COX, the conserved His-207 residue, and the position of the two fatty acid tails PGV307 and PGV607 under modified conditions. Figure 34B shows a superimposition of the crystal structures of control and DCCD-modified bovine heart COX where one fatty acid tail is shown in the control position (PGV267) and the altered position induced by DCCD (PGV307). PGV267 (WT, light blue) was calculated to 3.86 angstroms from Glu-90 while DCCD modification induces movement in the lipid to 7.15 angstroms from Glu-90 (PGV307, DCCD-modified, purple). Figure 34C shows the control and DCCD-modified superimposition along with the second fatty acid tail in the control position (PGV525) and the altered position induced by DCCD modification (PGV607). PGV525 (WT, dark purple) was calculated to 3.80 angstroms from Glu-90, while DCCD modification induces movement in the lipid to 4.48 angstroms from Glu-90 (PGV607, DCCD-modified, pink). These results indicate that significant lipid movements are induced by DCCD in bovine heart COX. This effect has the ability to block the channel which extends from the surface of subunit III to subunit I at the O₂ reduction site (Shinzawa-Itoh et al., 2007). X-ray structure analysis proposes that the role of the hydrophobic PG1 and PG2 fatty acid tails is to regulate O₂ transfer within the enzyme complex. Furthermore, results suggest that the palmitate tail ends could be induced to adopt a conformation in which the channel space is occupied by O₂ molecules introduced
Figure 34. The Crystal Structure of Subunit III and its Environment in Bovine Heart COX Under Control and DCCD-modified Conditions. Part A shows transmembrane helices within subunit III (yellow) and the Glu-90 residue which is modified with DCCD (gray). The conserved His-207 residue is shown 3.513 angstroms from Glu-90. The structures and positions of the lipids PGV307 (dark purple) and PGV607 (light purple) after DCCD modification at Glu-90 are shown 7.152 angstroms and 4.480 angstroms from Glu-90, respectively. Part B shows the overlaid crystal structures WT (green) and DCCD-modified (yellow) bovine heart COX. DCCD (gray) is bound to Glu-90. PGV267 (WT, light blue) was calculated to 3.86 angstroms from Glu-90 while DCCD modification induces movement in the lipid to 7.15 angstroms from Glu-90 (PGV307, DCCD-modified, purple). Part C shows the overlaid crystal structure of WT (green) and DCCD-modified (yellow) with DCCD (gray) bound to Glu-90. PGV525 (WT, dark purple) was calculated to 3.80 angstroms from Glu-90, while DCCD modification induces movement in the lipid to 4.48 angstroms from Glu-90 (PGV607, DCCD-modified, pink). Images and structures in A, B, and C were created by Dr. Chris Pokalsky using the Discover program.
externally or by interactions from the redox active metal sites. These induced conformational changes would, therefore, control the O₂ supply to the O₂ reduction site (Shinzawa-Itoh et al., 2007). This is one interpretation of our results within the proposed roles of subunit III in COX functioning.

Another result from our experiments correlates with the work of Fatima-Lucas et al. (2011), who suggested that electron and proton transfer are coupled in order for an electron to reach the CuB of the binuclear center. In their models, spontaneous proton transfer from Fe-OH (on heme a₃) to the CuB-OH occurs while reducing CuB, indicating that there is necessity of a proton to stabilize electron transfer (Fatima Lucas et al., 2011). The question is raised in DCCD- modified enzyme as to whether O₂ is transferred through the O₂ transfer channel due to the blockage by fatty acid tails. If little or even decreased oxygen amounts are available at the binuclear center to accept the proton transfer from Fe-OH, electron transfer will be perturbed and become unstable due to the coupling of the two processes. This provides us with one possible mechanism in which the movement of fatty acid tail ends in subunit III caused by DCCD modification at Glu-90 could affect the electron transfer activity of the enzyme occurring in subunit I.

In the catalytic cycle of cytochrome c oxidase, the electron transfer rate from heme a to heme a₃ is known to be the internal rate-limiting step during COX turnover (Hill and Greenwood, 1984; Mahapatro and Robinson, 1990). Furthermore, this rate of transfer from heme a to heme a₃ has been shown to be pH dependent and decreases at alkaline pH values (Riegler et al., 2005; Verkovsky et al., 1995). Figure 10 shows that bovine heart COX exhibits a steep decrease in activity above pH 8.0, with an average pKa of 7.9 (Riegler et al., 2005). When bovine heart COX is modified with 0.25 mM
DCCD, an acidic shift of 0.7 units in pKa is observed, indicating a change in the electron transfer activity mechanism as compared to the mechanism of control enzyme (Figure 10). pH dependence curves of both control and DCCD-modified data yield results which are inconsistent with one proton binding site in the enzyme.

To resolve the question of whether multiple proton binding sites exist in the electron transfer kinetics, Michaelis-Menten and Eadie-Hofstee plots were analyzed and exhibited results which confirmed the biphasic binding involved in bovine heart COX turnover for the control and DCCD-modified enzyme (Figure 11, 12). This parallels the biphasic binding properties observed for hydrogen peroxide by Riegler et al. (2005) for control bovine heart COX at pH 7.8 and 9.75. Hydrogen peroxide binding yields the P intermediate which would be sensitive to both electron transfer from heme $a$ to $a_3$ and also pH value. Control and DCCD-modified data were fitted with more consistent two-site saturation curves, and Eadie-Hofstee plots yielded an average pKa at site 1 of 7.6±0.1 and 7.7±0.2, respectively. This site does not seem affected by the introduction of DCCD to subunit III; however, site 2 yielded an average pKa of 8.8±0.2 and 9.3±0.1 for control and DCCD-modified enzyme, respectively. Michaelis-Menten analysis displayed this same trend, in that an alkaline shift was observed in site 2 when DCCD was bound to the enzyme. This alkaline shift is statistically significant and suggests that upon DCCD modification, proton-binding site 2 is affected and undergoes a conformational change which favors an environment of higher alkalinity. As previously shown, WT bovine heart COX turnover decreases significantly as alkalinity in the environment increases; however, the inhibition of electron transfer activity is highly decreased at pH 9.5 and 10.0 in DCCD-modified enzyme (Figure 13) (Riegler et al., 2005). This stimulated behavior in
electron transfer activity could be explained by the DCCD-induced alkaline shift (or an increased protonated form) observed in site 2 of the enzyme, allowing activity to increase in an alkaline environment due to the more favorable alkaline pKa and higher state of protonation of the proton-binding site. These results also support that DCCD bound to Glu-90 in subunit III creates an alteration in the mechanism of electron transfer activity occurring in subunits I and II of the enzyme.

A specific examination of the steady-state electron transfer from heme $a$ to heme $a_3$, a 2.6 fold decrease has been observed in the rate constant as pH is increased from 7.0 to 9.5 (Sadoski et al., 2001). This decrease in steady-state electron transfer at alkaline pH has been attributed to the slow rate of electron transfer from heme $a$ to heme $a_3$; it is not affected by the alkaline conformation of cytochrome $c$ nor a decreased interaction strength between cytochrome $c$ and oxidase. Furthermore, proton delivery through the K channel has been shown to be rate-limiting for the reaction with oxygen at alkaline pH, for K mutated enzymes display the same inhibition of activity (Riegler et al., 2005).

The steady-state heme $a$ reduction results observed for control bovine heart COX at pH 7.0 and pH 10.0 yielded average heme $a$ reduction levels of $18\%\pm1\%$ and $67\%\pm7\%$ (Table 5, Figures 22, 23, 24), respectively. This follows the published trend by Riegler et al. (2005) where heme $a$ reduction levels at pH 7.8 were $\sim 30\%$ and reduction levels at pH 9.75 were $\sim 60\%$. When the enzyme is modified with DCCD, heme $a$ is significantly reduced to a greater extent at both pH 7.0 and 10.0 as compared to the control results (Table 5), indicating that the transfer of electrons from heme $a$ to heme $a_3$ in subunit I becomes less efficient do the induction of DCCD in subunit III in physiological and alkaline environments. These results further suggest that the perturbation caused by
DCCD modification indirectly alters the mechanism involved in the specific electron transfer from heme \(a\) to the binuclear center.

After observing an alteration in electron transfer activity of the enzyme, circular dichroism spectroscopy of the Soret region was performed to determine if any variations in the heme environments were induced due to DCCD modification of the enzyme. The Soret CD spectrum of COX results from the chiral nature of its heme porphyrins and the asymmetric interactions of these hemes with their surroundings (Orii et al., 1977; Callahan and Babcock, 1983). Hemes in cytochrome \(c\) oxidase are not covalently bound, and the catalytic electron transfer from Cu\(_A\) to heme \(a\) to the binuclear center (heme \(a_3\)-Cu\(_B\)) is significantly affected by any electrostatic perturbations at the active site (Fatima Lucas et al., 2011). The strong electron withdrawing formyl group of heme \(a\) has the ability to alter redox and ligand binding, interactions of the porphyrin system with protein, and the optical absorption spectrum (Riistima et al., 2000; Caughey et al., 1975). The crystal structure of \(P.\) denitrificans proposes that the side chain of Arg-54 in helix I of subunit I forms a hydrogen bond with this formyl group (Riistima et al., 2000; Iwata et al., 1995). To determine whether modification of Arg-54 affects the optical spectrum of heme \(a\), Riistima et al. (2000) performed mutations of this residue substituting with glutamine (R54Q) and methionine (R54M). Their results showed that R54Q, which forms a weakened hydrogen bond with the formyl group at a less favorable angle, induced a blue shifted spectrum of heme \(a\) by 6 nm (605 nm to 597 nm) and electron transfer activity was decreased to \(\frac{1}{4}\) of that of WT. R54M, which does not hydrogen bond with the formyl group also induced a 16 nm blue shift (605 nm to 589 nm), almost shifting the spectrum to that of isolated heme \(a\); additionally, its electron transfer activity was
completely abolished (Riistima et al., 2000). Another conserved residue in *P. denitrificans*, Arg-474, interacts with a propionate of heme *a*, and when this site is mutated to an Asp, a blue shift of 2-3 nm in the optical spectrum of heme *a* occurs. The results provided by Riistima et al. (2000) indicate that the observed red shift seen in heme *a* of COX is mainly attributed to the specific hydrogen bonding interactions of the heme *a* formyl group and Arg-54 (*P. denitrificans*), while the electrostatic effect caused by the interaction between the propionate of heme *a* and Arg-474 partially contributes to this shift as well.

The CD spectra obtained from control and DCCD-modified bovine heart COX indicate a red shift in the Soret region when enzyme is DCCD-modified from 427.7±0.3 nm to 428.2±0.1 nm at pH 7.0 and from 429.2±0.2 nm to 429.6±0.1 nm at pH 10 (Table 4). Previously, CD spectra of oxidized bovine heart COX was shown to be 426 nm at pH 7.4 and red shifted by 1.5 nm when titrated to the pH value of 9.75 (Riegler et al., 2005).

According to the data presented by Riistima et al. (2000), a red shift in the spectrum of the heme environments may indicate a stronger hydrogen bonding interaction between the formyl group of heme *a* and the Arg-38 of bovine heart COX. Figure 35A is a model of the crystal structure of WT bovine heart COX heme *a* and depicts the hydrogen bond, nonbonding interaction, and hydrophobic interaction lengths between the formyl group and Arg-38. Figure 35B shows the crystal structure of heme *a* as well as the bond and interaction lengths upon the modification of DCCD. Shown in Table 6, DCCD induces a hydrogen bond between the formyl group and Arg-38 which is 0.006 angstroms closer than that of WT, and nonbonding interactions are 0.151
Figure 35. WT and DCCD-modified Heme a Structures and Formyl Group

Interactions in Bovine Heart COX. Part A shows the heme a structure of WT bovine heart COX and the interactions which occur between the formyl group of the porphyrin ring and the Arg-38 residue. Hydrogen bonding between the hydrogen of Arg-38 and the carbonyl of the formyl group is calculated and shown as 2.891 angstroms. Nonbonding interactions and hydrophobic interactions are calculated and shown as 3.287 angstroms, and 4.046 angstroms, respectively. Part B shows the heme a structure and the interaction between the formyl group of the porphyrin ring and Arg-38 of bovine heart COX when modified with DCCD at Glu-90. The hydrogen bond between the hydrogen of Arg-38 is calculated and shown as 2.886 angstroms. The nonbonding interactions and hydrophobic interactions are calculated and shown as 3.136 angstroms and 3.995 angstroms, respectively. Images and calculations were done using PDB267 in the Discover program and produced by Dr. Chris Pokalsky.
Table VI

Comparative Distances Between the Formyl Group of Heme α and Arg-38 in Bovine Heart COX

<table>
<thead>
<tr>
<th></th>
<th>Hydrogen bond (Å)</th>
<th>Nonbonding Interaction (Å)</th>
<th>Hydrophobic Interaction (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Bovine Heart COX</td>
<td>2.891</td>
<td>3.287</td>
<td>4.406</td>
</tr>
<tr>
<td>Bovine Heart COX +0.25 mM DCCD</td>
<td>2.886</td>
<td>3.136</td>
<td>3.995</td>
</tr>
<tr>
<td>Difference in distance between WT and +0.25 mM DCCD Bovine Heart COX</td>
<td>0.006</td>
<td>0.151</td>
<td>0.411</td>
</tr>
</tbody>
</table>

*Distances calculated using PDB file 267
angstroms closer than WT enzyme. The closer bond distance will increase electron
density at the formyl group and, therefore, cause the red shift observed. The hydrogen
bonding network between the proprionates of heme $a$, Arg-438 and Arg-439, which
correspond to the Arg-474 in $P. denitrificans$, was also analyzed and bond lengths
calculated. These results indicate no significant change in bond lengths upon
modification of DCCD, which suggests that it is the electrostatic perturbation at the
formyl group of heme $a$, which contributes to the red shift seen in the spectra.
Additionally, a larger red shift at pH 7.0 is seen in comparison to the red shift at pH 10.0,
which may indicate that alkaline pH partially inhibits the electrostatic alteration of the
formyl group. Furthermore, this may explain why DCCD-induced inhibition is decreased
in the alkaline environment. This is one example of a molecular mechanism of the red
shift of the Soret CD induced by DCCD in bovine heart COX.

**DCCD Modification in R. sphaeroides Cytochrome c Oxidase**

Glu-90 is located on helix 3 of subunit III and forms a triad complex with His-212
and Tyr-246 residues located on helix 6 of the subunit (Ogunjimi et al., 2000). Ogunjimi
et al. (2000) has also shown that this triad is indirectly involved in bovine heart COX
electron transfer activity. A mutation of Tyr-246 in $R. sphaeroides$ resulted in enzyme
which exhibited normal WT activity, indicating that the hydrogen bond from Glu-90 to
Tyr-246 is not necessary for normal oxidase functioning (Geyer, 2007). Additionally, a
mutation of Glu-90 (Glu90H) in $R. sphaeroides$ reduced steady state electron transfer
activity by 50%, yet a double point mutation of E90H/H212E to allow for the
maintenance of the hydrogen bonding to occur, only decreases enzyme activity by 13%
(Omolewu, 2013). These results suggest that this the spatial arrangement of the triad is less significant than the hydrogen bond between Glu-90 and His-212 which plays a significant role in the catalytic activity of the enzyme.

*R. sphaeroides* also contains two lipids which are part of the surroundings of Glu-90, similar to that of bovine heart COX (Varanasi et al., 2006). Figure 36A shows subunit III (green) of WT *R. sphaeroides* with Glu-90 on helix 3. Also shown are the lipids PEH2009 (light purple) and PEH2008 (dark purple) in their native position. Figure 36B shows subunit III of *R. sphaeroides* modified with DCCD (yellow) and the modified positions of PEH2009 and PEH2008. Recent molecular modeling from our lab reveals that when *R. sphaeroides* is modified with DCCD, C2I of PEH2009 shifts from 10.27 angstroms from OE2 of Glu-90 to 9.68 angstroms from Glu-90. Also in PEH2009, WT C2H shifts from 10.19 angstroms from OE2 of Glu-90 to 9.63 angstroms, and WT C3F shifts from 8.83 angstroms from OE2 of Glu-90 to 10.21 angstroms. In PEH2008, WT C2B shifts from 6.09 angstroms from OE2 of Glu-90 to 6.18 angstroms, WT C2G shifts from 10.48 angstroms from OE2 of Glu-90 to 10.59 angstroms, and WT C2I shifts from 11.35 angstroms from OE2 of Glu-90 to 11.23 angstroms. These results indicate DCCD induces significant movements in PEH2009 of *R. sphaeroides*, while little movement is observed in PEH2008 upon DCCD modification. When the bulky, hydrophobic DCCD molecule is bound to the bacterial Glu-90 residue, the significant movement of PEH2009 yields the potential for blockage in the O₂ transfer channel as seen in bovine heart COX, which could contribute to the decrease in electron transfer activity observed in the enzyme.
Figure 36. The Crystal Structure of Subunit III in *R. sphaeroides* and the Lipid Environment Surrounding Glu-90. Part A shows subunit III (green) of WT *R. sphaeroides* and the Glu-90 residue on helix 3. The lipids, PEH2009 (light purple) and PEH2008 (dark purple), are shown in their native positions. The image shows that C3F of PEH2009 is 8.83 angstroms from OE2 of Glu-90, while C2B of PEH2008 is 6.10 angstroms from OE2 of Glu-90. Part B shows subunit III with the DCCD modification at Glu-90 in *R. sphaeroides* (yellow) and the modified positions of the lipids PEH2009 (light purple) and PEH2008 (dark purple). C3F of PEH2009 is 10.21 angstroms from OE2 of Glu-90, while C2B of PEH2008 is 6.18 angstroms from OE2 of Glu-90. Images were produced by Dr. Chris Pokalsky using the Discover program and PDB1M56.
Previous work with WT *R. sphaeroides* COX has shown that steady state electron transfer activity has a pKa of 8.5 (Gilderson et al., 2003). When subunit III is removed in *R. sphaeroides*, the pKa results in an acidic shift to 7.2 with an accompanying 50% decrease in steady state electron transfer activity occurs (Cvetkov, 2010). The observed pH dependence of WT and 0.5 mM DCCD-modified *R. sphaeroides* indicated an acidic shift from 7.9 to 6.9, respectively (Figure 15). Like bovine heart COX, data were not consistent with one proton binding site, and Michaelis-Menten and Eadie-Hofstee analysis was performed to produce more reliable results (Figures 16, 17). These plots revealed biphasic proton binding as seen in bovine heart COX, with Michalis-Menten plots fitted with two-site saturation curves and Eadie-Hofstee plots revealing two pKa’s for two separate sites. Eadie-Hofstee analysis yielded pKa’s of 7.8±0.4 and 7.3±0.4 for control and DCCD-modified enzyme at site 1, respectively, although this is not a strong statistical significance. At site 2, control and DCCD-modified enzyme yield pKa’s of 9.4±0.1 and 9.3±0 (Table 4), which indicates that there is an acidic shift occurring at site 1 of the enzyme when modified with DCCD. It is likely that the two sites interact and regulate each other by conformational means. Potentially site 1 (low pKa site) controls the proton uptake at high pH values, and because it displays a small acidic shift, a slower proton uptake occurs at more alkaline pH values. Site 2 (high pKa site), which does not show variation between WT and DCCD-modified enzyme, may regulate proton uptake at low pH values. At low pH values there is a saturating amount of protons in the environment, causing the rate-limiting step to be independent of the proton concentration and proton uptake rate. Therefore, at low pH values, the electron transfer activity may not be dependent on the interaction of the two sites. This is one possibility which may
explain why a decrease is observed in the inhibition of electron transfer activity induced by DCCD at pH 6.5 and 7.0 (Figure 18). This suggests that when modified with DCCD, site 1 in \textit{R. sphaeroides} is perturbed while site 2 is unaffected. These data also indicate that a different catalytic mechanism is induced when \textit{R. sphaeroides} is modified with DCCD as compared to WT.

The acidic shift observed in these results are different from the alkaline shift which results from the E90H mutant where the E90:H212 interaction is disrupted, and they more closely mimic the trend seen in subunit III depleted COX (Omolewu, 2003; Cvetkov, 2010). This suggests that DCCD modification of Glu-90 is not just disrupting the hydrogen bond to His-212, but has another effect on the surrounding environment as well. An acidic shift implies that there is a decrease in the proton uptake pathway, the D channel. As previously mentioned, electron transfer from heme \textit{a} to the binuclear center has been found to be coupled with the transfer of a proton from Fe-OH to Cu\textsubscript{B}-OH, and this stabilizes the efficiency of electron transfer activity. Using this theory, in \textit{R. sphaeroides}, a slowed proton uptake may be the cause of decreased efficiency in electron transfer activity.

Due to an unknown reason, the heme \textit{a} reduction levels observed in WT \textit{R. sphaeroides} at pH 7.0 were higher than the previously reported value of 35\% at pH 8.0, though data was collected from 5 different trials of experiments which yielded a standard deviation of 2\% (Konstantinov et al., 1996). WT and DCCD-modified enzyme at pH 7.0 (Figure 28, 29) yielded heme \textit{a} reduction levels of 41±2\% and 36±2\% (Table 5, Figures 28, 29, 30). At pH 10.0, WT and DCCD-modified enzyme yielded heme \textit{a} reduction levels of 33±4\% and 47±5\% (Table 5, Figures 31, 32, 33), consistent with a decreased
efficiency of electron transfer activity from heme $a$ to the binuclear center when modified with DCCD. Though results from pH 7.0 WT enzyme remain inconclusive, heme $a$ reduction levels at alkaline pH show a significant increase in the reduction of heme $a$ upon modification of DCCD in $R. sphaeroides$ COX. This indicates some perturbation at the active site in subunit I induced by a modification of Glu-90 in subunit III.

To analyze the heme environments of $R. sphaeroides$ COX upon modification with DCCD, circular dichroism spectroscopy was also performed in the Soret region. At pH 7.0, the modification of DCCD induced a shift from 429.4±0.1 nm to 430.2±0.1 nm, and at pH 10.0, DCCD modification induced a shift from 431±0.5 nm to 431.9±0.4 nm (Table 4). The red shift induced by DCCD is much more significant at pH 7.0 than the suggested red shift induced by DCCD at pH 10.0, but even a small red shift in the Soret region could indicate electrostatic perturbations at the formyl group of heme $a$, as suggested in bovine. In addition the red shift may be attributed to a variation in the hydrogen bond network between the propionates of heme $a$ and conserved Arg residues. More molecular modeling and calculations are necessary in order to provide a more complete assessment of the mechanism; however, it indicates that the modification of Glu-90 in subunit III of $R. sphaeroides$ causes a perturbation in the heme environment located in subunit I of the enzyme.
A Comparative Analysis of Bovine Heart COX and *R. sphaeroides* COX with Emphasis on the Conserved Mitochondrial Encoded Subunits

Figure 37 shows a superimposition of the three conserved mitochondrial encoded subunits in native bovine heart COX (green) and WT *R. sphaeroides* COX (yellow) from their 3-D crystal structures. Molecular simulations were done to compare the two enzyme structures. A superimposition of subunit I from bovine heart and *R. sphaeroides* COX yields a structural homology of 75%. Subunit II alone yields 64% homology. When subunits I, II, and III are superimposed together, bovine heart and *R. sphaeroides* COX yield a structural homology value of 69%. This difference in structure as well as the additional nuclear subunits in the bovine COX explain the variation in the response to DCCD modification between the two enzyme forms.

As previously stated, both enzymes exhibit biphasic properties in proton binding sites in their activities. Whether these sites are ordered, independent, dependent, or allostERIC is beyond the scope of these experiments, however, determining their exact mechanism in the enzyme’s catalytic turnover would provide more insight into how DCCD is specifically affecting these proton binding sites. It could be that one site is important for proton uptake, while the other may be involved in allostERIC binding and structural regulation. In both forms of the enzyme, DCCD seems to affect one binding site, while the other is similar to that of its WT or control counterpart. Bovine heart COX exhibits an alkaline shift in site 2, indicating that when modified with DCCD, bovine heart COX has an increased affinity for protons. This corresponds to the decreased percent inhibition of electron transfer activity displayed at pH 9.5 and 10.0 where site 2 may be more favorable of an alkaline environment. *R. sphaeroides* COX exhibits an
Figure 37. A Comparison of the Conserved Mitochondrial Encoded Subunits of Bovine Heart and *R. sphaeroides* COX. The conserved, mitochondrial encoded subunits I, II, and III of bovine heart COX (green) and *R. sphaeroides* COX (yellow) have been superimposed to show their variation in structure, which potentially gives rise to their differences in activity. A 69% similarity value was calculated from the crystal structures. Chris Pokalsky created this image in the Discover program.
acidic shift at site 1, indicating that upon DCCD modification, *R. sphaeroides* COX has a decreased affinity for protons. This acidic shift in site 1 also corresponds to the decrease in the inhibition of activity at pH values of 6.5 and 7.0 where site 1 may become more favorable of an acidic environment.

Circular dichroism spectroscopy of the Soret region yields similar trends in bovine heart and *R. sphaeroides* COX. The bovine heart COX exhibits perturbation to a larger extent as compared to that of *R. sphaeroides* COX upon DCCD modification. The same similar trend is seen in the steady state heme *a* reduction experiments, with bovine heart COX exhibiting reduction of heme *a* to a higher extent upon DCCD modification at both physiological and alkaline pH values than the less differential *R. sphaeroides* heme *a* reduction values. The heme *a* reduction time traces visibly show that DCCD had a greater effect on bovine heart COX as compared to those of DCCD-modified COX from *R. sphaeroides*.

I hypothesize that upon DCCD modification at Glu-90 in subunit III, the decrease in electron transfer activity in bovine heart COX is likely due to the blockage of the O$_2$ transfer pathway by the repositioning of interacting lipids (Shinzawa-Itoh et al., 2007). A similar lipid movement occurs in *R. sphaeroides*, but only one lipid (PEH2009) shows significant movement induced by DCCD; therefore, this blockage at the O$_2$ transfer pathway is likely different as compared to that which occurs in bovine heart COX. This may also explain some of the observed variation in electron transfer activity inhibition as well as variation in the heme environments between bovine heart and *R. sphaeroides* COX.
Additionally, when *R. sphaeroides* COX is modified with DCCD, the results are consistent with slowed proton uptake by the D channel, which in turn would decrease the efficiency of electron transfer activity in the enzyme (Cvetkov, 2010). The stabilization and efficiency of electron transfer activity is considered to be coupled to the transfer of a proton from Fe-OH to Cu_B-OH (Fatima Lucas et al., 2011). In this event, it is likely that when bovine heart COX is modified with DCCD, O_2 is blocked from reaching the active site, and no proton transfer occurs, therefore electron transfer activity efficiency is decreased. When *R. sphaeroides* COX is modified with DCCD, it is likely that the slowed proton uptake by the D channel affects the efficiency of the electron transfer activity.

DCCD modification in both enzymes yields a similar outcome wherein the catalytic electron transfer activity is significantly decreased and the heme environment in subunit I becomes structurally perturbed. Observed results indicate that a mechanistic change in both enzymes is occurring in the catalytic cycle of subunit I due to a modification which is induced at subunit III. Results also suggest that this mechanistic change is different in bovine heart COX than in *R. sphaeroides* COX, however, one common insight is gained: the subunit III of cytochrome *c* oxidase, which lacks metal redox active centers, continues to reveal its significance to the catalytic mechanism and maintaining structural integrity of the enzyme.
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Å</td>
<td>Angstroms</td>
</tr>
<tr>
<td>A State</td>
<td>The oxy state of cytochrome c oxidase</td>
</tr>
<tr>
<td>$aa_3$</td>
<td>Hemes a and a$_3$ of cytochrome c oxidase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’ triphosphate</td>
</tr>
<tr>
<td>CcO</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism Spectroscopy</td>
</tr>
<tr>
<td>CHES</td>
<td>N-cyclohexyl-2-aminoethanesulfonic acid</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>Cu$^+$</td>
<td>Copper metal center</td>
</tr>
<tr>
<td>Cyt. c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>DCCD</td>
<td>N,N’-Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DM</td>
<td>Dodecyl β-D maltoside</td>
</tr>
<tr>
<td>F State</td>
<td>An oxyferryl intermediate of cytochrome c oxidase</td>
</tr>
<tr>
<td>FADH$_2$</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>H$^+/e^-$</td>
<td>Ratio of protons translocated per electrons transferred</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>O State</td>
<td>The oxidized state of cytochrome c oxidase</td>
</tr>
<tr>
<td>OD$_{660}$</td>
<td>Optical density measured at 660 nm</td>
</tr>
<tr>
<td>P. denitrificans</td>
<td>Paracoccus denitrificans</td>
</tr>
<tr>
<td>P$_R$ State</td>
<td>An oxyferryl intermediate of cytochrome c oxidase</td>
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<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PGV</td>
<td>(1R)-2{<a href="hydroxy">[[2S]-2,3-dihydroxypropyl]oxy</a>phosphoryl]oxy}-1-[(palmitoyloxy)methyl]ethyl(11E)-octadec-11-enoate</td>
</tr>
<tr>
<td>R State</td>
<td>The reduced state of cytochrome c oxidase</td>
</tr>
<tr>
<td>R. sphaeroides</td>
<td>Rhodobacter sphaeroides</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide</td>
</tr>
<tr>
<td>TMPD</td>
<td>N,N,N’-tetramethyl-phenylenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminoethane</td>
</tr>
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<td>TX-100</td>
<td>Triton 100</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype enzyme</td>
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</table>
References


75. Sadoski, R. C., Zaslavsky, D., Gennis, R. B., Durham, B., Millet, F. 2001. Exposure of bovine cytochrome c oxidase to high Triton X-100 or to alkaline
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