STUDIES ON CRITICAL PROTON PATHWAY RESIDUES IN THE $A\text{A}_3$-TYPE CYTOCHROME C OXIDASE FROM $R\text{HODOBACTER SPHAEROIDES}$

BY

KRITHIKA GANESAN

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biophysics and Computational Biology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2009

Urbana, Illinois

Doctoral Committee:

Professor Robert B. Gennis, Chair
Professor Antony Crofts
Professor Yi Lu
Associate Professor Satish Nair
Abstract

Aerobic organisms derive energy needed to sustain life from foodstuffs through the process of cellular respiration. Respiration is performed by a series of soluble and multi-subunit membrane proteins that form the ‘electron-transport chain’, found in the inner mitochondrial membrane of eukaryotes and the cytoplasmic membrane of aerobic prokaryotes. Cytochrome c oxidase, the terminal member of this respiratory chain, catalyzes the four-electron reduction of oxygen to water and uses the free energy of this reaction to translocates protons across the membrane. The oxidase accomplishes oxygen reduction by drawing electron and protons from the opposite sides of the membrane and transfers the pumped protons against the membrane potential through very specific routes within the enzyme. Several years of research have thrown some light on the electron and the proton-input pathways, catalytic cycle intermediates and the timing of the proton-pumping steps. However, the coupling of the oxygen chemistry to proton pumping is not yet understood at a molecular level and several grey areas like proton/water-exit channel, residues controlling the unidirectionality of the pump and a universal proton pumping mechanism are being actively researched. The focus of this thesis is on amino-acid residues in the catalytic subunit-I that are important to the delivery of protons to the active site for the reduction of oxygen and proton pumping. Chapter 2 describes the characteristics of mutations at the residue Met-107 near the critical proton transferring residue in the D-pathway, Glu-286. The aim of the study is to check if Met-107 has a role in assisting Glu-286 in the delivery of protons. Chapter 3 describes the characteristics of mutations at Glu-286 with respect to their effects on the environment of the active-site. This study aims to provide experimental evidence for
the long range hydrogen-bonded connectivity between Glu-286 and the active site. Chapter 4 adds to the experimental evidence on the well studied residue Lys-362, for its critical role in transferring protons through the K-pathway. The question of the number of protons delivered through this pathway has been addressed in this chapter. Chapter 5 describes some of the conditions required for the formation of the unique covalent cross-link found at the active site of all heme-copper oxidases. We have also speculated on the radical chemistry at the active site that may have lead to its formation.
Dedicated to

My Family
Acknowledgments

I would like to express my thanks and gratitude to my advisor, Professor Robert Gennis for his guidance and support throughout this work. I have greatly benefitted from his wealth of knowledge and experience and will always be inspired by his enthusiasm and passion for science and life in general.

I thank my committee members Prof. Antony Crofts, Prof. Colin Wraight, Prof. Yi Lu and Prof. Satish Nair for their insightful comments and suggestions.

I’d like to use this opportunity to also thank all the past and present members and the short-term visitors of the Gennis Lab. I sincerely thank Prof. Alexander Konstantinov, Dr. Tatiana Vygodina and Prof. Jonathan Hosler for their invaluable inputs on my research. I’m grateful to Dr. Carrie Hiser for her kind assistance over e-mail on several molecular biology techniques. I’d like to thank Dr. Joel Morgan, Dr. Dan Han, Dr. Ashtamurthy Pawate and Dr. Kara Weiss for training me in various laboratory techniques. I thank Dana Robinson for his work on mass spectrometry. Many thanks to Dr. James Hemp, Dr. Laura Pace, Dr. Deborah Berthold, Dr. Jiapeng Zhu, Dr. Ahmet Vakkasoglu, Dr. Lai Lai Yap, Ke Yang, Myat Lin, Hyun Ju Lee, Young Ahn, Hanlin Ouyang, Eric Han, Calvin Chang, Myongsin Yi, Sylvia Choi and my undergraduate student Lori Haymon for their friendship and collegiality. I had a great time working with all of you, thank you! I’d like to especially appreciate Myat Lin for being ‘Mr.Reliable’, and I sincerely wish him all the success in the pursuit of his ambitions.

I’d like to thank my collaborators and hosts during my visits to other laboratories- Prof. Denis Rousseau and Michelle Yu at the Albert Einstein College of Medicine, Prof. Peter Brzezinski, Linda Ojemyr and their research group at the
Stockholm University. I’m also grateful to Prof. Per Siegbahn and Angela Ljungstrom for their generous hospitality.

I’d like to thank the administrative staff members Cynthia Dodds, Louise Cox, James Poepsel and Jeri Kyle for their prompt assistance over the years.

A word of thanks to my best friends on campus- Dr. Moushumi Sen Sarma, Johannes Russer, Pratibha Ramanu, Ananth Prasad and their families for the innumerable pleasant memories carved over gourmet food and wine!

Finally I would like to express my gratitude to my family. All my accomplishments have been possible only due to the unconditional love and unflinching support of my dear parents Lalitha and Ganesan, my loving sister Janani and my beloved husband Raman. I feel fortunate and thankful for having them in my life and I love them all dearly.
### Table of contents

List of abbreviations ........................................................................................................... xi

Chapter 1: Background and significance ........................................................................... 1

1.1 Introduction .................................................................................................................. 1

1.2 Heme-copper oxidase superfamily .............................................................................. 2

1.3 *Rhodobacter sphaeroides* cytochrome *aa*₃ ............................................................... 3

1.4 Overall structure of cytochrome *c* oxidase ............................................................... 4

1.5 Electron transfer pathway ........................................................................................... 6

1.6 Proton transfer pathways ............................................................................................ 6

1.7 Catalytic cycle ............................................................................................................. 9

1.8 Mechanism of proton pumping ................................................................................... 11

1.9 Scope of this thesis ..................................................................................................... 14

1.10 Figures ...................................................................................................................... 16

1.11 References ............................................................................................................... 23

Chapter 2: Mutations at Met-107 site suggest the residue is not essential for the gating of protons .............................................................................................................. 33

2.1 Introduction ................................................................................................................ 33

2.2 Materials and methods .............................................................................................. 35

2.2.1 Mutagenesis ......................................................................................................... 36

2.2.2 Cell growth and protein purification ........................................................................ 36

2.2.3 UV/Vis spectroscopy ............................................................................................. 38

2.2.4 Steady-state activity .............................................................................................. 38

2.2.5 Reconstitution of oxidase in proteoliposomes ....................................................... 39

2.2.6 Proton-pumping .................................................................................................... 39
2.2.7 Oxidation kinetics .................................................................40
2.2.8 Reduction kinetics ...............................................................41
2.2.9 Preparation of fully reduced CO-bound cytochrome c oxidase ...41
2.2.10 Optical flow-flash measurements ........................................42
2.3 Results ..................................................................................42
2.3.1 UV/Vis spectroscopy ............................................................42
2.3.2 Steady-state activity ..............................................................43
2.3.3 Proton-pumping .................................................................43
2.3.4 Reduction kinetics ...............................................................44
2.3.5 Flow-flash kinetics ...............................................................45
2.4 Discussion .............................................................................46
2.5 Figures and tables .................................................................50
2.6 References ............................................................................60

Chapter 3: Substitutions at glutamate-286 in \( {aa}_3 \)-type cytochrome c oxidase affect the environment of the catalytic binuclear center ..................67

3.1 Introduction ...........................................................................67
3.2 Materials and methods ..........................................................70
3.2.1 Site-directed mutagenesis ....................................................70
3.2.2 Protein purification ..............................................................71
3.2.3 Steady-state kinetics ............................................................71
3.2.4 Reconstitution of oxidase in proteoliposomes .......................71
3.2.5 Resonance Raman spectroscopy ...........................................72
3.3 Results ..................................................................................73
3.3.1 Analysis of the optical spectra ..............................................73
3.3.2 Effects of mutation on catalytic activity and proton pumping....74
3.3.3 Resonance Raman spectra of the as-isolated enzymes .......... 74
3.3.4 Resonance Raman spectra of the fully-reduced enzymes ....... 77
3.3.5 Resonance Raman spectra of the fully-reduced CO-bound enzymes ................................................................................. 77
3.4 Discussion .................................................................................... 78
3.5 Figures .......................................................................................... 83
3.6 References .................................................................................... 93

Chapter 4: On the delivery of protons through K-pathway during the reduction of the aa3-type cytochrome c oxidase from Rhodobacter sphaeroides ................................................................................................ 100

4.1 Introduction .................................................................................... 100
4.2 Materials and methods ................................................................. 103
4.2.1 Site-directed mutagenesis ......................................................... 103
4.2.2 Protein purification ................................................................. 103
4.2.3 Measurement of cytochrome c oxidase activity ...................... 104
4.2.4 Measuring the rate of reduction of heme a3 ......................... 104
4.2.5 EPR spectroscopy ................................................................. 105
4.3 Results .......................................................................................... 105
4.3.1 The rate of reduction of heme a3 in K362M ......................... 105
4.3.2 EPR spectrum of dithionite-reduced K362M ....................... 106
4.3.3 The rate of reduction of heme a3 in E101A and T359A .......... 107
4.3.4 Characteristics of K362T compared to K362M ................. 108
4.4 Discussion .................................................................................... 109
4.5 Figures .......................................................................................... 114
4.6 References .................................................................................... 121
Chapter 5: Identification of key players involved in the formation of the His-Tyr covalent crosslink in the cytochrome c oxidase

5.1 Introduction ................................................................. 126
5.2 Materials and methods .................................................. 129
  5.2.1 Overexpression and purification of oxidases ...................... 129
  5.2.2 Trypsin digestion and sample preparation for FTMS .......... 129
  5.2.3 Mass spectrometry and data analysis .......................... 129
5.3 Results .............................................................................. 130
5.4 Discussion ......................................................................... 132
5.5 Figures ............................................................................... 136
5.6 References ......................................................................... 146
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Å</td>
<td>angstrom ($10^{-10}$ meters)</td>
</tr>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BNC</td>
<td>binuclear center</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>CcO</td>
<td>cytochrome $c$ oxidase</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide m-chlorophenylhydrazine</td>
</tr>
<tr>
<td>CO</td>
<td>carbon monoxide</td>
</tr>
<tr>
<td>COV</td>
<td>cytochrome oxidase vesicle</td>
</tr>
<tr>
<td>COX</td>
<td>cytochrome $c$ oxidase</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DDM</td>
<td>dodecyl-$\beta$-D-maltoside</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>E</td>
<td>single-electron reduced state of cytochrome $c$ oxidase</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>F</td>
<td>Ferryl-oxo state of cytochrome $c$ oxidase</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier-transform Infrared</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>KPi</td>
<td>Potassium phosphate</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MS/MS</td>
<td>tandem mass spectrometry</td>
</tr>
<tr>
<td>N-side</td>
<td>negative side of the membrane</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer (10⁻⁹ meters)</td>
</tr>
<tr>
<td>NTA</td>
<td>nitrilotriacetic acid</td>
</tr>
<tr>
<td>O</td>
<td>oxidized state of cytochrome c oxidase</td>
</tr>
<tr>
<td>O_H</td>
<td>oxidized state of ‘activated’ cytochrome c oxidase</td>
</tr>
<tr>
<td>P</td>
<td>peroxo form of cytochrome c oxidase</td>
</tr>
<tr>
<td>P-side</td>
<td>positive side of the membrane</td>
</tr>
<tr>
<td>PLS</td>
<td>proton loading site</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl methyl sulfonyl fluoride</td>
</tr>
<tr>
<td>R</td>
<td>reduced-form of cytochrome c oxidase</td>
</tr>
<tr>
<td>Sp, sp</td>
<td>spectinomycin</td>
</tr>
<tr>
<td>St, st</td>
<td>streptomycin</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>Tet</td>
<td>tetracycline</td>
</tr>
<tr>
<td>TMPD</td>
<td>N,N,N’,N’-tetramethyl-1,4-phenylenediamine</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type oxidase</td>
</tr>
</tbody>
</table>
Chapter 1: Background and significance

1.1 Introduction

Erwin Schrödinger famously asked “What is life?” and helpfully added, “Life seems to be orderly and lawful behavior of matter, not based exclusively on its tendency to go over from order to disorder, but based partly on existing order that is kept up.”

Keeping up order requires constant input of energy. Aerobic organisms derive the energy needed to sustain life from foodstuffs through the process of cellular respiration. Early steps in respiration, glycolysis and Kreb’s cycle, conserve this energy in food by creating high-energy reducing equivalents, NADH and FADH$_2$. Energy in these reducing equivalents is very efficiently transformed to a proton gradient in a process called “oxidative phosphorylation”. This process involves feeding electrons through a set of membrane protein complexes called the “respiratory chain” or the “electron-transport chain”. The resultant proton gradient drives another membrane protein complex, the ATP synthase, to produce ATP, a form of energy that can be readily used in organisms for life-sustaining processes. This process of producing ATP using an electrochemical transmembrane gradient generated by the oxidation of high-energy molecules is known as the “chemiosmotic theory” and was proposed by Peter Mitchell in 1969.

The major components of the electron-transport chain are four membrane protein complexes: NADH-ubiquinone oxidoreductase (Complex I), succinate-ubiquinone oxidoreductase (Complex II), ubiquinol-cytochrome $c$ oxidoreductase (Complex III) and cytochrome $c$ oxidase (Complex IV). This respiratory chain is typical of eukaryotes and is present in the inner mitochondrial membrane (Figure 1.1).
Oxidative phosphorylation begins with the transfer of electrons from NADH through Complex I or FADH$_2$ through Complex II to reduce ubiquinone to ubiquinol. Complex III transfers the electrons from quinol to cytochrome $c$. The last member of this chain, cytochrome $c$ oxidase, transfers the electrons from cytochrome $c$ to oxygen, irreversibly reduces it to water and thereby completes a cycle of aerobic respiration. Complexes I, III and IV conserve the energy released during catalysis into an electrochemical proton gradient, by transferring protons across the inner mitochondrial membrane. The ATP synthase or Complex V, couples the backflow of protons across the membrane to the synthesis of ATP from ADP. The universal energy currency, the ATP molecule, keeps life going.

1.2 Heme-copper oxidase superfamily

The terminal oxidases of bacteria and archaea, present in the cytoplasmic membrane, also catalyze the reduction of oxygen to water. All terminal oxidases conserve the most of the energy released from this exergonic reaction by translocating protons across the lipid bilayer. The overall reaction carried out by terminal oxidases across all three domains of life can be generalized as:

$4e^-_P + 4H^+^-_N + O_2 \rightarrow 2H_2O$; active site chemistry

$4H^+_N \rightarrow 4H^+_P$; proton pumping

The subscripts N and P refer to the negative (cytoplasmic or matrix) and the positive sides (periplasmic or intermembrane) of the membrane. These terminal oxidases from bacteria, archaea and eukaryota are grouped to form the heme-copper oxidase superfamily (HCO). All members have a highly homologous catalytic subunit with six invariant histidine residues that serve to coordinate two heme moieties and a copper ion. The presence of a crosslinked co-factor at the active site is also conserved across species. At present, the HCOs are divided
into three major subgroups: the A-, B- and C-families as shown in Figure 1.2 (but see (6)). The three subfamilies respectively account for about 72%, 3% and 24% of the current genomic data. The A-type is seen in all domains of life whereas the B- and C-type are strictly prokaryotic. X-ray crystal structures are available for the A-type and the extremophilic B-type (7) oxidases. The A-family is further divided into two subfamilies: the A1- and A2-type based on the conservation of the proton channel residues (8). For all A1-type heme-copper oxidases, the D-channel ends at a glutamate residue: Glu\(^{1-286}\), which is considered to be the branch point of substrate protons and pumped protons (9). However, in the A2-type subfamily, a tyrosine residue whose hydroxyl group occupies the spatial position of the carboxyl group of the glutamate residue presumably carries out the function of Glu\(^{1-286}\) (10). The A1-type oxidase, henceforth referred to as \textit{aa}_3, is the most extensively studied among the HCO superfamily and is also the subject matter of this thesis.

1.3 \textit{Rhodobacter sphaeroides} cytochrome \textit{aa}_3

The \textit{aa}_3 oxidase used in this thesis was isolated from \textit{Rhodobacter sphaeroides}, a gram-negative non-sulfur purple bacterium. It has the ability to grow under aerobic, microaerophilic or photosynthetic conditions. The respiratory chain of \textit{Rhodobacter sphaeroides} is branched (see Fig 1.3) and its \textit{aa}_3 oxidase is an excellent model system to study the more complex mitochondrial enzyme (11). \textit{Rhodobacter sphaeroides} lends itself to easy genetic manipulation and the field owes much of its knowledge of the \textit{aa}_3 to the studies performed on the recombinant enzyme isolated from this organism.

The \textit{aa}_3-oxidase is encoded by the cta (cox) operon. This operon is spread over two regions of the chromosome, one encoding the ctaD (sub I) gene product and the other encoding ctaC (sub II), ctaE (sub III), orf1, and orf3. Most of the work done in
this thesis uses the strain where the chromosomal copy of the ctaD gene is deleted (12). The ctaD gene was cloned and expressed on a plasmid (12) and subsequently histidine-tagged to facilitate rapid purification (13). The recombinant enzyme thus purified has his-tagged subunit I expressed from a plasmid assembled with subunits II, III and IV expressed from the chromosome.

1.4 Overall structure of cytochrome c oxidase

The aa₃-oxidase has several high-resolution X-ray crystal structures: of the 13-subunit bovine mitochondrial enzyme (14), of the 4-subunit aa₃-type from *Rhodobacter sphaeroides* (15), *Paracoccus denitrificans* (16, 17) (18) and of the bo₃-type from *Escherichia coli* (19). The structure of the aa₃-oxidase from *R. sphaeroides* is described below.

Cytochrome c oxidase from *R. sphaeroides* has four subunits and has a total molecular mass of ~125 KDa (15). SU-I is the largest and the best conserved subunit across species. It has twelve transmembrane helices and houses three out of the four redox-active metal centers: the low-spin (six-coordinated) heme *a*, the high-spin (five-coordinated) heme *a₃* and a copper atom, Cuₙ. The latter two, Fe-*a₃* and Cuₙ are only ~5 Å apart (14),(15),(16),(20) and constitute the binuclear catalytic center (BNC) where the binding and reduction of oxygen take place. The redox centers are ligated to six highly conserved histidine residues – heme *a* (H102, H421), heme *a₃* (H419) and Cuₙ(H333, H334, H284). One of the Cuₙ ligands, H284 is covalently cross-linked to a nearby tyrosine residue, Y288 and this post-translational modification, first observed in the bovine aa₃ X-ray crystal structure, is unique to the oxidases (16),(14). There are also binding sites for two non-redox active metals. At the interface of SU-I and SU-II, an Mg²⁺ ion is seen ligated to residues D412 and H411 and three water molecules (15). Its role is unclear and is thought to help with the exit of the product
water molecules (21). SU-I also has a Ca\(^{2+}\) ion binding site whose purpose is not yet known (15). More importantly, this subunit has the two-proton input channels, the D-pathway and the K-pathway, that supply protons for chemistry and pumping during the catalytic cycle. Both pathways are essential for activity, but the D-pathway is responsible for all of the pumped protons in addition to at least two chemical protons (9, 22).

SU-II has two transmembrane helices and a soluble domain on the P-side of the membrane. The globular periplasmic domain has the binding site for the \(e^-\) donor cytochrome \(c\) as well as the first \(e^-\) accepting site, the dinuclear Cu\(_A\) center (3). The Cu\(_A\) site is formed by two mixed-valence copper ions (Cu\(^{1.5+}\), Cu\(^{1.5+}\)), which transfer one \(e^-\) at a time. SU-I and SU-II have been shown to be necessary and sufficient for oxygen reduction coupled to proton-pumping.

SU-III is also well-conserved and is very hydrophobic. Its seven transmembrane helices are separated to two groups of helices separated by a V-shaped cleft. This region interfaces with SU-I and has conserved lipid binding domains across species. SU-III enzyme is very active but undergoes suicide-inactivation after multiple turnovers (23). It is believed that SU-III facilitates rapid proton transfer through the D-pathway which shortens the lifetime of reactive oxygen intermediates formed during catalysis and maintains the integrity of the binuclear center, especially Cu\(_{B}\)(24-26).

SU-IV is a single transmembrane helix that interfaces with both SU-I and SU-III and its presence was not known until the crystal structure of Rhodobacter sphaeroides was solved (15). It does not appear to be necessary for catalytic activity but may play a regulatory role \textit{in vivo}. 
Cytochrome c oxidase has been actively studied since its discovery by Warburg in 1924. Research carried out using whole cells, mitochondria and enzyme purified from cows (Bos taurus) revealed a lot about its subunit composition, redox centers, their order and the rates of internal electron transfer, their spectroscopic signatures, ligand binding etc. In 1977, cytochrome c oxidase was shown to be a proton-pump (27) and it led researchers to ponder how the oxygen reduction chemistry is coupled to the pumping of protons, a question that remains unanswered to this day (22, 28). The application of genetic engineering techniques and the availability of multiple X-ray crystallographic structures have allowed researchers to investigate at the molecular level and a brief summary of the wealth of information about the catalysis gained over the years will be presented in the following section.

1.5 Electron transfer pathway

Electrons are delivered one at a time by reduced cytochrome c, which docks at the interface of SU-I and SU-II via electrostatic interactions. The first acceptor in the aa3 enzyme is the dinuclear CuA center in SU-II (29). The electrons are relayed to heme a and then to the heme a3/CuB binuclear center in SU-I. The distances of CuA from the heme a iron and heme a3 iron are 19.5 Å and 22 Å respectively. Although the two heme centers are equidistant from CuA, rapid electron transfer has not been observed between CuA and the BNC metals (30).

1.6 Proton transfer pathways

Since the oxidase is an integral membrane protein, there is a need for specific pathways on the N-side to transfer protons from the bulk solution to the active site through the hydrophobic interior. Currently, it is believed that the A1- and A2-subfamilies of oxidases alone have two conserved proton-input pathways, the D- and
the K-pathway. The canonical D-pathway described below is present only in the A1-subfamily of oxidases.

The D-pathway is clearly identified by the string of crystallographically resolved water molecules in the X-ray crystal structures. It begins at an aspartate residue (D132) at the N-side of the membrane. It then continues upwards for ~24 Å along a hydrogen-bonded network of protonatable residues and water molecules that include N207, N139, N121, S142, S200, S201 up to E286. When either D132 or E286 are mutated to non-protonatable residues, proton uptake through D-pathway is severely blocked and there is no significant enzyme turnover (31, 32). Mutating any of the three conserved asparagines does not affect oxygen reduction activity but decouple proton pumping from chemistry (33, 34). The decoupling mechanism caused by mutations 20 Å away from E286 is not yet understood and is actively being researched (35, 36). Although the influence of these mutations in altering the pKa of E286 has not yet been generalized, the environment of E286 is certainly affected as revealed by FTIR studies (37). These mutations however, do reconfirm that all four pumped protons are delivered through the D-pathway. The highly conserved glutamate-286 residue acts as a proton donor/acceptor and is believed to play an important role in appropriately directing protons that are to be used for chemistry or pumping. The region between E286 and the binuclear center is very hydrophobic and there are many hypotheses about the intervening hydrogen-bonded connectivity. Computational studies have suggested that putative water molecules transiently line up in this cavity establishing connectivity with the binuclear center 12 Å away (38), (39).

The K-pathway is named after a highly conserved lysine residue (K362) in the middle of the pathway. The N-side entrance is located at a glutamate residue (E101)
in SU-II and extends through K362, T359, farnesyl hydroxyl group of heme $a_3$ up to Y288 in SU-I (40, 41). Only a few water molecules have been resolved in the earlier crystal structures. However, a recent high-resolution crystal structure has resolved four additional water molecules at the top of the K-pathway in the reduced form of the enzyme (42). Mutagenic studies have proposed that the K-pathway supplies one or two chemical protons during the reduction of the catalytic site and this recent structure reconfirms the same. K-channel mutants have been shown to exhibit oxidation kinetic profiles similar to that of the wild-type and are therefore not associated with proton pumping (43).

From the crystal structures of the bovine oxidase, an additional proton pathway called the H-pathway has been proposed to exist (14). This is based on the conformational changes of D51 residue in the oxidized and the reduced states of the enzyme and also on the absence of proton pumping in the asparagine mutant, D51N (44). These residues are neither conserved nor do their mutations affect the proton-pumping machinery in the $aa_3$ oxidase from *Rhodobacter sphaeroides* (45). However, the bovine oxidase has 10 additional subunits which probably have a very involved role in the regulatory roles and hence the presence of an additional channel cannot be ruled out in the eukaryotic oxidases.

The pumped protons also need an exit pathway that extends beyond the D-pathway to be released to the P-side. After reaching E286, the pumped protons are thought to be connected to heme $a_3$ propionates via transient water chains (39, 46). The domain above the heme propionates has several polar, charged residues and water molecules and is connected to bulk solution through hydrogen-bonded networks. Mutations on a pair of highly conserved arginine residues R481 and R482 in that region have been shown to not affect the exit machinery (47). Although the D-
propionates of the hemes are also putative acceptors of pumped protons, a recent study has negated the involvement of D-propionate of heme $a_3$ in the process (48). Computational studies have identified other putative PLS candidates although these are yet to be supported with experimental evidence.

1.7 Catalytic cycle

The catalytic cycle of the $aa_3$ oxidase is shown in the Fig. During the reduction of oxygen to water, the active site cycles through different intermediate states which have distinctive UV-Visible, IR and Raman spectra. These intermediates are denoted by a single letter code, $O$ (fully oxidized enzyme), $R$ (fully reduced enzyme), $A$ (oxygen-bound two-electron reduced enzyme), $P$ (peroxy species) and $F$ (ferryl species) and subscript numerals indicate the number of electrons present in the active site. In the figure, the active site is depicted to include the high-spin heme $a_3$, Cu$_{b}$ and the tyrosine residue, Y288. The mechanism described below starts with the two-electron reduced enzyme ($R_2$).

$R_2 \rightarrow A_2$: In this 2-electron reduced state, heme $a_3$ and Cu$_{b}$ are reduced and heme $a$ and Cu$_{A}$ are oxidized. The tyrosine residue Y288 is protonated. Oxygen rapidly binds to the 2-electron reduced state to form the A-intermediate with a time constant of $\sim 8 \mu s$. This intermediate has a characteristic peak at 595 nm in the UV-visible spectrum.

$A_2 \rightarrow P_{2(M)}$: As soon as oxygen binds, the O=O bond is immediately cleaved resulting in the formation of $P_{2(M)}$ intermediate within $\sim 300 \mu s$ (49). This intermediate has a characteristic peak at 607 nm in the P - O difference spectrum. This reaction requires four electrons, two come from heme $a_3$, one from Cu$_{b}$ and one proton and an electron are given by residue Y288. The crosslink of Y288 with a Cu$_{b}$ ligand H284 supposedly lowers the midpoint potential and the pKa of the tyrosine facilitating the
extraction of a proton and an electron for breaking the O=O bond (50, 51). The formation of the P\textsubscript{m-} intermediate changes the active site into having heme a\textsubscript{3} in the oxoferryl state, a hydroxyl ion bound to Cu\textsubscript{b}\textsuperscript{2+} and Y288 as a neutral tyrosyl radical. This R\textsubscript{2} → P\textsubscript{2(M)} transition in neither associated with any proton uptake from solution nor any proton pumping. Resonance Raman spectroscopy identified the oxoferryl state of the heme a\textsubscript{3} in the P\textsubscript{m-} state (52) and the breakage of the oxygen bond was confirmed by exchangeability of oxygen isotope with solvent water (53).

If the heme a is reduced at this time, the fourth electron for splitting the O=O bond comes from heme a instead of the tyrosine residue. The P state formed in this scenario is designated P\textsubscript{R} and is formed within 50 \(\mu\)s after the A state is formed. This state is very unstable and forms the F intermediate within 120 \(\mu\)s with the delivery of a proton from E286 (54).

P\textsubscript{M2} → F\textsubscript{3}: Upon the introduction of the third electron into the catalytic site, via Cu\textsubscript{A} and heme a, and the transfer of a proton from E286 to active site, the F-state is formed with a time-constant of a few milliseconds (55). E286 gets rapidly reprotonated from the bulk solution and the tyrosyl radical is reduced to tyrosinate. This transition is associated with proton pumping and both these protons arrive through the D-pathway. The P\textsubscript{R} to F transition involves the transfer of only a proton from E286 to the binuclear center and is done under 150 \(\mu\)s. This step is also associated with a pumped proton (56).

F\textsubscript{3}→O\textsuperscript{2-}: Injection of the fourth electron into the active site results in the formation of the fully oxidized state and another proton is taken up from the bulk solution (57). In this state, the ferryl heme a\textsubscript{3} is reduced to ferric heme a\textsubscript{3} bound to a hydroxide. This transition is associated with a pumped proton (58, 59). The O\textsuperscript{2-} state is
also referred to as the O\textsuperscript{1} state or the “activated” oxidized state and is different from the resting oxidized state in terms of its ability to pump protons upon reduction (60).

\( \text{O}^- \rightarrow \text{E}_1 \rightarrow \text{R}_2 \): These transitions are referred to as the reductive half of the catalytic cycle. The kinetics of the entire transition \( \text{O}^- \rightarrow \text{E}_1 \rightarrow \text{R}_2 \) has been studied in detail by Wikstrom’s group and that led to the proposal of the activated oxidized state (60, 61). Current hypothesis is that two protons are taken up during the reduction of the binuclear site (one each from the D-and the K-channel) and each transition is associated with the pumping of one proton that comes through the D-channel. It is also believed that this proton translocation is possible during reductive phase only if it is immediately preceded by the oxidative phase (62). This effect however, has not yet been universally observed (63).

1.8 **Mechanism of proton pumping**

Cytochrome oxidase reduces \( \text{O}_2 \) to water by taking protons and electrons from the opposite sides of the membrane. Apart from the charge separation created this way, it also translocates 1 \( \text{H}^+/\text{e}^- \) across the entire span of the membrane by coupling this to the active site chemistry (64). Despite our understanding of the formation of the intermediates in the catalytic cycle and the steps involved in proton-pumping, the mechanism by which this coupling is established is not yet understood. Over the years, many mechanisms that directly correlated the proton pumping to a redox center have been disproved. Cu\text{A}-based proton pumping mechanism was discarded upon the discovery of Cu\text{A}-less enzymes like \( \text{bo}_3 \)- oxidase being able to pump protons and the Cu\text{A}-site mutants in the \( \text{aa}_3 \)- oxidases retaining the pumping ability (65). Mechanisms proposed by Yoshikawa based on redox changes at heme \( \text{a} \) are not convincing since the H-channel pathway equivalent does not appear to be present in the prokaryotic oxidases (45). The histidine-cycle model based on the assumption that two protons are
pumped during P→F and F→O was sidelined after subsequent experimental evidences disproving the same (66).

Currently, the importance of the Glu-286 at the end of D-channel is receiving major attention. One of the major questions that are being actively researched is how the unidirectionality in the proton transfer is achieved and proposals have been put forth based on the side-chain movement of Glu-286. It has to be kept in mind that this cannot be the universal mechanism for the HCO- superfamily, since the B-type oxidases that lack this residue and the K-channel pump protons as well (67). Nevertheless, the findings based on changes in E286 are very reasonable for the A-type oxidases.

For a proton-pump like oxidase, there has to be a PLS (proton loading site) that is accessible to the N-side and the P-side through proton-pathways but not at the same time. Such a control can be achieved by local conformational changes or pKa changes of the residues involved which in turn can be coupled to specific internal electron and proton transfer. The catalytic cycle has 6 distinct steps: O→E, E→R, R→A, A→P, P→F and F→O. Of these, proton pumping is associated only with four steps: O→E, E→R, P→F and F→O. The electron coupled proton transfers in each of these steps occur at different rates and the proton-input is done using two separate pathways. However, fundamentally, the four one-electron transfer steps from heme $a$ to the BNC coupled to uptake of chemical proton into the BNC and translocation of a pumped proton across membrane is the same. Therefore, we can assume proton-pumping is coupled to the reduction of oxygen using the same mechanistic principles in all four steps. The major steps in the mechanism are as follows:
i. Electron entry into heme $a$ increases the proton-affinity of the PLS leading to its protonation from the N-side of the membrane. Since these are “pumped” protons, this protonation always occurs through E286.

ii. Protonated PLS raises the redox potential of the BNC, this leads to the transfer of electron from heme $a$ to the active site. This also severs contact with the N-side through subtle conformational rearrangements around E286 preserving unidirectionality.

iii. Electron transfer into the active site increases its proton affinity and a chemical proton is delivered to the BNC. This proton can come from either D- or the K-pathway.

iv. The chemical proton so delivered into the active site decreases the pKa of the PLS leading to the expulsion of the pumped proton towards the P-side of the membrane.

This mechanism assumes that there are no proton back-leaks from the P-side to the N-side of the membrane. The residue E286 has long been proposed to act as a proton-sorter appropriately directing the protons to the PLS or the active site. Recent computational studies (68-70) have provided supporting evidence for the role of the glutamate residue as a valve which prevents any proton back-flow from the PLS to the D-pathway. The theory is that as soon as E286 delivers protons to the PLS, the deprotonated E286 immediately flips down towards the D-pathway thereby assuring the proton stays at the PLS. There seems to be inherent instability for the deprotonated glutamate to be in the “up” position. This ensures that at no point in time there is continuous proton conductivity across the entire membrane (22, 71).

The identity of the PLS has not yet been assigned unambiguously. The candidates that are frequently considered are the D-propionates of heme $a$ and heme
α₃, the A-propionate of heme α₃ and the residue H334, which is also a Cu₃ ligand since all these are highly conserved across all families of oxidases (72, 73). Experimental evidence suggests that the former two are unlikely to be the PLS themselves although they may still play an important role in delivering the proton from the D-pathway to the putative PLS (48). Hence, current models favor either the A-propionate of heme α₃ or the H334 residue as the putative pump site (22, 62, 73).

1.9 Scope of this thesis

There are many unanswered questions in the field; the most researched being the search for a universal proton pumping mechanism across members of the heme-copper oxidase superfamily. Some of the other areas that are under study include the proton-exit pathway, role of subunit III, the residues involved in controlling the unidirectionality of proton transfer or “the gate”, the specific need for the K-pathway, the role of the crosslink cofactor at the active site, the site of radical formation etc., This thesis deals with mutations in the proton channels in subunit I, mostly in the neighborhood of the active site. The major questions dealt with in this work are outlined below.

In Chapter 2, the possibility of Met-107, a residue hydrogen-bonded to E286, being part of a putative gating region is explored by site-directed mutagenesis. The mechanism implicating Met-107 as part of the gating region involves conformational changes involving E286, R481, R482 and the heme α₃ D-propionate and these changes apparently ensure there is no direct connectivity between the N- and the P-side at any time during the catalytic cycle. The flow-flash kinetics was done in collaboration with Linda Ojemyr and Dr. Peter Brzezinski at the Stockholm University, Sweden.
Chapter 3 presents the effect on the binuclear site environment when substitutions are made to the Glu-286 residue at the end of the D-pathway. The earlier substitutions and studies on Glu-286 have helped us understand its role in proton-transfer through the D-pathway to the binuclear center during the oxidative half of the catalytic cycle. In this work, we have used Resonance Raman spectroscopy to provide evidence for the hydrogen bonded connectivity between Glu-286 and the binuclear center. Resonance Raman experiments were done in collaboration with Dr. Michelle Yu and Dr. Denis Rousseau at the Albert Einstein College of Medicine, New York.

Chapter 4 deals with the question of the number of protons delivered to the active site during catalysis through the K-channel. It is known that K-channel is used for the uptake of protons during the reductive half of the catalytic cycle but it is not clear if one or two protons are delivered using this pathway. We have attempted to resolve this question by analyzing site-directed mutants in the K-channel using EPR and stopped-flow spectroscopy.

Chapter 5 deals with the unique post-translational modification present in the active site in all terminal oxygen reductases in the heme-copper superfamily. We have studied select proton and electron transfer pathway mutants using high-resolution mass spectrometry to investigate their effects on the His-Tyr crosslink. We have presented some of the conditions required for the formation of this covalent modification. This work was done in collaboration with my lab colleague Dana Robinson, using instrumentation in the laboratory of Dr. Neil Kelleher at the University of Illinois at Urbana-Champaign.
Figure 1.1: A schematic representation of the mitochondrial respiratory chain present in the inner mitochondrial membrane. I: NADH dehydrogenase, II: succinate dehydrogenase, III: cytochrome $bc_1$ complex, IV: cytochrome $c$ oxidase, V: ATP synthase, cyt $c$: cytochrome $c$, CoQ: ubiquinol.
Figure 1.2: Overview of the three major groups of the Heme-copper oxidase superfamily. LS: six co-ordinate low-spin heme, HS: five co-ordinate high-spin heme, black arrows represent the proton input pathway, red arrows represent the proton pumping.
**Figure 1.3:** Respiratory chain complexes in the cytoplasmic membrane of *Rhodobacter sphaeroides*. Q: quinols, $bc_1$: cytochrome $bc_1$ complex, PSRC: photosynthetic reaction center, $c_2/c_4/c_5$: cytochrome $c_2$, $c_4$, $c_5$. 
Figure 1.4: Structures of the hemes A, B, C and O. The \textit{aa}_5^-\textit{ type oxidase in}

\textit{Rhodobacter sphaeroides} has two A-type hemes and its electron donor cytochrome \textit{c}
has one C-type heme.
**Figure 1.5:** X-ray crystal structure of the cytochrome c oxidase from *Rhodobacter sphaeroides*, with the four subunits shown in different colors as indicated. PDB ID: 1M56. Figure was prepared using VMD software.
Figure 1.6: Electron and proton transfer pathways in cytochrome c oxidase from *Rhodobacter sphaeroides*. The residues and water molecules (red spheres) of the D- and the K-pathways are shown. Residue E101 is from subunit II, all others are from subunit I. Figure was prepared using VMD software.
Figure 1.7: Catalytic cycle of cytochrome c oxidase
1.11 References


Chapter 2: Mutations at Met-107 site suggest the residue is not essential for the gating of protons

2.1 Introduction

Cytochrome $c$ oxidase catalyzes the reduction of oxygen to water and generates charge separation across the membrane by taking up electrons and protons from the opposite sides of the lipid bilayer. Additionally, it also acts as a proton-pump and translocates $1 \text{H}^+/\text{e}^-$ across the entire span of the membrane. In the $aa_3$-type cytochrome $c$ oxidase from $Rhodobacter sphaeroides$, the binuclear center is located about two-thirds of the membrane thickness from the N-side of the membrane. Hence, the enzyme needs specific pathways to transfer protons from the bulk solution to the binuclear center as well as another pathway leading to the P-side of the membrane for the pumped protons. Two proton-input pathways, called the D- and the K-pathways, leading from the cytoplasmic side to the active site have been identified based on mutagenic studies and by examining the high-resolution X-ray crystal structures ($1w3$).

The K-pathway has two structurally resolved water molecules and is named after the highly conserved lysine residue, K362 ($4$). The K-pathway is used for proton transfer to the binuclear center during the reductive half of the catalytic cycle ($5$).

The D-pathway begins at a conserved aspartate D132 and it leads through a string of hydrogen-bonded water molecules to Glu-286 which is $\sim 25$ Å above. Mutations at D132 removing the carboxylic group (D132A and D132N) lead to decreased turnover and abolishment of proton pumping ($6$). Similar effects are observed when another conserved residue G204, in the middle of the D-channel is mutated ($7$). Both these mutations especially retard the uptake of proton through the D-pathway during the F→O transition ($6-8$).
Between D132 and G204, there is a ring of three conserved asparagine residues N139, N207 and N121. Mutation of any of these asparagines to an aspartate does not affect the oxygen reduction activity but completely decouples proton pumping (9, 10). The mechanism of decoupling was believed to be coupled to pKa changes of the Glu-286 residue at the top of the D-channel (11, 12) but the contradictory behavior of N139T mutant (13) has added to the mystery of the decoupling mechanism. Nevertheless, these mutational studies have made it very clear that all the pumped protons apart from chemical protons are supplied through the D-pathway. It is obvious that at some point within the enzyme, the chemical and the pumped protons will take different routes to their destinations.

At the end of the D-pathway is the highly conserved Glu-286 residue. From the above mutational studies on the other D-channel residues, it is evident that both chemical and pumped protons taken up through the D-pathway reach till Glu-286. Mutations at E286 like E286Q and E286A themselves are very inactive and show no proton pumping ability (14-16). This behavior makes it difficult to trace the path of the protons, chemical or pumped, beyond the Glu-286 residue. Therefore, it is very likely that Glu-286 is the “branch point” for the chemical or the pumped protons (17-19). It appears that E286 delivers the protons to the PLS or the binuclear site depending on the redox state of the binuclear center, pKa values of the neighboring proton-acceptors and/or the dynamics of water chains, all of which are only very vaguely understood at this point (20, 21).

When the crystal structure of the mutant oxidase E286Q from *Rhodobacter sphaeroides* was resolved, it was seen that the hydrogen bonding between E286 and the carbonyl oxygen of the M107 residue was disrupted in the mutant (3). This led to the hypothesis that E286 and the surrounding residues are involved in forming a
“gate” tightly regulating the movements of the chemical and the pumped protons. From the structure, the residues in the vicinity of E286 implicated to be part of proton gating are M107, W172, R481, R482 and the propionate side chain of heme a. The hypothesis for gating is as follows: when an electron from heme a was relayed to the BNC, it triggered E286 to transfer a substrate proton to the catalytic site. This deprotonation event caused local structural changes involving M107 and W172 which increased the pKa of the putative “pump site” and opened up connectivity to the D-pathway followed by proton transfer to BNC from E286. Upon deprotonation of E286, local structural changes closed the connectivity to PLS and the reprotonation of E286 from N-side through the D-pathway restarted this cycle of events. This gating region undergoes mild structural rearrangements, thereby switching between an ‘input’ and an ‘output’ conformation, alternating the access to the N- and P-sides of the membrane (22). Since the hypothesis is based on the disruption of the hydrogen bond between M107 and E286 in the E286Q mutant, we decided to get a better insight into the functioning of the proposed gate by making substitutions to the Met-107 side-chain.

2.2 Materials and methods

Plasmids and strains used in this study are summarized in Table 2.1. The mutants were constructed using the Quikchange site-directed mutagenesis kit from Stratagene. Primers used in cloning were obtained from the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign. The genes were partially sequenced at the Keck Center to verify the mutation. All chemicals used are of the highest grade available. Standard DNA manipulations were done as described by Sambrook et al.,(23).
2.2.1 Mutagenesis

The mutagenic primers used are listed in Table 2.2. The plasmid pJS3-X6H, containing the modified ctaD gene (encoding subunit I) with six-histidines at its C-terminus to facilitate one-step affinity purification, was used as the template for site-directed mutagenesis (24). The mutagenesis protocol recommended by Stratagene was followed. After the introduced mutation was confirmed by sequencing, the ctaD fragment bound by EcoRI and HindIII sites was transferred from the plasmid pJS3-X6H to the E. coli -R. sphaeroides shuttle vector, pRK-415, by ligation. The expression plasmid pRK-415, was introduced into R. sphaeroides JS100 strain, by biparental conjugation using E. coli S-17-1 as the donor. JS100 maintains a deletion of subunit I gene, in which the ctaD gene has been replaced with a streptomycin/spectinomycin resistance cassette (25). The plasmid pRK-415, containing either the wild-type or mutated ctaD gene, allows for the expression and assembly of oxidases when transferred to JS100.

E. coli strains XL-10 Gold, TOP 10 and S-17-1 cells were grown at 37°C in LB medium. Plasmids pJS3-X6H and pRK-415 were maintained respectively in the presence of the following antibiotics: ampicillin (100 ug/ml) and tetracycline (15 ug/ml). R. sphaeroides strain JS100 was grown aerobically at 30 °C in baffled flasks at 220 rpm. pRK-415 was maintained in JS100 at a tetracycline concentration of 1 μg/ml.

2.2.2 Cell growth and protein purification

Wild-type and mutant strains were grown at 30 °C in 2L baffled flasks containing 1L Sistrom’s minimal medium (26). Cultures were shaken at 220 rpm and were harvested by centrifugation in the late-log phase. Cell pellets were re-suspended in 50mM potassium phosphate pH 8.0 buffer. 50mg/ml DNaseI and protease-inhibitor
cocktail were added to the cell suspension prior to cell-breakage and cells were broken using a microfluidizer (Watts Fluidair, Inc.). Unbroken cells and debris were removed by centrifugation at 30,000 g for 30 min.

The cell membranes were pelleted by ultracentrifugation of the supernatant for 4 hr at 200,000 g. The membranes were stored at -80 °C. All steps further down were done on ice or at 4 °C. The membranes were thawed and re-suspended into 10mM Tris, 40mM KCl, pH 8.0 buffer. The detergent dodecyl-β-D-maltoside (Anatrace), was added to a final concentration of 1% for solubilization. After stirring at 4 °C for 90 min, the unsolubilized membranes were removed by ultracentrifugation at 200,000 g for 40 min.

Ni²⁺-NTA resin was added to the solubilized membranes at a ratio of 1ml packed resin/ 4mg of oxidase. Batch-binding was carried out for 1 hr, in the presence of 10mM Imidazol to prevent non-specific binding. The slurry was loaded on to a gravity column and washed with the following buffers: 10mM Tris, 40mM KCl, 10mM Imidazole, 0.05% DDM, pH 8.0 until it dripped clear followed by 5 column volumes of 10mM Tris, 40mM KCl, 20mM Imidazole, 0.05% DDM, pH 8.0 The enzyme was eluted with 10mM Tris, 40mM KCl, 100mM Imidazole, 0.05% DDM, pH 8.0 and the green fractions were pooled together. The enzyme was concentrated using Amicon concentrator with a cut-off of 100 kDa. Imidazole was removed by exchanging three times into the buffer 25mM Hepes-KOH, 0.05% DDM, pH 7.4 The concentrated protein was aliquoted, flash-frozen using liquid N₂ and stored at -80 °C (24, 27).

Enzyme used for proton-pumping measurements was further purified using anion-exchange chromatography. Briefly, 5 mg of affinity-purified oxidase was diluted to 2ml with Buffer A (10 mM potassium phosphate, 1mM EDTA, 0.1% DM,
pH 7.2) and loaded onto a DEAE-5PW column (Toso-Haas) connected to an FPLC system (AKTA Basic, Amersham). The column was washed with Buffer A and the oxidase was eluted over 4 column volumes, during 15-45% gradient of Buffer B (Buffer A + 1M KCl). The two peaks containing the three-subunit oxidase were pooled together and concentrated for proton-pumping measurements (27).

2.2.3 UV/Vis spectroscopy

Protein concentrations were determined by recording their visible spectra using a Shimadzu UV/Vis-2101PC spectrophotometer. If as-isolated oxidase appeared partly reduced, they were oxidized by adding 100 \( \mu \)M of potassium ferricyanide. Reduced spectra were recorded after the addition of a few granules of sodium dithionite. The extinction coefficients used were \( \Delta E_{606-640\text{nm}} = 40 \text{mM}^{-1}\text{cm}^{-1} \) for dithionite-reduced spectra and \( \Delta E_{605-630\text{nm}} = 24 \text{mM}^{-1}\text{cm}^{-1} \) for dithionite-reduced minus ferricyanide-oxidized difference spectra.

2.2.4 Steady-state activity

The enzymatic activity of cytochrome oxidases was measured by following the rate of oxygen consumption using an oxygen-meter (Yellow Springs Instruments, model-53). To the water-jacketed chamber, 1.8 ml of potassium phosphate buffer (50mM, pH 6.5 or pH 8.0, 0.1% DDM), ascorbate (10 mM), TMPD (0.5 mM) and horse-heart cytochrome c (30 \( \mu \)M) were added and the background oxygen uptake was recorded. The addition of oxidase (10-100 nM) caused enzymatic turnover and the resultant decrease in the concentration of oxygen. The turnover number (moles of \( e^- \) per sec/ mole of oxidase) was estimated from the slope of the oxygen consumption traces.
While using reconstituted cytochrome oxidase vesicles, the potassium
ionophore valinomycin and the uncoupler CCCP were added sequentially after the
addition of the COVs to the assay buffer (100 mM HEPES-KOH, pH 7.4).

2.2.5 Reconstitution of oxidase in proteoliposomes

A lipid solution (Asolectin type II-S, Sigma) was prepared at a concentration
of 80mg/ml with 2% (wt/vol) cholate in 100mM K+Hepes (pH 7.4). This was
sonicated using W-375 sonicator (Mixonix, Inc., Farmingdale, NY) at 50% duty cycle
for 30 seconds with a 60- second break in between until the solution was clear enough
to read through. When sonicated to clarity, DEAE- purified oxidase was added to the
lipid/detergent solution to a final concentration of ~4-8 µM. Cytochrome c oxidase
was incorporated into these vesicles by slow cholate depletion using the Bio-Beads
SM2 (Bio-Rad) method (28, 29). The Bio-beads were added at regular intervals as
shown in the Table 2.4. The COVs were pipetted out carefully and the stray Bio-
Beads were removed by centrifuging at 13000 rpm in a microcentrifuge for 10 min.
This reconstituted COV solution was dialyzed against 60mM KCl (pH ~7.4) to
remove the buffer used earlier.

The proton-permeability of these COVs was determined by measuring enzyme
activity in the presence and absence of ionophores, valinomycin (5µM) and
carbonylcyanide p-chloromethoxy phenylhydrazone (10µM). The ratio of these two
values is the RCR (Respiratory Control Ratio) and it was typically ~6, indicating
tight-enough vesicles to measure proton translocation.

2.2.6 Proton-pumping

Proton pumping was estimated using an SX.17-MV model stopped-flow
spectrophotometer from Applied Photophysics equipped with a diode array detector.
Proton pumping of the COVs was measured by monitoring the absorption changes of phenol red at 557 nm, the isosbestic point of reduced and oxidized cytochrome c \(\text{(30)}\). Horse heart cytochrome c was pre-reduced with dithionite, desalted using PD-10 column in 100 mM HEPES-KOH, pH 7.4 and then concentrated, aliquoted and frozen in liquid nitrogen for future use.

Stopped-flow measurements were done by mixing a solution of 60 mM KCl, 2 µM valinomycin, and 0.4 µM COVs (pH 7.4) with a solution containing 60 mM KCl, 20 µM reduced cytochrome c, and 40 µM phenol red (pH 7.4). The experiment was repeated with 5 µM CCCP added to the COV solution. The data were analyzed using SPLINE function of MATLAB (The Mathworks, Inc.). Evaluation of the proton pumping was accomplished by comparing the proton consumption determined in the presence of protonophore CCCP to the proton release in its absence (the potassium ionophore valinomycin is present to discharge membrane potential)\(\text{(30)}\).

2.2.7 Oxidation kinetics

Rapid kinetics measurements were made with Applied Photophysics stopped-flow spectrophotometer equipped with a diode-array detector. A solution containing 6-8µM oxidase and ~100µg/ml catalase in 50mM potassium phosphate (pH 8.0 and 0.1% DM) was placed in a modified syringe barrel at the loading unit. After rendering it anaerobic by directing a gentle jet of water-saturated argon gas over the sample, 400µM dithionite was added to fully reduce the oxidase. After full reduction, this was rapidly mixed with oxygen saturated buffer (~1mM O\(_2\)) loaded onto the other syringe. Oxygen present in sufficient excess oxidizes the fully reduced enzyme and prevents re-reduction by eliminating dithionite. Any hydrogen peroxide that may be generated by dithionite will be removed by catalase, eliminating interference from any peroxidase activity \(\text{(7)}\).
Absorbance changes were recorded at single wavelengths as a function of time. The rate constants and amplitudes of the transient absorbance changes during the reaction were analyzed by fitting the data to a multi-exponential function using the ProK software (Applied Photophysics, UK).

2.2.8 Reduction kinetics

Before proceeding with reduction kinetics, oxidase (~6µM) was incubated with 100µM potassium ferricyanide in the presence of 10µg/mL polylysine for 30min to ensure full oxidation of the sample. The kinetics of reduction of oxidized CcO was measured under anaerobic conditions in an argon atmosphere by mixing a solution of hexaaminerruthenium(10mM) and sodium dithionite(30 mM) in a 1:1 ratio. Upon mixing, CuA and heme a get reduced within the dead time of the instrument and heme a3 reduction kinetics can be monitored (7).

The collected data were analyzed using the Pro-K software (Applied Photophysics, UK).

2.2.9 Preparation of fully reduced CO-bound cytochrome c oxidase

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

The flow-flash method was used to study the reaction between fully reduced cytochrome c oxidase and oxygen. The experimental setup used is described in (4). Approximately 10 µM of purified oxidase was exchanged to the buffer 100 mM HEPES pH 7.5, 0.1% DDM, 50 µM EDTA and transferred to an anaerobic cuvette. Phenazine methosulfate (PMS) was added to a final concentration of 1 µM and the sample was deoxygenated by exchanging with N\textsubscript{2} on a vacuum/gas line. The sample was reduced by addition using 2 mM ascorbate and formation of the fully reduced state after ~1 h of incubation was confirmed by the optical absorption spectrum of the sample. N\textsubscript{2} was exchanged CO and the binding of CO to the enzyme was verified using optical spectroscopy.

The fully reduced CO-bound oxidase, in the buffer 100 mM HEPES, 0.1% DDM, and 50 µM EDTA (pH 7.5), was mixed in a 1:5 ratio in a modified stopped-flow apparatus (Applied Photophysics, U.K.), with an O\textsubscript{2}-saturated buffer of the same composition. Approximately 200 ms after the samples had been mixed, the CO ligand was dissociated by an 8 ns laser flash at 532 nm (Quantel, Brilliant B) and the enzyme reaction with O\textsubscript{2} was monitored optically as absorbance differences at single wavelengths. Data were analyzed using the ProK software (Applied Photophysics). The flow-flash experiments were done in collaboration with Linda Ojemyr in Dr.Peter Brzezinski’s group at Stockholm.

2.3 Results

2.3.1 UV/Vis spectroscopy

The hemes of the wild-type \textit{aa}_{3}-oxidase characteristically absorb at 424 nm (Soret) and 600 nm (\textit{\alpha}-band) in the oxidized form of the enzyme. Upon reduction
with dithionite, these bands shift to 445 and 605 nm respectively. In the reduced form, the hemes $a$ and $a_3$ absorb almost equally in the Soret region but in the visible region, the dominant absorbance is due to heme $a$ (~80%). The dithionite-reduced spectra of the oxidases are shown in Figure 2.2. Among the mutant oxidases, the UV/Vis spectra of the mutant M107I is similar to the wild-type in the reduced form but oxidized Soret is blue-shifted to 421 nm. M107A absorbs similar to the wild-type in both redox states. In the mutant M107V, oxidized and reduced Soret bands are at 421 nm and 442 nm respectively; the $\alpha$-band is also blue-shifted to 602 nm. The reduced M107C oxidase has peaks at 442 nm and 603 nm.

The environment of the hemes appear to be perturbed, it is more likely to be that of heme $a$, since the methionine residue is in its vicinity.

### 2.3.2 Steady-state activity

The oxygen reduction activities of the mutants are listed in the Table 2.3. The steady-state activity of all mutants at this site is considerably lower than that of the wild-type. The isoleucine substitution has the highest activity at ~33%. The reduction of oxygen is reduced to ~23%, 20% and 4% compared to wild-type in M107A, M107V and M107C respectively. Despite being conservative substitutions, it is interesting that these affect the turnover significantly.

### 2.3.3 Proton-pumping

To test if the mutant oxidases retain proton-pumping ability, the DEAE-purified oxidases were reconstituted into liposomes. The intactness of these vesicles is important to maintain proton-motive force and this determinable property is called RCR (Respiratory Control Ratio). This is the ratio of steady-state oxidase activity in the presence of protonophores to that of controlled activity in its absence. This ratio
for all the reconstituted mutant oxidases was at least 5 or more, indicating good integrity of the proteoliposomes.

Proton-pumping was determined by tracking the changes in the absorbance of the pH sensitive dye, phenol red. The proteoliposomes in air-oxygenated buffer were mixed with reduced cytochrome c in the presence of the dye. The changes in the absorbance of phenol red are monitored at 557 nm which is an isosbestic point of cytochrome c. In the presence of valinomycin alone, a decrease in the phenol red absorbance is captured and this is proportional to the protons pumped during turnover. In the presence of both valinomycin and CCCP, the increase in the dye absorbance corresponds to the substrate protons consumed during turnover. Since one proton is pumped per substrate proton in the wild-type, the ratio of these two amplitudes is used to determine the protons pumped by the mutant oxidases.

From the results shown in the Figure 2.3 and the Table 2.5, it can be seen that the mutants M107I, M107A and M107V pump protons with the same stoichiometry as that of the wild-type. When treated with valinomycin alone, the acidification caused by proton-pumping in M107C is slightly lower than that of wild-type. We conclude that M107C also pumps protons albeit with a lower efficiency. Overall, the mutations at this site do not seem to drastically affect the proton-pumping ability which implies that D-pathway is quite robust and is not affected to any significant extent in these mutant oxidases.

2.3.4 Reduction kinetics

To measure the rate of reduction of the hemes, the oxidized enzyme is mixed 1:1 with an excess of reductant (dithionite plus hexaammineruthenium). Upon mixing, the reduction of heme $a$ is complete for the wild-type during the dead-time of the instrument and the kinetics of heme $a_1$ reduction is monitored. This assay helps
determine if the introduced mutation has affected the rate of electron transfer to the
binuclear center.

Interestingly, the rate of reduction is significantly affected in all the Met-site
mutants (see Figure 2.4). Compared to wild-type with a rate constant of \( \sim 145/\text{sec} \), the
rate of reduction is slowed down by approximately three-fold in the M107I mutant.
\( \text{(k= \sim 45/sec)} \). The kinetics of reduction have been altered to the same extent, to
approximately one-sixth to that of wild-type, in the mutants M107A (k=\sim 22/sec) and
M107V (k=\sim 23/sec). The slowest rate is seen in M107C (k= \sim 19/sec) where it has
been reduced to one-eighth of the rate observed in the wild-type. Although minor
alterations in the heme environment were observed in the UV/Vis spectra, this major
change in the reduction rates of all mutants is quite surprising.

2.3.5 Flow-flash kinetics

The reduction of oxygen to water by oxidase is a very rapid reaction, and so in
order to capture all the reaction intermediates, a technique invented by Gibson and
Greenwood called “flow-flash” is used (31). The enzyme is first reduced
anaerobically and is bound to CO; the reduced enzyme is thus prevented from
reacting with oxygen until CO is removed. The reduced CO-bound enzyme is mixed
with oxygen-saturated buffer in the ratio 1:5 in the stopped-flow cuvette and after a
set delay time, the laser is triggered by the stop-syringe to photo-dissociate CO and
initiate data acquisition. The entire kinetics of oxygen reduction starting from the
binding of oxygen to form intermediate-A to the fully oxidized enzyme can thus be
monitored.

For the wild-type enzyme, the rate constants for the formation of the
intermediates A, P_{R}, F and O are approximately 10 \( \mu \)s, 50 \( \mu \)s, 140 \( \mu \)s and 1.6 ms when
done at neutral pH. From the results of the oxidation kinetics shown in the Figure 2.5,
the following can be inferred: the rate of formation of the A-intermediate (absorbance increase at 595 nm) in both mutants is same as the wild-type enzyme. For M107I, the formation of P-intermediate is similar to wild-type (decrease at 595 nm) but the transitions to F and the O-intermediate appear to be slowed down by a factor of two in this mutant. For M107C, with the exception of the A-intermediate, the transition to all other intermediates is retarded. The absorbance decrease at 445 nm corresponds to the oxidation of both $a$-hemes. The absorbance changes at 445 nm points to an overall slowdown in the rate of oxidation in the mutant enzymes (Panel C).

The decrease at 605 nm corresponds primarily to heme $a$, since it contributes to most of the absorbance in the $\alpha$-region (32). Both M107I and M107C exhibit slow oxidation of heme $a$ as seen from absorbance traces in Panel D.

Taking this data in light of proton pumping and reduction kinetics, the slowdown in oxidation kinetics appears more likely due to retardation in electron transfer rather than proton transfer through the D-channel.

### 2.4 Discussion

The aim of this work was to test if the hydrogen bonding interaction between the side chain of E286 and the peptide carbonyl group of M107 is essential for the gating of protons through the D-pathway without back-leak. As stated in the introduction, the disruption of hydrogen-bonding between these two groups in the E286Q mutant crystal structure was suggestive of such a role. It is important to note that Met-107 is not a highly conserved residue in the A1-type oxidases. Nevertheless, we decided to introduce mutations at this site since our goal was to determine if impairment in hydrogen-bonding will lead to decoupling of activity from proton-pumping.
All mutations introduced at this site, though conservative, lead to lowering of the oxygen reduction activity. However, the stoichiometry of proton-pumping is virtually unaffected in Ile-107, Ala-107 and Val-107 whereas it is slightly lowered in the Cys-107 mutant enzyme. Since the oxygen chemistry and proton pumping ability are not greatly affected in these mutants, it denotes that proton transfer through the D-pathway is not affected to any significant extent, especially with respect to Glu-286 residue.

The lowered steady-state activities in the mutants are probably due to slowed down electron delivery to the BNC rather than due to any problems in the proton delivery through Glu-286. We infer this from the kinetics of reduction and oxidation of the enzymes. The slow-oxidation rates seen in the mutants reveal retarded oxidation of heme $a$. This could be attributed to slow proton delivery only in those cases of mutants with very low activity and no proton pumping. This does not seem to apply to the Met-107 site mutants.

Compared to the wild-type, the rates of reduction are reduced to ~3-fold, 6-fold and 8-fold respectively in the mutants Ile-107, Ala/Val-107 and Cys-107 oxidases. This result is most striking since the steady-state activities are also reduced to comparable levels.

Even so, the presence of the methionine residue seems necessary for the optimal functioning of the oxidase since mutations at Met-107 do affect the enzymatic turnover. When the neighborhood of Met-107 residue is considered, it is in a very hydrophobic cavity and is in proximity to heme $a$. Besides hydrogen-bonding to Glu-286, Met-107 is in van der Waals contact with the neighboring Trp-172 which in turn is in van der Waals contact with heme $a$. It is plausible that substitutions made at Met-
107 may have affected the environment of heme $a$ to the extent of affecting rapid electron transfer to the binuclear center.

Therefore, we conclude that the Met-107 mutants do not affect proton sorting at Glu-286 but seems to rather affect the midpoint potential of heme $a$. Its hydrogen-bonding interaction with Glu-286 does not appear to be vital in controlling proton delivery and this notion is supported by results from other mutagenic studies.

The most compelling evidence comes from the work where Glu-286 was “relocated” from helix VI to helix II. The double mutant E286A/I112E was shown to reduce oxygen and pump protons. The efficiency of turnover was only 5% of the wild-type and the proton pumping stoichiometry for the double mutant was also lower. However, the reduction activity increased 10-fold when compared to the single mutant E286A. This result underscored the need for a protonatable residue at the top of the D-pathway whose position need not be restricted to helix VI alone (33, 34).

Moreover, the idea that the control of proton delivery rests with the side-chain of Glu-286 is furthered by the results of more recent studies. These suggest that the side-chain of Glu-286 isomerizes to the “up” position or the “down” position depending on the number of water molecules present in the hydrophobic cavity above. Also, upon deprotonation, the anionic side-chain flips to the “down” position rapidly thereby disconnecting contact with the P-side. The protonation dependent isomerization of the glutamate side-chain on its own appears to act as a valve to maintain unidirectionality of proton transfer (20, 35).

Additionally, the mutagenic studies on the other neighboring residues namely W172, R481 and R482 that are considered part of the putative gating region also reveal effects similar to M107-mutants on the functionality of the oxidase. W172 (W164 in Paracoccus) mutant has low oxygen reduction activity and has reduced
proton translocation ability. This mutation had also affected the midpoint potential of the hemes (36). Although some mutants at R481 affected the electron transfer or the oxidative half of the catalytic cycle to a significant extent (37, 38), a non-conservative mutant R481L pumps protons with the same stoichiometry as the wild-type enzyme (39). Therefore, it appears that both R481 and its hydrogen bond partner, the D-propionate of heme $a_3$ do not play an important role in the proton pump mechanism.

The results of all these studies taken together negate the hypothesis proposed in (3). None of the proposed “gating” residues namely M107, W172, R481, R482 and the heme $a_3$ D-propionate appear to have a major role to play in the proton pumping mechanism although all these are important for the efficient functioning of the oxidase.

These results also implicate that the potential PLS site might be a part of the binuclear center itself, probably the A-propionate of heme $a_3$ or H334 as has been suggested (39-42).

This study also supports the finding that the timely delivery of the protons rests mostly on the inherent dynamics of the Glu-286 side-chain protonation and deprotonation events in response to the redox changes in the heme centers and pKa values of the nearby proton acceptors. The side-chain shifts seen in the E286Q crystal structure appear to be related to the mutation itself and do not reflect on the actual functioning of the proton gating or pump mechanism in the $aa_3$-type oxidase.
2.5 Figures and tables

Figure 2.1: Structure of the \textit{aa}_3-oxidase defining the residues M107, W172, R481 and R482 considered part of the putative gating region. The residues D132 and E286 signify the start and the end of the D-pathway. This figure was made using the software VMD (43) with PDB entry 1M56.
<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Parent</th>
<th>Key features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL-10 Gold (Escherichia coli)</td>
<td>Tet⁺ Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lacI] ΔM15 Tn10 (Tet⁺) Amy Cam⁺</td>
<td>Stratagene</td>
<td></td>
</tr>
<tr>
<td>S17-1 (Escherichia coli)</td>
<td>E. coli 294 recA (F⁻ thi pro hsdR), derivative of IncPα plasmid pRP4, integrated in the chromosome, Tp⁺</td>
<td>(44)</td>
<td></td>
</tr>
<tr>
<td>JS100 (R. sphaeroides)</td>
<td>Ga</td>
<td>ΔetaD(coxl)::sm/sp</td>
<td>(25)</td>
</tr>
<tr>
<td>Plasmid pUC19 pJS3(X6H)</td>
<td>Amp⁺, coxl (ctaD) with six-His tag at C-ter</td>
<td>(24, 45)</td>
<td></td>
</tr>
<tr>
<td>Plasmid pRK415</td>
<td>Tet⁺, oriT, lacZ</td>
<td>(46)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.1:** List of bacterial strains and plasmids used in this work.
<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>M107Ifor</td>
<td>5'-gatcaccgcacgggctctgatctttctggtggttcattcc-3'</td>
</tr>
<tr>
<td>M107Irev</td>
<td>5'-ggaatgaccagcagaaagatcagattgctgggctgtggtcattcc-3'</td>
</tr>
<tr>
<td>M107Afor</td>
<td>5'-gatcaccgcaacgggctctgatggccttctggtggtcattcc-3'</td>
</tr>
<tr>
<td>M107Arev</td>
<td>5'-ggaatgaccagcagaaccacagtgcagcgggctgtggtcattcc-3'</td>
</tr>
<tr>
<td>M107Vfor</td>
<td>5'-gatcaccggcagccccgtgctgttctctggtcattcc-3'</td>
</tr>
<tr>
<td>M107Vrev</td>
<td>5'-ggaatgaccagcagaaccacagtgcagcgggctgtggtcattcc-3'</td>
</tr>
<tr>
<td>M107Cfor</td>
<td>5'-gatcaccgccacgggctctgatgttctctggtcattcc-3'</td>
</tr>
<tr>
<td>M107Crev</td>
<td>5'-ggaatgaccagcagaacacatcaggatgcggtggtcattcc-3'</td>
</tr>
</tbody>
</table>

**Table 2.2:** List of primers used in the Quickchange mutagenesis.
Figure 2.2: UV-Visible spectra of dithionite-reduced enzymes: wild-type, M107I, M107A, M107V and M107C. The Soret and the $\alpha$-bands are the same as wild-type in M107I and M107A, but these bands are blue-shifted in the mutants, M107V and M107C.
<table>
<thead>
<tr>
<th></th>
<th>Turnover number at pH 6.5 (e/sec)</th>
<th>Relative to WT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1500 ± 50</td>
<td>100%</td>
</tr>
<tr>
<td>M107I</td>
<td>490 ± 30</td>
<td>~33%</td>
</tr>
<tr>
<td>M107V</td>
<td>285 ± 30</td>
<td>~20%</td>
</tr>
<tr>
<td>M107A</td>
<td>340 ± 40</td>
<td>~23%</td>
</tr>
<tr>
<td>M107C</td>
<td>55 ± 15</td>
<td>~4%</td>
</tr>
</tbody>
</table>

Table 2.3: The steady-state oxygen reduction activity of wild-type and the M107-site mutant oxidases determined polarographically using Clark-type electrode. The buffer used was 50 mM potassium phosphate with 0.05% DDM at pH 6.5. Ascorbate and TMPD were used to keep cytochrome c reduced.
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Bio-beads (mg/ml)</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>-20</td>
<td>0</td>
<td>RT</td>
</tr>
<tr>
<td>0</td>
<td>33.2</td>
<td>4</td>
</tr>
<tr>
<td>30</td>
<td>33.2</td>
<td>4</td>
</tr>
<tr>
<td>60</td>
<td>33.2</td>
<td>4</td>
</tr>
<tr>
<td>90</td>
<td>33.2</td>
<td>4</td>
</tr>
<tr>
<td>120</td>
<td>66.5</td>
<td>4</td>
</tr>
<tr>
<td>180</td>
<td>66.5</td>
<td>4</td>
</tr>
<tr>
<td>240</td>
<td>133.0</td>
<td>RT(*)</td>
</tr>
<tr>
<td>270</td>
<td>133.0</td>
<td>RT</td>
</tr>
<tr>
<td>300</td>
<td>266.0</td>
<td>RT</td>
</tr>
<tr>
<td>360</td>
<td>266.0</td>
<td>RT</td>
</tr>
<tr>
<td>420</td>
<td>Ready</td>
<td>RT,ice</td>
</tr>
</tbody>
</table>

**Table 2.4:** Addition of Bio-beads SM2 to the COVs in asolectin/cholate solution. At 240 min, 0.5 ml of 100 mM K⁺HEPES per ml of the COV solution was added to the mixture. The Bio-beads were dried by aspiration before weighing.
Figure 2.3: Proton pumping determined by monitoring the pH changes of the indicator dye, phenol red. Increase and decrease in absorbance at 557 nm signifies alkalization and acidification of the medium respectively. Panels B, C and D show that the mutants M107I, M107A and M107V pump protons with the same efficiency as the wild-type. Panel A shows that the proton-pumping stoichiometry of M107C is lower than that of wild-type.
<table>
<thead>
<tr>
<th></th>
<th>(H^+ / e^-)</th>
<th>RCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>0.8</td>
<td>7</td>
</tr>
<tr>
<td>M107I</td>
<td>0.8</td>
<td>6</td>
</tr>
<tr>
<td>M107V</td>
<td>0.7</td>
<td>5</td>
</tr>
<tr>
<td>M107A</td>
<td>0.75</td>
<td>6</td>
</tr>
<tr>
<td>M107C</td>
<td>0.55</td>
<td>4</td>
</tr>
</tbody>
</table>

**Table 2.5:** Proton-pumping stoichiometry of the M107-site mutants compared to the wild-type. RCR is the ratio of enzyme activity in the absence and in the presence of uncouplers. Higher RCRs indicate better “tightness” of the vesicles.
Figure 2.4: Kinetics of reduction of wild-type and Met-107 site mutants as followed by a stopped-flow spectrophotometer. Reduction was carried out by mixing oxidase rapidly with a mixture of two reductants, dithionite and ruthenium hexamine. The rate constants of reduction for the wild-type, M107I, M107A, M107V and M107C are 145 s\(^{-1}\), 46 s\(^{-1}\), 22 s\(^{-1}\), 23 s\(^{-1}\) and 19 s\(^{-1}\) respectively.
**Figure 2.5:** Flow-flash kinetics of fully reduced wild-type, M107I and M107C oxidases.

Reactions were monitored at (A) 595 nm (B) 580 nm (C) 445 nm and (D) 605 nm. Traces are scaled to 1 µM reacting enzyme.
2.6 References


native processing site, shows involvement of the C-terminus in cytochrome c binding, and improves the assay for proton pumping, *Biochemistry* 40, 1606-1615.


electron and proton transfer in cytochrome c oxidase, *Biochemistry* 44, 10457-10465.


Chapter 3: Substitutions at glutamate-286 in \textit{aa}_{3}\text{-type}
cytochrome \textit{c} oxidase affect the environment of the
catalytic binuclear center

3.1 Introduction

Cytochrome oxidase is the membrane bound terminal enzyme found in the
electron transfer chain of all aerobic organisms. This enzyme is a member of the
heme-copper superfamily, which is characterized by the presence of a binuclear center
consisting of a high-spin heme and an associated copper atom Cu_{B}. Here, the
reduction of oxygen into two molecules of water is coupled to the translocation of
protons across the membrane \((I)\) \((2)\). Since the binuclear center is located in the
hydrophobic interior of the enzyme, a specific pathway for protons to reach the
catalytic site is required. Based on X-ray crystallographic structures \((3-5)\) and
mutagenesis experiments, two proton transfer pathways, the D- and the K-pathway
have been identified in the \textit{aa}_{3}\text{-type oxidase in} \textit{Rhodobacter sphaeroides.}

The D-pathway runs from D132$^I$ (\textit{Rhodobacter sphaeroides} numbering) near
the N-side of the membrane, through a series of hydrophilic residues and structurally
ordered water molecules, to a conserved glutamate E286$^I$ in the middle of the
membrane domain. Since this pathway appears to transfer all four “pumped” protons
and two to three “chemical” protons, E286 is proposed to act as the “proton sorter” or
a “valve”, directing protons to the proton loading site (PLS) and the binuclear center
(BNC) during catalysis without backflow\((6-8)\). The essentiality of this conserved
 glutamate in the \textit{A1}-type oxidases has been established by prior mutagenesis studies.
When the glutamate residue is replaced by an aspartate, oxygen reduction activity is halved but the efficiency of proton pumping is retained (9, 10). All other variants like glutamine, alanine and cysteine in \textit{bo}_{3}\text{-type} and \textit{aa}_{3}\text{-type} oxidases show abolition of proton pumping (9, 11-13). The catalytic activities of glutamine and alanine mutants are severely impaired whereas \sim 15\% activity is retained in the E286C mutant from the \textit{bo}_{3}\text{-oxidase} in \textit{Escherichia coli}(9). Moreover, the activity-impaired mutants accrue P-intermediate during the reaction of the fully reduced enzyme with oxygen (11, 14). These experiments attest to the importance of proton transfer through E286 for oxygen reduction activity. Studies on other D-channel mutants have also revealed that Glu-286 is essential for transferring pumped protons through the D-pathway (15-17).

Since the D-pathway is used to transfer all four pumped protons and at least two chemical protons, Glu-286 at the end of the pathway probably acts as the branching point, directing protons to the proton loading site (PLS) and the binuclear center (BNC) during catalysis without backflow(6-8). The string of water molecules in the D-pathway seen in the X-ray structures extend from D132 up to E286 and the pathway for the protons beyond E286 is not clear since there are no crystallographically resolved water molecules in the hydrophobic cavity. The hydrophobic cavity above E286 has been suggested to contain transient chains of water molecules to form hydrogen-bonded pathways that connect to the PLS or the BNC (18-22).

The prediction of molecular dynamics studies that the cavity may hold about four water molecules (23) is supported by a recent X-ray structure, which has revealed one crystallographically resolved water molecule in this intervening region(5). It is possible that the other water molecules are not ordered enough to be observable in the
X-ray structures or are only transiently present. Although not yet experimentally observed, this network seems to be the likeliest method of getting protons from E286 to the BNC which is ~12 Å away. The protonation/deprotonation of Glu-286 has been captured by FTIR experiments and its side-chain has been postulated to communicate with the BNC using the hydrogen-bonded network in the cavity with protonation-dependent conformational isomerizations (24). In a recent X-ray crystal structure of N131D mutant (N139D in *Rhodobacter sphaeroides*) the glutamate side-chain has been trapped in an alternate conformation providing some evidence to this up-down flipping mechanism (25). In order for proton translocation to occur, Glu-286 has been proposed to go through cyclic conformational isomerization where it alternates connecting to water chain in the D-pathway and waters in the hydrophobic cavity above (19, 26).

There are a number of computational studies that have looked in to the dynamics of the E286 side-chain in response to its protonation state, number of water molecules in the cavity etc., Tuukankanen et al (23) showed that the protonated Glu-286 flipped “up” only when the cavity above was filled with at least four water molecules. In the absence of water molecules, the stable conformation seemed to be the one seen in crystal structures, pointing to the D-pathway. Kaila et al (27) in their simulations with anionic glutamate side-chain demonstrated that it immediately flipped down to the D-pathway and upon protonation, flipped up readily to connect to the waters above. These above results point to how Glu-286 may prevent back-leak by flipping down immediately upon deprotonation and also the importance of the water molecules in the cavity above (28). Pisliakov et al (29) also discuss the role of these waters in enabling the unidirectionality of proton transfer from E286 to the D-propionate of heme $a_3$. Nevertheless, how the hydrogen bonded connectivity is
achieved between E286 and either destination is yet to be demonstrated experimentally.

Previous work in E286Q mutants revealed some interesting features that were not investigated further. It was noted in (10, 30) that the absorption spectra of E286Q in the Rhodobacter system taken under resting and steady-state conditions show a perturbed heme $a_3$ region absorbing at 595 nm. Junemann et al reported that their enzyme preparation has two populations one of which exhibited the redox-inactive 595-nm group. They found that its presence lowered the population of the mutant enzyme reactive to external ligands (10). Konstantinov et al suggested that the heme $a_3$ in E286Q is in the low-spin form and is probably bound to an external ligand (30). The E286Q mutant from *Rhodobacter sphaeroides* was crystallized by Svennson-Ek et al., and the mutant structure showed loss of hydrogen bonding between Gln-286 and the peptide backbone of the neighboring Met-107 residue. Interestingly, it also revealed subtle changes in the environment of the binuclear center, the most notable being the shift of the D-propionate of heme $a_3$ by 1 Å.

In this work, we have described our characterization of two mutations of the glutamate-286 residue, E286C and E286A, in $aa_3$-type cytochrome $c$ oxidase from *Rhodobacter sphaeroides*. Using Resonance Raman spectroscopy for our characterizations, we have presented the first experimental evidence for a hydrogen-bonded network around E286 that influences the conformation of the binuclear center.

### 3.2 Materials and methods

#### 3.2.1 Site-directed mutagenesis

The mutation E286C and E286A in subunit I were introduced using the Quikchange site-directed mutagenesis kit (Stratagene) using the following primers:
E286Cfor: 5′-GGTTCTCCGACCCGTGCCTACATCATCGTGC-3′,  
E286Crev: 5′-GGTTCTCCGACCCGTGCCTACATCATCGTGC-3′ and  
E286Afor: 5′-GGTTCTCCGACCCGTGCCTACATCATCGTGC-3′ and  
E286Arev: 5′-GGTTCTCCGACCCGTGCCTACATCATCGTGC-3′. The genes were partially sequenced to verify the introduced mutations. Please refer Section 2.2.1 for details about creating site-directed mutants in *Rhodobacter sphaeroides*.

### 3.2.2 Protein purification

Histidine-tagged wildtype and mutant cytochrome oxidases were expressed and purified from *Rhodobacter sphaeroides* as described previously (31, 32). Further purification for proton-pumping was done by fast protein liquid chromatography using DEAE-5PW column (33).

### 3.2.3 Steady-state kinetics

Steady state activity was measured polarographically using a YSI model 53 oxygen meter. The reaction mix contained 1.8 mL of 50mM potassium phosphate buffer pH 6.5 with 0.1% DDM, 10 mM ascorbate, 0.5 mM TMPD and 30 µM horse heart cytochrome c. Upon the addition of oxidase, oxygen consumption was monitored and the enzyme turnover number was calculated.

### 3.2.4 Reconstitution of oxidase in proteoliposomes

A lipid solution (Asolectin type II-S, Sigma) was prepared at a concentration of 80mg/ml with 2% (wt/vol) cholate in 100mM K+ Hepes (pH 7.4). After sonicating to clarity, DEAE-purified oxidase was added to a final concentration of ~4 µM. CcO was inserted into these vesicles by slow cholate depletion using Bio-Beads (BioRad)(34). The COVs were spun at 13000 rpm for 10 min to remove Bio-Beads and
then dialyzed against 60mM KCl (pH ~7.4). The integrity of COVs was determined by measuring enzyme activity in the presence and absence of ionophores, valinomycin (5µM) and carbonylcyanide p-chloromethoxy phenylhydrazone (10µM). Proton pumping assay was performed according to the procedure described in Section 2.2.6

3.2.5 Resonance Raman spectroscopy

Resonance Raman spectroscopy is a definitive tool for assigning the oxidation, spin, and coordination states in heme proteins (35-38).

A 413.1-nm krypton ion laser (Spectra Physics, Mountain View, CA) served as the excitation source for the resonance Raman experiments. Samples were prepared in a custom quartz cell (2 mm path length, sample volume ~150 µl), designed to optimize anaerobic conditions, facilitate optical absorption measurements, and allow long Raman acquisitions. Optical spectra were recorded on a UV-2100U spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) with a spectral slit of 1 nm on the same sample measured by Raman spectroscopy. During Raman measurements, the laser beam was focused on a cell, spinning at 3000-6000 rpm to avoid local heating of the sample. A camera lens focused the Raman scattered light onto an entrance slit (100 µm) of a polychromater (Spex, Metuchen, NJ), where it was dispersed by a grating with 1200 grooves/mm and collected by a charge-coupled-device camera (Princeton Instruments, Trenton, NJ) cooled to 77 K. To eliminate Rayleigh scattering and laser fluorescence lines, holographic notch filters (Kaiser, Ann Arbor, MI) and band-pass filters were employed. A neutral density filter allowed fine adjustments of laser power, which was maintained at 3-5 mW at the level of the sample. The spectral slit width was 5 cm⁻¹. Frequency shifts were calibrated.
using indene (100-1700 cm\(^{-1}\) region), purchased from Sigma-Aldrich, as a reference with an accuracy of ±1 cm\(^{-1}\).

Samples for Raman contained 50 µM enzyme (RS aa\(_{3}\) WT, E286C, or E286A) in 25 mM HEPES, 0.1% DM, 1 mM EDTA at pH 7.4. The resting, reduced, and CO-bound forms of the enzyme, were prepared as follows: the resting oxidized form was unchanged after purification, the fully-reduced form was thoroughly purged under Argon gas before exposure to 10x sodium dithionite, and the CO-bound form was made by introducing CO gas (Matheson, E. Rutherford, NJ) to fully-reduced enzyme. Optical absorption acquired before and after the Raman measurements ensured stability of the samples.

### 3.3 Results

#### 3.3.1 Analysis of the optical spectra

Absolute optical spectra of the as-isolated and dithionite-reduced spectra of wild-type and the mutant E286 oxidases are presented the in Figure 3.2. In the fully reduced form of E286C oxidase, the Soret and the visible bands are blue-shifted to 441nm and 601nm, compared to 444nm and 605nm in the wild-type enzyme. In the dithionite-reduced form of the E286A enzyme, the Soret and the visible bands are blue-shifted to 442 and 602 nm respectively. In the difference spectrum of fully oxidized wildtype minus ferricyanide-oxidized E286C enzyme, a broad absorbance band centered at 595nm is seen. This feature is present in the difference spectra of the reduced enzymes as well (Figure 3.3). These broad absorbance bands at 595nm are also observed in the difference spectra of the wild-type and the E286A oxidase. This characteristic was observed earlier by Junemann et al in their report on E286Q mutant from *Rhodobacter sphaeroides* (10). As seen in E286Q, this band appears to be
redox-insensitive judging from its presence in both the reduced and oxidized difference spectra with the wild-type oxidase.

### 3.3.2 Effects of mutation on catalytic activity and proton pumping

The replacement of glutamate with cysteine and alanine residue reduces the catalytic activity to < 0.05% of the wild-type enzyme. The E286A mutant oxygen reduction activity is consistent with the findings in the bo$_3$ (9, 11, 12) but the activity of E286C in the aa$_3$-type is inactive when compared to the equivalent mutant in bo$_3$. oxidase.

Nevertheless, these results support the general observation that replacement of the glutamate with a nonprotonatable residue greatly diminishes catalytic turnover. As expected for a D-channel mutant whose oxygen reduction activity is affected markedly, proton pumping assay performed on vesicles reconstituted with the E286C and E286A mutant enzymes reveal that proton pumping functionality is abolished as well (see Figure 3.4 and 3.5).

### 3.3.3 Resonance Raman spectra of the as-isolated enzymes

The presence of the “595” band has been reported earlier by (10, 30). Junemann et al noted that this “595 nm” compound in E286Q was redox-inactive since it was present in the difference spectra of reduced E286Q minus reduced WT enzyme as well as oxidized E286Q minus oxidized WT forms. Konstantinov et al (30) suggested that the state of heme $a_3$ in the E286Q mutant from *Rhodobacter sphaeroides* was affected and was probably bound to a ligand.

Since we have observed that other substitutions like cysteine and alanine also cause the “595 nm” absorbance, we were motivated to investigate its effect on the binuclear centers of the E286C and E286A mutant oxidases using Resonance Raman
spectroscopy. Our aim was to unambiguously identify the spin and the co-ordination state of heme \( a_3 \) in these mutants. Since the optical spectrum of the already characterized glutamine substitution also has the “595nm species” (10), the findings in the E286C and E286A mutant oxidases will help form a general picture of the effects of Glu-286 substitution on the binuclear center.

The high frequency resonance Raman spectra of the resting forms of WT, E286C, and E286A are shown in Figure 3.6. The most compelling change is seen in the spin-state marker, \( \nu_2 \), which shifts to the position of low-spin (LS) heme \( a_3 \). The \( \nu_2 \) position at 1572 cm\(^{-1}\) corresponds to the high-spin pentacoordinate marker of heme \( a_3^{3+} \) (HS 5C) (39). In the wild-type oxidase, this marker has comparable intensity to the low-spin hexacoordinate (LS 6C) marker of heme \( a_3^{3+} \) at 1588 cm\(^{-1}\), which is consistent with a 1:1 stoichiometry of HS heme \( a_3 \) to LS heme \( a \). In the E286C and E286A enzymes, \( \nu_2 \) of heme \( a_3 \) shifts to 1591 cm\(^{-1}\), a position consistent with CN\(^-\) bound low-spin heme \( a_3 \) (39), which is dramatically illustrated in the difference spectra of E286C and E286A relative to wild-type. A change from high- to low-spin would cause the anti-bonding orbitals \( d_{z^2} \) and \( d_{x^2-y^2} \) to empty, the Fe-ligand bond order to increase, and the length of the Fe-ligand bond to shorten, resulting in an increase of frequency. This spin-state change would also impact the Fe-N bond lengths, causing the heme core to contract. Therefore, a decrease in core size is anticipated with a shift from high-spin to low-spin heme \( a_3 \).

The marker most sensitive to core-size, \( \nu_3 \), depicts a shift in frequency for heme \( a_3 \) while heme \( a \) remains unperturbed. The \( \nu_3 \) mode of heme \( a_3 \) exhibits changes for E286A but not E286C, shown by a negative feature at 1481 cm\(^{-1}\) in the difference spectra and a positive broadened positive feature around 1503-1511 cm\(^{-1}\). A plausible explanation is that the glutamate to cysteine mutation conserves polarity of the
residue, preserving a remnant population of high-spin heme \( a_3 \) through charge interactions. The glutamate to alanine mutation is non-conservative, and it may have eliminated polar interactions and so completely perturbs the binuclear center.

Because \( \nu_4 \) is dominated by the heme iron oxidation state, its frequency shift in the mutants is smaller than that of \( \nu_2 \) or \( \nu_3 \), but is apparent in the difference spectra. The \( \nu_4 \) mode at 1372 cm\(^{-1}\) indicates an Fe\(^{3+} \) oxidation state (39) of heme \( a \) and \( a_3 \) in WT, E286C, and E286A. Moreover, the mode exhibits a subtle blue shift in E286C and E286A relative to wild-type, evidenced only in the difference spectra. The subtlety of this change is expected due to a slight sensitivity of \( \nu_4 \) to spin-state changes despite its predominant sensitivity to changes in oxidation state. This finding is consistent with heme \( a_3 \) transforming from HS, 5C to LS 6C while heme \( a \) remains LS 6C.

The formyl markers of \( a \) and \( a_3 \) show perturbations consistent with changes in their hydrogen-bonding environments. The heme \( a_3 \) formyl marker shifts down from 1672 cm\(^{-1}\) in WT to 1661 cm\(^{-1}\) in the mutants, indicating an increase in the strength of hydrogen-bonding. In contrast, the formyl marker of heme \( a \) shifts up from 1648 cm\(^{-1}\) in WT to 1656 cm\(^{-1}\) in the mutants, which signifies a decrease in its hydrogen-bonding environment. The formyl groups of heme \( a_3 \) and \( a \) are 14 Å and 19 Å away from E286, respectively, implying that E286 has hydrogen-bond interactions along a network that affects the formyl groups.

The space between E286 and heme \( a_3 \) constitutes part of the proposed oxygen pathway, which is hydrophobic and does not contain crystallographic water molecules (3). However, 3-4 water molecules are proposed to exist transiently during turnover (40) via molecular dynamics simulations. This work gives spectroscopic evidence of
a hydrogen-bond network around E286 that influences the conformation of the binuclear center.

3.3.4 Resonance Raman spectra of the fully-reduced enzymes

Similar changes are seen in the fully-reduced mutants, shown in Figure 3.7. In both E286A and E286C, the $v_2$ mode of heme $a_3$ at 1566 cm$^{-1}$ shifts to 1591 cm$^{-1}$, consistent with a change from HS 5C to LS 6C. The $v_4$ mode at 1354 cm$^{-1}$ is consistent with the Fe$^{2+}$ oxidation state (39), with a small shift to higher frequency seen in the mutants. The $v_3$ modes of both hemes exhibit changes at 1473 and 1491 cm$^{-1}$, likely shifting to a broadened peak around 1510 cm$^{-1}$. The formyl marker of heme $a_3$ shifts down from 1660 to 1633 cm$^{-1}$, indicating increased hydrogen-bonding to the formyl carbonyl. In contrast, the formyl marker of heme $a$ shifts up from 1607 to 1620 cm$^{-1}$ which indicates the loss of hydrogen-bonding. These findings reinforce the conclusions drawn in our studies on the resting state.

3.3.5 Resonance Raman spectra of the fully-reduced CO-bound enzymes

Figure 3.8 shows the high frequency region of spectra of the CO-bound forms. Because the CO-adduct forces the heme $a_3$ iron into a low-spin configuration, the markers affected by spin-state appear unperturbed. The formyl marker of heme $a_3$ shifts down from 1665 to 1658 cm$^{-1}$ but shifts of the formyl marker of heme $a$ are within the level of noise. In addition, the in-plane asymmetric mode, $v_{30}$, at 1152 cm$^{-1}$ loses resonance in E286A and decreases in resonance in E286C.

The low frequency region of the reduced form is shown in Figure 3.9. The $v_{Fe-His}$ of heme $a_3$ at 213 cm$^{-1}$ loses resonance in the mutants as shown in the difference spectra. This change is consistent with a hexacoordinate state of heme $a_3$ in which the iron is pulled back into the plane of the heme by a distal axial ligand that opposes the
proximal histidine. Other changes are seen in $\nu_8$ of heme $a_3$ at 337 cm$^{-1}$, the propionate mode at 365 cm$^{-1}$, $\nu_7$ at 679 cm$^{-1}$, and $\nu_{16}$ at 745 cm$^{-1}$. Similar changes, particularly in the loss of resonance of the propionate mode, are seen in the resting enzyme (Figure 5).

The low frequency region of the CO-bound forms is shown in Figure 3.10. Two CO-bound configurations have been described for the $aa_3$-oxidase from *Rhodobacter sphaeroides* (41). Both the $\alpha$- and $\beta$-forms populate in wild-type, shown by bands at 495 and 520 cm$^{-1}$ while only the $\beta$-form populates in the mutants. The bending mode at 573 cm$^{-1}$, associated with the 520 cm$^{-1}$ form is only seen in the wild-type enzyme, further suggesting that this form does not populate in E286C or E286A. These observations are best illustrated in the difference spectra. The $\alpha$- and $\beta$-forms have been attributed to different Fe$_{a3}$-Cu$_{B}$ distances in the two conformations, with a much shorter distance existing in the $\alpha$-conformer (41). The steric constraint compresses the Fe-C bond and forces the Fe-C-O adduct into a highly bent position. These changes translate to a higher Fe-CO stretching frequency of the $\alpha$-form at 520 cm$^{-1}$. In contrast, the Fe-C-O adduct in the $\beta$-form has an almost linear position and a much lower-energy Fe-C bond at 495 cm$^{-1}$. Therefore, the E286C and E286A enzymes appear to have larger Fe$_{a3}$-Cu$_{B}$ distances in the binuclear center. Such space could accommodate a distal axial ligand, resulting in a hexacoordinate conformation of heme $a_3$.

3.4 Discussion

In the resting and reduced forms of the mutant oxidases E286A and E286C, it can be seen that heme $a_3$ is converted to six-coordinate low-spin. In the dithionite-reduced forms, a small fraction of high-spin heme $a_3$ is seen but most of the population is in the low-spin hexa-coordinate form. This implies significant
perturbation of the binuclear center since heme \( a_3 \) is penta-coordinate high-spin in the wild-type oxidase in both forms of the enzyme. Additionally, signals corresponding to the formyl groups of hemes \( a \) and \( a_3 \) are also shifted indicating that the hydrogen bonding environment is affected. How the mutations at Glu-286 site can cause such changes in the heme \( a_3 \) coordination and its hydrogen bonding environment are the questions that will be addressed below in this section.

In the E286 mutant oxidases, the loss of oxygen reduction activity can be attributed solely to the block in proton transfer at the end of the D-channel (9, 11, 42, 43). A number of kinetic and spectroscopic studies have provided evidence for its role in proton delivery (24, 42, 43). Modeling studies have suggested that E286 transfers protons by undergoing an up-to-down conformational switch (8, 19, 23, 26, 27). In all X-ray crystal structure of the \( aa_3 \)-type oxidases, E286 points “down,” which is thought to characterize the recipient state of the enzyme, in which E286 receives protons through the D-pathway (3-5, 44, 45). The “up” position of E286 has not been resolved by X-ray crystallography but has been demonstrated in several computational studies (23, 27). In the “up” position, E286 is hypothesized to be hydrogen bonded to the BNC or PLS by a transient network of water molecules and modeling studies have predicted the presence of up to four molecules in this hydrophobic cavity (23, 27).

A recent X-ray crystallographic structure of the oxidase by Ling et al has resolved one water molecule in this region (5). Ling et al reported that E286 is within hydrogen bonding distance to this resolved water molecule, which can further hydrogen-bond to the carbonyl oxygen of Gly283 or the \( \text{Cu}_\text{b} \) ligand, \( \text{OH}^- \). In the crystal structure of the E286Q mutant from \textit{Rhodobacter sphaeroides}, changes in the
environment of Glu-286 were observed but interestingly they also observed a 1 Å shift in the heme $a_3$ moiety (3).

Our study supports the above observations by providing strong spectroscopic evidence for a hydrogen-bonding network linking E286 to the binuclear center. In the Raman spectra of the resting forms of E286A and E286C, the carbonyl stretching mode of heme $a_3$ formyl group shows a significant shift compared to the wild-type. The hydrogen-bonding environment of formyl group of heme $a$ also appears to be perturbed although to a lesser extent. In the reduced forms of E286A and E286C oxidases, the alteration in the hydrogen-bonding environment of heme $a_3$ is more pronounced than heme $a$ as seen from the $\nu_{C=O}$ mode shifts. The loss of resonance of the propionate mode in the reduced forms of the mutant oxidases is also indicative of alterations in the heme environments. These results clearly show the effects of substitutions at Glu-286 on the hydrogen-bonding environment of heme $a_3$ whose formyl, D-propionate and A-propionate groups are respectively 16, 12 and 17 Å away from the side-chain. Such an effect is perhaps due to the disturbances in the water network between the Glu-286 and the BNC in the mutant oxidases. This network would presumably traverse the distal pocket of heme $a_3$, which is in line with simulations suggesting the existence of 3-4 transient water molecules during turnover. The physiological form of the enzyme is thought to have a hydrated distal pocket due to a continual production of two waters for every molecule of oxygen reduced. These waters have been suggested to have an important role in the mechanism of proton pumping and are thought to be the difference between “resting” and the “pulsed” forms of the oxidase (6, 46, 47). These waters are probably essential to establish the hydrogen-bonding network between Glu-286 and heme $a_3$ of the BNC. Changes to the
Glu-286 environment presumably alter this intervening network which then exhibits itself as long-range effects on the heme $a_3$ environment.

The other major observation is the conversion of the penta-coordinate high-spin heme $a_3$ to hexa-coordinate low-spin form in both the reduced and resting states of the mutant oxidases. This indicates that the heme $a_3$ at the active site of the mutants has picked up a strong axial ligand. Konstantinov et al reported that in the mutant E286Q, heme $a_3$ appeared to be ferrous low-spin bound to a sixth axial ligand (30). Our data supports their observation of spin change and we can conclude that in general, the substitution with a non-protonatable residue at Glu-286 leads to the conversion of heme $a_3$ to six-coordinate low-spin state.

Junemann et al (10) reported that the reactivity of E286Q with external ligands like $\text{H}_2\text{O}_2$ was very low. Konstantinov et al showed that the “peroxidase half-reaction” of the performed with $\text{H}_2\text{O}_2$ and reduced cytochrome $c$ was inhibited in E286Q (30). This is interesting because K362M, a mutant that severely inhibits the uptake of protons through the K-channel exhibited peroxidase activity (48). $\text{H}_2\text{O}_2$ is thought to make up for some deficiency in the proton delivery to the active site. The lack of reactivity of $\text{H}_2\text{O}_2$ with E286Q is perhaps limited due to the presence of this unknown axial ligand bound to heme $a_3$.

This leads us to the question of the identity of the sixth ligand. It is very unlikely to be one among the three histidine ligand of Cu$\beta$, since the crystal structure of the E286Q mutant did not show any shifts in these side-chain positions (3). We know that when oxygen binds to reduced heme $a_3$, the resulting compound A has an absorbance peak at 595 nm. Since the mutant oxidases also have a broad absorbance band in the 595 nm region, we would like to propose that it is some form of an oxy-species. When dithionite-reduced enzyme is purged with oxygen, the enzyme
reoxidizes to the O-state with the 595 nm form, as seen from our Raman spectra. We think that oxygen gets slowly reduced to water through an unknown mechanism that leaves an oxy-species bound to the heme $a_3$ at all times.

To summarize, we have characterized the activity, optical, and vibrational characteristics of E286A and E286C oxidases from *Rhodobacter sphaeroides*. Both substitutions at Glu-286 site exhibit similar characteristics, which may help understand the general mechanism by which such mutants affect oxygen reduction. The mutant oxidases have perturbed binuclear centers with hexacoordinate low-spin heme $a_3$ seemingly bound to an unknown oxy-species. Mutations at Glu-286 affect the hydrogen-bonding environment of heme $a_3$ significantly. This is a very relevant finding since current theories implicate that Glu-286 coordinates functionally important water chains to the active site and the proton-loading site, which has been putatively assigned to the A-propionate of heme $a_3$. 
3.5 Figures

**Figure 3.1:** Schematic showing the position of glutamate-286 in the aa$_3$-type oxidase. E286 is at the end of the D-pathway and is 16 Å and 19 Å away from the formyl groups of heme a$_3$ and heme a respectively. This figure was prepared using the PDB file 1M56 and VMD software.
Figure 3.2: Panel A shows the oxidized spectra of wild-type (---), E286A (----) and E286C (-.-.-) oxidases. Panel B shows the dithionite reduced spectra of wild-type (---), E286A (----) and E286C (-.-.-) oxidases.
Figure 3.3: Optical difference spectra of the wild-type and E286C mutant cytochrome c oxidases. (A) Difference spectrum of ferricyanide-oxidized E286C \textit{minus} oxidized wild-type enzyme (………). (B) Difference spectrum of dithionite reduced E286C \textit{minus} dithionite-reduced wild-type enzyme(—). Both figures show the broad absorbance band at the 595 nm region in both forms of the E286C oxidase. Similar absorbance bands are also seen for E286A mutant oxidase.
Figure 3.4: Phenol red absorbance followed at isosbestic wavelength 556.8 nm shows that the wild-type pumps protons but not the E286C mutant enzyme. COVs were mixed with reduced cytochrome c and 80 µM phenol red in an Applied Photophysics stopped-flow spectrophotometer. In the presence of valinomycin alone, proton pumping is detected as a decrease in absorbance due to acidification of phenol red. With the addition of CCCP, the uncoupled enzyme shows only alkalinization as seen from the increase in absorbance of phenol red.
Figure 3.5: Phenol red absorbance followed at isosbestic wavelength 556.8 nm shows that the wild-type pumps protons but not the E286A mutant enzyme. COVs were mixed with reduced cytochrome c and 80 µM phenol red in an Applied Photophysics stopped-flow spectrophotometer. In the presence of valinomycin alone, proton pumping is detected as a decrease in absorbance due to acidification of phenol red. With the addition of CCCP, the uncoupled enzyme shows only alkalinization as seen from the increase in absorbance of phenol red.
Figure 3.6: Resonance Raman spectra of as-isolated wild-type (A), E286C (B), and E286A (C) RS $a_3$ in the high-frequency region. Difference spectra of E286C minus wild-type (D) and E286A minus wild-type (E) are also shown. The heme $a_3$ formyl marker shifts down while the formyl marker of heme $a$ shifts up in the mutants. The high-spin heme $a_3$ also shifts up, indicating a conversion to low-spin. The excitation frequency was 413.1 nm, and the power delivered at the sample was 5 mW. 50 µM protein was prepared in 25 mM HEPES, 0.1% DM, 1 mM EDTA at pH 7.4.
**Figure 3. 7:** Resonance Raman spectra of dithionite-reduced wild-type (A), E286C (B), and E286A (C) RS $a_3$ in the high-frequency region. Difference spectra of E286C minus wild-type (D) and E286A minus wild-type (E) are also shown. The spectroscopic conditions are the same as in Figure 3.6. The heme $a_3$ formyl marker shifts down while that of heme $a$ shifts up in the mutants. The high-spin heme $a_3$ marker is regained but a small population appears to remain in the low-spin conformation.
Figure 3.8: Resonance Raman spectra of CO-bound wild-type (A), E286C (B), and E286A (C) RS $a_{3}$ in the high-frequency region. Difference spectra of E286C minus wild-type (D) and E286A minus wild-type (E) are also shown. The spectroscopic conditions are the same as in Figure 3.6 except 3 mW power was delivered at the sample. The heme $a_{3}$ marker shifts down in frequency in the mutants. No change is seen in the spin-state as CO-bound heme $a_{3}$ exists in a low-spin conformation.
Figure 3.9: Resonance Raman spectra of dithionite-reduced wild-type (A), E286C (B), and E286A (C) RS $a_3a_3$ in the low-frequency region. Difference spectra of E286C minus wild-type (D) and E286A minus wild-type (E) are also shown. The spectroscopic conditions are the same as in Figure 3.6. The Fe-His stretching mode is missing in the mutants, which is consistent with a 6C heme $a_3$ conformation.
Figure 3.10: Resonance Raman spectra of CO-bound state wild-type (A), E286C (B), and E286A (C) RS $a a_3$ in the low-frequency region. Difference spectra of E286C minus wild-type (D) and E286A minus wild-type (E) are also shown. The spectroscopic conditions are the same as in Figure 3.6. The mutants predominantly form the 495 cm$^{-1}$ CO-bound conformation. The changes in $v_{16}$ and $v_{7}$ are of unknown origin.
3.6 References


15. Han, D., Morgan, J. E., and Gennis, R. B. (2005) G204D, a mutation that blocks the proton-conducting D-channel of the aa3-type cytochrome c oxidase from Rhodobacter sphaeroides, *Biochemistry* 44, 12767-12774.


Chapter 4: On the delivery of protons through K-pathway during the reduction of the \( aa_3 \)-type cytochrome \( c \) oxidase from \( Rhodobacter sphaeroides \)

4.1 Introduction

Cytochrome \( c \) oxidases are membrane proteins that catalyze the reduction of oxygen to water with the concomitant generation of a proton electrochemical gradient across the membrane. The electrons and protons used in the chemical reaction come from opposite sides of the membrane (1). Protons are delivered to the active site buried in the hydrophobic interior through proton-conducting channels. The X-ray crystal structures of the \( aa_3 \)-type cytochrome \( c \) oxidases reveal the presence of two proton-input channels and they are named as the D-and the K-pathways (2-4).

The K-pathway, named after a highly conserved residue Lys-362 in subunit I, begins at Glu-101 in subunit II and continues through highly conserved residues in subunit I like Lys362, Thr-352, Thr-359, Ser-299 and Tyr-288. Tyr-288 is also considered to be a part of the active site since it is covalently cross linked to a histidine residue His-284, a ligand of the active site metal \( Cu_b \). The hydroxyl group of heme \( a_3 \) is also thought to be part of this pathway since it is within hydrogen-bonding distance to a water molecule near T359.

An intriguing feature of the K-pathway as seen from several crystal structures in various redox and ligand-bound states is that it does not have a well-ordered string of water molecules as observed for the D-pathway (2-6). It is possible that the bridging water molecules are too disordered to be captured in a crystal structure. However, several studies have shown that it is the D-pathway that is a major supplier
of protons to the catalytic site (7-12). The K-pathway is believed to provide either one or utmost two “chemical” protons and these transfers appear to be restricted to the reductive half of the catalytic cycle (13). The need for a specific pathway to supply just a proton or two has been mystifying and has not yet been unambiguously described.

Many K-pathway mutants have been well characterized and they behave distinctly from the D-pathway mutants. Common features shared by the K-pathway mutants are low steady-state oxygen reduction activities and decelerated rate of reduction of the binuclear center. This has been demonstrated by site-directed mutants made on the conserved K-pathway residues: E101-II, K362 and T359. Mutation of E101 to any residue including aspartate reduced the oxygen reduction activity to a large degree (14, 15) and it was confirmed to be the entry point using CO-flash photolysis (16). Replacement of Thr-352 and Thr-359 side chains with alanine resulted in lowered oxygen reduction activity primarily due to decelerated rate of reduction (17). Mutations made at S299 imply that it may not be directly involved in proton transfer but may be important to stabilize the environment around K362 through hydrogen-bonding with a crystallographically resolved water (16).

The residue K362 is buried in a hydrophobic environment and is ~15 Å below the axial ligand of heme $a_3$. Substituting the lysine side-chain with methionine or alanine completely abolishes steady-state turnover (17) and the rate of reduction of the binuclear center is severely retarded (18, 19). However, the reaction of fully reduced K362M mutant with oxygen is mostly unaffected as shown by several optical and electrometric studies (7, 20). These studies showed that the loss of activity in K362M mutants is not due to impairment in the oxidative-half of the catalytic cycle.
When treated with hydrogen peroxide, the peroxidase activity of the otherwise inactive K362M mutant was shown to be comparable to the wild type enzyme (19).

The effects of the K-channel mutant seem to be mainly related to the reduction of the enzyme (21, 22). When CO-photolysis is done on the wild-type, electron transfer from heme $a_3$ to heme $a$ is associated with the release of a proton through the K-channel. CO-photolysis of mixed valence K362M showed that proton-transfer coupled to electron back-flow from heme $a_3$ to heme $a$ was impaired (23). Injection of single electron into oxidized K362M studied using electrometry showed that K-pathway has a role in the first step of the reductive phase, O→E transition (24). These results taken together indicate that the K-channel is used to deliver protons to the binuclear center only during the reductive half of the catalytic cycle.

Recent X-ray crystal structure of the reduced form of *Rhodobacter sphaeroides* oxidase resolved to 2.15 Å revealed shifts in helix VIII and resolved water molecules at the top of the K-pathway (6). However the connectivity between the “dry” lower half and the upper-half going through lysine-362 is not obvious from the structure. Computational modeling has predicted that the lysine side chain may swing closer to the binuclear center for charge compensation although such structural rearrangements are yet to be captured by X-ray crystallography (25).

Additionally, the number of protons taken up during reduction is under debate. It has been shown that the reductive phase is associated with the net uptake of two protons into the enzyme from the N-side (26). Studies on the K-pathway mutants clearly show the uptake of at least one proton and the other proton is believed to come from D-pathway although the evidence is not as convincing (13, 24).

The work presented here is aimed at answering two questions. Firstly, it addresses the number of protons that are taken through the K-pathway during the
reductive phase and secondly, the role played by the lysine side chain in effecting this transfer. To address the former, we followed the kinetics of reduction of K-channel mutants K362M, E101A and T359A using stopped-flow spectrophotometer. We also used EPR spectroscopy to characterize the dithionite-reduced form of the K362M mutant enzyme. To understand the role of side chain, we have made a comparison of polar threonine substitution (K362T) against non-polar methionine (K362M) at the site of lysine-362.

4.2 Materials and methods

4.2.1 Site-directed mutagenesis

The mutation K362T in subunit I was introduced into the plasmid pJS3(X6H) using the Quikchange site-directed mutagenesis kit (Stratagene) using the following primers:

K362Tfor: 5'- CCACCGGATCACCATC TTCTCCTG- 3' and
K362Trev: 5' CAGGAGAAGATGGTGATGCCGGTGG-3’.

The gene was partially sequenced at the W.M. Keck Center for Comparative and Functional Genomics to verify the mutation. This insert with mutation was ligated to pRK415 before conjugation into *Rhodobacter sphaeroides*. For further description of the mutagenic procedures, please refer Section 2.2.1.

4.2.2 Protein purification

The cells were grown in Sistrom’s medium as described previously and for EPR samples, the cells were grown in the presence of higher magnesium (1200 µM) and lower manganese (0.5 µM) concentrations (27). Protein purification of the histidine-tagged oxidases was done as described in Section 2.2.2. Protein samples
used for EPR were dialyzed against buffer containing 10 mM EDTA. These samples were then concentrated and exchanged to EDTA-free buffer.

4.2.3 Measurement of cytochrome c oxidase activity

Steady-state measurements of the activity of CcO were made polarographically using a YSI model 53 oxygen meter. The reaction mix contained 1.8 mL of 50mM potassium phosphate buffer pH 6.5 with 0.1% DDM, 10 mM ascorbate, 0.5 mM TMPD and 30 µM horse heart cytochrome c. Upon the addition of oxidase, oxygen consumption was monitored and the enzyme turnover number was calculated.

4.2.4 Measuring the rate of reduction of heme a₃

The kinetics of reduction was monitored spectroscopically using an Applied Photophysics SX-18MV stopped-flow spectrophotometer equipped with a photodiode array detector under anaerobic conditions.

Sodium dithionite in argon-sparged buffer was rapidly mixed in a 1:1 ratio with CcO in the same anaerobic buffer to give a final concentration of 2mM dithionite and 2 µM wild-type or mutant CcO. Before mixing with dithionite, CcO was incubated with 100 µM ferricyanide for 15 min to ensure full oxidation of the sample. The buffer used was 50 mM BTP adjusted to pH 8 containing 0.05% DDM.

In some experiments, hexaaammine ruthenium (III) was used at a final concentration of 50 µM along with dithionite. The data sets were collected in triplicates. Time-resolved spectra thus obtained were analyzed using the Pro-K software from Applied Photophysics and OriginPro 8.
4.2.5 EPR spectroscopy

X-band EPR spectra were recorded with a Varian 122 spectrometer equipped with an Air Products Helitran cryostat maintained by University of Illinois NIH EPR Research Center.

The experimental conditions are as follows: temperature, 15K; microwave power, 20mW; modulation amplitude, 10G; recording time, 60s; microwave frequency, 9.0448 GHz. Oxidase samples were 90-100 µM in 25mM HEPES-KOH, pH 7.4, 0.05% dodecyl maltoside.

EPR spectroscopy is a useful tool to study the redox centers in cytochrome c oxidase. Out of the four metal centers, only two are EPR-visible in the oxidized form of the enzyme. The low spin heme \( a \) gives rise to signals at \( g = 3 \) and the Cu\( \alpha \) center at \( g = 2 \); the high-spin heme \( a_3 \) and Cu\( \beta \) are EPR-invisible owing to antiferromagnetic coupling between these sites. However, this coupling may be broken when one of these metals are reduced or by the binding of an external ligand. During redox titrations, transient high-spin ferric heme \( a_3 \) signal at \( g = 6 \) owing to partial reduction of the enzyme have been observed \((28)\). High-spin heme \( a_3 \) signal has also been observed in the oxidized form upon the addition of NO \((29)\).

4.3 Results

4.3.1 The rate of reduction of heme \( a_3 \) in K362M

The mutant K362M has been reported to significantly slow down the reduction of heme \( a_3 \) in the binuclear center since the delivery of the charge compensating proton through K-channel is obstructed \((18, 19)\). The reduction kinetics of K362M in comparison with the wild-type is shown in the Figure 4.2. A 1000-fold excess of sodium dithionite was rapidly mixed with the enzyme to reduce it under
anaerobic conditions. The reduction of both hemes in the wild-type was essentially complete within 2 sec. The reduction of K362M took >1000 sec to go to completion (19) and this has been attributed to complete inhibition of heme $a_3$ reduction in this mutant.

The fully reduced spectrum of the mutant enzyme obtained after 1000s is shown in the inset of Figure 4.2. In order to estimate the extent of reduction, we assumed that heme $a$ and heme $a_3$ absorb equally in the Soret region, i.e., they contribute to 50% of absorption when measured at $A_{445nm}$ - $A_{460nm}$. Therefore, one-half of this $A_{445nm}$ - $A_{460nm}$ value for the fully reduced K362M enzyme must represent the endpoint of heme $a$ reduction and it corresponded to $t=0.2s$. This is interesting since the fast rise in absorbance seen at $A_{445nm}$ - $A_{460nm}$ up to $t=2s$ must then correspond to heme $a_3$ reduction. Therefore, the data shown in Figure 4.2 is suggestive of the reduction of heme $a$ being completed within 0.2s and the reduction of heme $a_3$ occurring in two phases. The extent of reduction estimated between $t=0.2s$ and $t=2s$ amounted to 28% of the total absorbance at $A_{445nm}$ - $A_{460nm}$, in other words about 50% of heme $a_3$ reduction. It can be inferred that one electron enters the binuclear center and is probably equilibrating between the metals Fe$_{a3}$ and Cu$_{b}$.

### 4.3.2 EPR spectrum of dithionite-reduced K362M

In order to substantiate the above supposition, we checked the EPR signatures of the dithionite-reduced mutant enzyme. It was expected that the single electron entering the binuclear center would break the anti-ferromagnetic coupling between Fe$_{a3}$ and Cu$_{b}$ rendering them EPR visible.

The EPR spectra of the oxidized K362M enzyme and the dithionite-reduced forms of K362M and wild-type are shown in the Figure 4.3. The EPR spectrum of oxidized K362M is essentially the same as that of the wild type oxidase (11). The
signals at g=2.8, 2.3 and 1.6 are due to heme \( a \) and those at g= 2.2, 2 and 1.9 are due to Cu\(_A\). The signals seen at g6 and g4.3 are generally attributed to negligible concentrations of high spin Fe\(_{a3}\) and denatured material. The EPR spectrum of the oxidized enzyme is indicative of the intactness of the binuclear center in K362M.

To the anaerobic oxidized K362M enzyme, dithionite solution was added to a final concentration of ~2mM (25-fold excess), mixed well and frozen in liquid nitrogen immediately after 5s. Interestingly, the EPR spectrum of dithionite-reduced K362M enzyme shows high spin heme \( a_3 \) signal at g=6 (Figure 4.3) as predicted for one-electron bearing binuclear center. This behavior contrasts that of the wild-type, where quick reduction renders the binuclear center diamagnetic.

This EPR result supports the idea that an electron can enter the binuclear center easily, in the K362M oxidase. Our group is the first to show evidence for this phenomenon although this scenario has been suggested by Junemann et al(30). Their group had reported earlier that the first reducing equivalent resided primarily on Cu\(_B\) leaving the spin-uncoupled heme \( a_3 \) EPR visible.

The results of EPR and stopped-flow reduction when taken together allows us to interpret that the first electron indeed enters the binuclear center and equilibrates between the two metal centers in the active site. This is also supported by the intensity of the high-spin heme \( a_3 \) signal estimated relative to myoglobin standard (see Figure 4.3) which corresponded to ~30%. Since this initial electron transfer into the binuclear center is coupled to proton-uptake from the K-pathway, we propose that this proton is most likely donated by Y288 at the active site (see Discussion).

### 4.3.3 The rate of reduction of heme \( a_3 \) in E101A and T359A

E101 and T359 are the other two highly conserved residues in the K-pathway that impair the rate of heme \( a_3 \) reduction when substituted with alanine. A 1000-fold
excess of sodium dithionite and a catalytic amount of ruthenium hexammine II (50 µM) were rapidly mixed with the enzyme to reduce it under anaerobic conditions. Under these conditions, the reduction of heme \( a \) is complete during the dead time of the stopped-flow instrument and the reduction of heme \( a_3 \) can be monitored.

From the kinetics of reduction shown in Figure 4.4, it is apparent that for both E101A and T359A mutants, the rate of reduction is slowed down but approximately 90% of heme \( a_3 \) is reduced when \( t=5s \). This comparatively faster rate of reduction seen in E101A and T359A is to be expected since their steady-state activities are \(~8\%\) and \(~30\%\) respectively \( (14, 17) \).

### 4.3.4 Characteristics of K362T compared to K362M

K362A and K362H are the two other substitutions made previously at the site of lysine-362 \( (17) \). The alanine mutant also has no significant oxygen reduction activity like the methionine substitution and the histidine mutant affected the assembly of the oxidase. We introduced the threonine mutation in order to investigate the effect of a polar side-chain in place of lysine.

The optical spectra of the reduced and the oxidized form of the K362T mutant are shown in the Figure 4.5. The partial reduction feature seen in K362M is also seen in the K362T mutant. The steady-state oxygen reduction activity of K362T is negligible and is similar to K362M which also has no measurable activity.

The kinetics of reduction of K362T oxidase compared to K362M is shown in Figure 4.6. It is interesting to note that K362T exhibits very similar rate of heme \( a_3 \) reduction as seen for K362M. Since the substitution of polar threonine has also failed to support the delivery of protons through the pathway, it implies that the lysine side-chain contributes to more than just being a polar residue enabling hydrogen-bonded connectivity through the pathway.
4.4 Discussion

Whether the K-pathway delivers one or two protons to the binuclear center during the reductive-half of the catalytic cycle has been a puzzling question. We think the work presented here provides some evidence to agree that the pathway is used for the uptake of two “chemical” protons.

Bloch et al (22) showed using electrometry that there is no membrane potential generation in the reductive phase in K354M (K362 equivalent in Paracoccus denitrificans) but it is comparable to the wild-type during the oxidative phase.

Adelroth et al (1998) showed that in the mixed valence K362M, the proton release to the bulk solution coupled to the oxidation/reduction of heme $a_3$ was impaired (23). In other words, during the E→R step of the catalytic cycle, when the second electron is delivered to the BNC, a proton is taken up from the bulk solution through the K-pathway. Our results add to the above to show that the reduction of the binuclear center with the second electron is kinetically affected when lysine-362 is mutated to methionine or threonine.

Ruitenberg et al (2000) demonstrated that when a single electron is injected into the oxidized enzyme, proton uptake via K-pathway occurred during O→E transition (first electron delivery). Using electrometry, they observed that the 175 $\mu$s phase in wild-type corresponding to reduction of heme $a_3$ (or one-electron delivery) was slowed down to 900 $\mu$s in the K362M mutant (K354M in Paracoccus denitrificans). The O→E transition appeared to have been slowed down in the K362M oxidase (24). We propose that the first electron that enters the binuclear site is perhaps accompanied by a proton transfer event through K-pathway occurring on a 900 $\mu$s timescale. Our optical and EPR spectroscopy data support their observation of a half-reduced binuclear center using electrometry.
As for the accompanying proton, we can be certain it does not come through the D-pathway, especially through E286, since the reduction kinetics of the glutamate-site mutants are closer to the wild-type behavior. Therefore, the proton donor for this event has to be restricted to the top-half of the K-pathway, above the lysine-362 residue. These constraints point to tyrosine-288 as the likeliest donor. Y288 is at the end of the K-pathway and is also part of the active site. It has a unique covalent crosslink with a nearby histidine residue H284 that reportedly lowers its pKa value rendering it a good proton donor (31).

It may be of interest to note that all of the above data on K362-mutants were collected using the “resting” enzyme rather than the “pulsed” form. In the “resting” enzyme it is reasonable to presume Y288 to be preloaded with a proton and capable of the proton transfer event supposed above. This point is of importance since in the “pulsed” state of the enzyme (O_H state), Y288 is reportedly in the anionic form. Under such conditions, electron entry for the O_H→E transition may be harder to accomplish.

Gorbikova et al (2008) monitored the protonation state of the Y288 residue (Y280 in Paracoccus denitrificans) during R_4→O_H transition in N131V mutant using time-resolved FTIR spectroscopy (32). According to their data, under physiological conditions, Y288 is expected to be fully deprotonated in the O_H state. Additionally, they reported that Y288 may probably be protonated in the E- and R states and demonstrated the deprotonation of Y288 during the P_m- and F-states experimentally.

The protonation state of Y288 gives us pertinent clues about the number of protons delivered through the K-pathway. All data taken together, we can envision the following events. Y288 gets protonated during the O_H→E transition according to the FTIR data. We do not know if Y288 retains this proton through the R-state and deprotonates during P-state for breaking the oxygen bond. There is experimental
evidence from electron-backflow experiments that a proton is taken up from bulk solution through the K-pathway during E→R transition. This uptake of proton (proton release in backflow experiments) during E→R transition is blocked in the K362M mutant. Since Y288 is already protonated in the E-state, we may presume that in the R-state, Y288 gets deprotonated followed by quick reprotonation through the K-pathway.

Very little information is available on the protonation of Y288 during the O_H→E transition. We would like use some unexplained data from the past experiments to help resolve this question. When Adelroth et al (1998) studied the uptake of protons during R_4→O single turnover experiment, they saw a proton-uptake event on the 5 ms timescale after F→O transition in the wild-type enzyme, which was absent in the K362M oxidase (23). The authors explained that although 5 ms is a lot longer than the enzymatic turnover, the event might occur on a physiologically relevant time scale during steady-state turnover. It seems reasonable to propose that this proton uptake through the K-pathway in the latter part of F→O transition is important for the reprotonation of Y288 in the E-state.

Although proton transfer to the binuclear center is restricted to the reductive-half of the catalysis, significant movement through the K-pathway during oxidative-half has been observed. After the deprotonation of Y288 to form Pm-state, some proton movements through the K-channel are thought to occur. Lepp et al (2008) reported that electrogenicity observed during A→ P_m transition is due to charge transfer within the K-pathway (33). They hypothesized that protonated K362 moves closer to the catalytic site for charge compensation and an earlier computational study done on bovine oxidase (34) claimed that K362 exhibits substantial protonation at neutral pH.
In such a scenario, we speculate that the following events happen through the K-pathway during Pm→F→O→E transition. During Pm state, protonated K362 provides charge compensation to the deprotonated Y288. During the late F→O transition, a proton is taken up from the bulk solution probably protonating E101 at the entrance. The delivery of an electron into the fully oxidized enzyme to form E-state will cause Y288 to be protonated by K362 which in turn gains a proton from E101. During E→R transition, Y288 probably gets deprotonated and quickly reprotonated. This sequence of events suggests that not one but two protons are delivered through the K-pathway. Such a scheme would mean that the oxidase uses the K-channel for the reductive-half and the D-channel for the oxidative-half of the catalytic cycle. Figure 4.7 shows the state of Tyr-288 and the participation of the K-channel during the catalytic cycle.

The nature of proton transfer through the K-pathway leads us to the role played by the side-chain of Lys-362 residue. As known from the crystal structures, the K-channel appears to have few water molecules to ensure rapid connectivity (2-4). A possibility that the side-chain of Lys-362 residue flips upwards and downwards the K-channel has been proposed. Such a hypothesis is supported by computational studies and also by recent electrometric data.

Our replacement of lysine side-chain by threonine in the K362T mutant has enabled us to understand the importance of the lysine side-chain. If indeed the Lys-362 residue flips up and down within the K-pathway at different times during the catalytic cycle, then the residue at site-362 needs to not only be polar but also be able to get re/deprotonated. Our observations made on the K362T mutant support this hypothesis. The K362T mutant, possessed of a polar side-chain, exhibits the same behavior as the non-polar methionine in K362M oxidase.
Also, Qin et al (2009) reported that four water molecules were observed at the top of the K-pathway in their high-resolution structure of dithionite-reduced oxidase (6). The proposed movement of the lysine-side chain may possibly explain the “relative dryness” observed in the middle of the K-pathway. Based on the crystal structures, Qin et al (2009) also proposed that the top of the K-pathway opens in to the BNC in the reduced form and is shut out during the oxidized form by way of a strong hydrogen bond between Y288-OH and heme $a_3$ farnesyl-OH. The BNC opening up to K-pathway during reduction supports our proposal of the K-channel delivering both protons for reduction.

Our observations on the other two K-channel mutants T359A and E101A are in line with the findings of other research groups. The slower rate of reduction of heme $a_3$ in T359A implies that even slight disorders to the hydrogen-bonded network at the top of the K-pathway will noticeably affect the proton transfer coupled to electron transfer. Our result is supportive of the study where the rotation of T359 side-chain has been shown to be important to facilitate hydrogen-bonded connectivity (35). The rate of reduction in E101A is affected to a greater extent as compared to T359A. This not only reinforces the role of E101 as the entry point of K-pathway but also shows the significance of having an acidic residue at the mouth of the pathway for the fast uptake of protons from the bulk solution.

From our work on the K-pathway mutants, we have shown that this pathway is used to deliver two protons to the binuclear center, in contrast to what is generally believed. Our work also supports the notion that the lysine side-chain flips up and down in the K-pathway during the catalytic cycle depending on its protonation state.
Figure 4.1: Schematic showing the important residues lining the K-pathway in the aa3-type oxidase. The K-pathway has fewer crystallographically resolved water molecules. This figure was prepared using VMD.
Figure 4.2: Kinetics of reduction of the heme groups in WT and K362M. Stopped-flow measurements at 445-460nm with 4mM dithionite in one syringe were mixed 1:1 at RT with enzyme in 50mM BTP buffer (pH 8) and 0.05% DDM in the other syringe. Traces have been normalized to 1µM. The solid trace and the dotted kinetic traces for K362M show data collected over 20s and 1000s timescales.
Figure 4.3: The figure shows EPR spectra of 90µM oxidized K362M (A); K362M reduced with 2mM dithionite for 5sec (B); WT reduced with 2mM dithionite for 5sec (C); myoglobin standard for high-spin heme quantification (D) Conditions: temperature, 15K; microwave power, 20mW, modulation amplitude, 10G; microwave frequency, 9.0448 GHz.
**Figure 4.4:** Kinetics of reduction of the heme groups in WT, T359A and E101A:

Stopped-flow measurements at 445 minus 460nm with 4mM dithionite and 100μM hexaammine ruthenium in one syringe were mixed 1:1 at RT with enzyme in 50mM BTP buffer (pH 8) and 0.05% DDM in the other syringe. Traces have been normalized to 1μM. Heme α reduction is mostly done during the dead time.
Figure 4.5: The optical spectra of the oxidized and the dithionite-reduced form of the K362T mutant oxidase. Upon the addition of dithionite, the mutant oxidase takes ~30 min to go to the fully reduced form similar to the wild-type.
**Figure 4.6:** Kinetics of reduction of the heme groups in K362 site mutants- K362M and K362T. Stopped-flow measurements at 445 nm minus 460nm with 4mM dithionite and 100µM hexaammine ruthenium in one syringe were mixed 1:1 at RT with enzyme in 50mM BTP buffer (pH 8) and 0.05% DDM in the other syringe. Traces have been normalized to 1µM.
Figure 4.7: This figure shows the catalytic cycle of the oxidase. The pathways through which the chemical protons during intermediate transitions are taken up are indicated. Proton pumping also occurs during the same transitions and all four protons are taken up through the D-pathway.
4.6 References


from Rhodobacter sphaeroides probed by the effects of site-directed mutations on time-resolved electrogenic intraprotein proton transfer, *Proc Natl Acad Sci U S A* 94, 9085-9090.


compound F: resolution of partial steps by transient spectroscopy, Biochemistry 37, 14910-14916.


*Biochemical Society Transactions* 13, 548-560.


Chapter 5: Identification of key players involved in the formation of the His-Tyr covalent crosslink in the cytochrome c oxidase

5.1 Introduction

Cytochrome c oxidase couples the one-electron oxidation of cytochrome c to the four-electron reduction of oxygen to water and conserves the free energy by translocating protons across the inner mitochondrial or the bacterial cytoplasmic membrane. The catalytic site where the reduction of oxygen is carried out is a binuclear center, which is composed of a high-spin heme, heme $a_3$ and a copper center, Cu$_{B}$, which has three histidine ligands (H284, H333 and H334). The X-ray crystal structures of the bovine and the bacterial oxidases in the A-type oxidases first revealed a post-translational modification that is unique only to the heme-copper superfamily (1, 2) and was later verified experimentally by mass-spectrometry (3).

One of the Cu$_{B}$ ligands H284 was found to be covalently cross-linked to a nearby tyrosine residue, Y288 and this modification was subsequently observed in the crystal structure of the B-type oxidases (4). Until recently, it was believed that the C-type oxidases function without the crosslink owing to the absence of the conserved motif Gly-His-Pro-X-Val-Tyr in the sequence alignments. Computational modeling studies (5) in conjunction with mass spectrometry (6), (7) have revealed that the conserved tyrosine has been moved to a different helix but is still spatially allowed to be covalently cross-linked to the histidine residue (H284 equivalent in cbb3 oxidase).

This covalent linkage between the C$_{ε2}$ of Tyr-288 side-chain and N$_{ε2}$ of H284 side-chain is seen in the crystal structures of oxidized, fully reduced and ligand-bound
forms of the enzyme (1), (2), (8), (9) and is therefore considered an essential part of the active site. Mutation of tyrosine to phenylalanine has been reported to abolish the formation of crosslink and result in loss of CuB from the BNC (10) (see also (11)). This covalently linked Tyr-288, which is conserved across the heme-copper oxidase superfamily, is also at the end of the proton-input pathway, the K-channel. Therefore, this residue has been proposed to play a structural as well as a functional role in the oxidases.

As soon as oxygen binds to the reduced binuclear center, the O=O bond is irreversibly broken without the release of reactive oxygen intermediates. Out of the four electrons supplied by the oxidase in one-step, two are taken from heme a3, one from CuB and one proton and an electron presumably from the cross-linked Y288, leaving behind a neutral tyrosine radical (12), (13), (14). EPR signal of the radical seen during Pm formation or upon treatment of oxidase with H2O2 has generally been assigned to Y288 rather than residues like W280 and Y167 which have also been implicated to be source of the radical in the past (15), (16), (17). The proximity of Y288 to the catalytic site and its unique covalent modification with a CuB ligand make it the likeliest donor of the electron for O=O bond splitting (12), (14), (6), (7). The His-Tyr covalent linkage has been studied in vitro using model organic compounds and the findings range from the covalent crosslink lowering the pKa of the tyrosine residue, increasing its midpoint potential in the radical state, forming phenoxyl radicals during single turnover etc., (18), (19), (20), (21). Although tyrosyl radicals have been detected in EPR during the catalysis, not much is known about the regeneration of tyrosine or tyrosinate. It is also not known if during Pm- formation, it is a hydrogen-atom transfer or an electron transfer followed by proton transfer or vice-versa.
The role of Y288 in proton delivery is undisputed and has been clearly shown by recent FTIR studies in conjunction with electrometry, where the deprotonation of this tyrosine residue during Pm-formation in E286Q mutant was reported (22).

The properties of the crosslink that convert the Tyr-288 residue into a facile proton and electron donor have rightly received a lot of attention but, very little is known about the origin of the crosslink. It is generally believed to be a post-translational modification that is formed after the very first or initial few turnovers of the oxidase (3),(23),(24),(25). In other enzymatic systems with covalently linked amino acids, the metals in the active site in the presence of oxygen auto-catalytically induce covalent linkages among susceptible residues like Tyr, Cys, His, Trp and Met, to form the functional enzyme(26),(27),(28). In enzymatic systems like catalase peroxidase and galactose oxidase, holoproteins lacking the covalent linkage were synthesized by depleting the growth medium of the active site metals, Fe or Cu, respectively. Once the metal centers were reconstituted in-vitro, cross-links formed spontaneously in both systems, thus establishing that their post-translational event is a self-processing one (29),(30).

In the oxidases too, the cross-link formation is perceived as a self-processing event. There is no direct way to test it since in-vitro reconstitution of metals has been shown to be unsuccessful for oxidases (31, 32). Since for a turnover, supply of protons and electrons is necessary, we decided to test for the crosslink processing reaction by affecting the proton and electron transfer pathways in the enzyme. This in conjunction with very sensitive mass spectrometry technique will enable us to understand the conditions required for the formation of the covalent linkage.
5.2 Materials and methods

5.2.1 Overexpression and purification of oxidases

To counter poor expression of the mutant oxidases, the pRK415-expression plasmids with the mutations in subunit II, H260N and LpM were freshly conjugated to the subunit II deletion strain YZ200 using *Escherichia coli* strain S17-1. The bacterial strains with plasmids harboring the mutations in subunit II (H260N, LpM) and subunit I (D132N, D132N/K362M) were grown as described in Section 2.2.2. The histidine-tagged proteins were purified twice using IMAC chromatography and ion-exchange chromatography for MS/MS spectroscopy as described in Section 2.2.2. The ∆Cu₉ mutant protein was gifted by Dr. Jon Hosler at the Mississippi Medical Center. The expression vectors bearing mutations of H260N and LpM were kindly sent to us by Dr. Shelagh Ferguson-Miller at the Michigan State University, East Lansing.

5.2.2 Trypsin digestion and sample preparation for FTMS

Approximately 20 µM of the purified enzyme was digested with 20 µg of sequencing-grade trypsin in a 100mM ammonium bicarbonate/ acetonitrile mixture at 37 °C. Post-digestion, the low mass peptides were removed using a gel filtration spin column. Detergent was removed from the digest using methanol/chloroform precipitation. The detergent-free pellet was immediately resuspended in 500 µL of 75% acetic acid and used for mass spectrometric analysis.

5.2.3 Mass spectrometry and data analysis

The samples were run on a custom-built Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer at the Kelleher laboratory in the University of...
Illinois. The detergent-free trypsin digests were introduced into the spectrometer using electrospray ionization. The peptide of interest was isolated using broadband scans and then it was subjected to collisionally activated dissociation (CAD) for MS/MS spectrometry. The data generated were analyzed using THRASH algorithm (33) and ProSightPTM (34). These mass spectrometry measurements were done in collaboration with my colleague Dana Robinson and Dr.Neil Kelleher at the University of Illinois at Urbana-Champaign.

5.3 Results

Subunit I of the $aa_2$-type oxidase in *Rhodobacter sphaeroides* has 25 cleavage sites for trypsin as shown in the Figure 5.3. Trypsin cleaves at the C-terminus of the positively charged amino acids, lysine and arginine. The crosslink of interest is present in the helix VI of subunit I between H284 and Y288. This post-translational modification is present in the tryptic peptide N258-K307. Tandem mass spectrometry (MS/MS) results obtained to determine the presence of crosslink in the trypic digests of the subunit I of the wild-type and mutant $aa_2$-type oxidases are presented below:

**Wild-type**

The predicted mass of the tryptic peptide N258-K307 with crosslink (5531.85 Da) matches with the experimental mass obtained for the tryptic fragment in the wild-type (Figure 5.4). The crosslink was present in 100% of the enzyme population (6).

**H260N**

This residue H260 in subunit II serves as a ligand to the dinuclear Cu$_\alpha$-site. Electron transfer from Cu$_\alpha$ to heme $a$ is believed to occur via a route involving H260, peptide bond between R481/ R482 and several hydrogen bonds leading up to the heme $a$ propionates (31). When His-260 is substituted with asparagine, oxygen reduction activity is reduced to 1% of wild-type and the Cu$_\alpha$ band at 830 nm is
diminished. The redox-potential of CuA was raised by 118 mV compared to the wild-type resulting in very slow electron transfer from CuA to heme a (35).

In this mutant, electron transfer to the BNC is slowed down to a significant extent but is sufficient to allow enzyme-turnover. The MS/MS results show that the H284-Y288 crosslink is present in this mutant (Figure 5.6).

**LpM**

This is the “loop mutant” in the subunit II of the aa₃-type oxidase, where the entire Cuₐ-binding loop from residues 252-265 (Cys-Ser-Glu-Ile-Cys-Gly-Ile-Ser-His-Ala-Tyr-Met-Pro-Ile) is replaced with blue-copper binding loop from azurin (Cys-Ser-Glu-Pro-Gly-His-Ser-Ala-Leu-Met-Lys-Gly) (31). But this substitution resulted in the abolishment of any copper incorporation into the oxidase. Hence this oxidase lacks the Cuₐ-site to receive electrons from cytochrome c and as expected, has no measurable oxygen reduction activity.

The mass spectrometry results show that the tryptic peptide N258-K307 has a mass of 5533.86 Da, which is approximately 2 Da more than that of the wild-type peptide (Figure 5.7). This result clearly indicates the absence of any post-translational modification at the BNC and is supported by MS/MS sequencing data.

**D132N/K362M**

D132 is the residue at the entrance of the D-channel and K362 is a key residue for the K-channel. This double mutant blocks the entry of proton through either channels and the oxygen reduction activity of the enzyme is negligible (36).

The mass spectrometry data on this double mutant shows that an intact crosslink is present at the active site of this enzyme (Figure 5.8). Although the proton transfer is blocked for any measurable oxygen reduction activity during steady-state, the post-translational modification has not been affected.
\textbf{ΔCu_{111} mutant}

The wild-type \textit{aa}_{1}oxidase was expressed and purified from \textit{Δ}cox11 strain. The product of \textit{cox11} gene is the protein cox11p, which is involved in the assembly of Cu_{111} into the BNC of the oxidase. When the wild-type expression plasmid was expressed in the \textit{Δ}cox11 strain, the resulting enzyme did not have Cu_{111} in the active site. This was confirmed by metal analysis and EPR spectroscopy (32).

MS/MS results on this \textit{ΔCu_{111}} mutant very clearly show that the mass of the tryptic peptide N258-K307 is 2 Da higher than that of the wild-type tryptic peptide (Figure 5.9). Therefore we conclude that the active site in this enzyme lacks the post-translational crosslink modification.

\section*{5.4 Discussion}

From the MS/MS results, it is obvious that mere slowing down of electron and proton transfer to the active site as exemplified in the cases of H260N and D132N/K362M mutants do not prevent the formation of the crosslinked cofactor at the active site. Although these enzymes have negligible oxygen reduction activity, the presence of a crosslink in them suggests that it is sufficient for this post-translational modification to occur. Unlike the electron delivery, it is harder to block proton delivery to the active site of the oxidase since it is connected to the bulk solution through proton input and exit channels (37). Since radical chemistry involves the oxidation of amino acids in the vicinity of the catalytic site, specific proton delivery for the cross-link formation does not seem essential.

In the case of the LpM mutant or the \textit{ΔCu_{111}} mutant, electrons are unable to be transferred to heme \textit{a} and this prevents the formation of the covalent cross-link at the active site. The complete loss of crosslink due to a block in electron-transfer pathway also negates the possibility of the involvement of any assembly enzyme in catalyzing
the post-translational modification. The H-Y crosslink is not seen in the \( \Delta \text{Cu}_{\text{II}} \) mutant and this indicates that the presence of Cu_{II} at the active site is vital for the covalent modification.

This puts oxidase in the company of a host of other metalloproteins that undergo auto-catalyzed radical chemistry to form one or more covalent crosslinks among residues in the active site vicinity (27). The amino acids most susceptible to form these cross-linked cofactors are cysteine, methionine, tryptophan, tyrosine and glycine, and this is generally catalyzed by a metal (Fe, Co, Cu and Mn) in the presence of oxygen. Upon modification, these protein-derived cofactors contribute to the catalysis in many ways the unmodified amino acids cannot. Commonly, they have been found to provide electrophilic sites for substrate interaction or stabilize free-radical intermediates (26).

This is significant because, we can then be certain that in cytochrome oxidases too, the covalent modification after radical chemistry converts the tyrosine-288 residue to form stable tyrosyl radical during the catalytic cycle. Many research groups have localized the radical detected during Pm-formation on the Tyr-288 residue although it is not yet universally accepted (15, 17). The fact that the active-site tyrosine is conserved across the entire heme-copper superfamily and mutations at the site affect both structure and function is indicative of involved contribution to catalysis beyond the delivery of protons.

**Radical chemistry at the active site of the oxidase**

Here, we speculate on the events that cause the covalent cross-link at the active site. In heme proteins and copper proteins, as in the instances of catalase peroxidase (KatG) (29) and galactose oxidase (23, 30) respectively, the active site heme or copper individually could generate the cross-linked amino acids during
biosynthesis. From our results, we know that Cu_{b} is important to the radical chemistry. Despite the lack of evidence, we are certain that heme \( a_{3} \) is essential for the biosynthesis of cross-linked amino acids owing to the nature of the reaction in the oxidases.

During the catalysis of cytochrome \( c \) oxidase, Cu_{b} and heme \( a_{3} \) have been shown to provide one and two electrons respectively. It is possible that during the very first turnover, when oxygen binds to the reduced binuclear center, it gets reduced with three electrons resulting in the formation of a reactive hydroxyl radical. This hydroxyl radical may oxidize the neighboring tyrosine and histidine residues enabling the formation of the cross-link. This cross-link thus formed may help in the catalytic function of the mature enzyme by facilitating electron donation and stabilizing the radical formed at Tyr-288 (Figure 5.10).

The above hypothesis is quite reasonable since there are supportive instances in the literature (38, 39). In general, it is accepted that •OH is capable of causing modifications to the primary structure of proteins and it has especially been demonstrated that hydrogen abstraction by hydroxyl radical produces tyrosyl radical (40). These tyrosyl radicals may then react with the nearby amino acids to form stable covalent bonds.

It is evident that it is the His-Tyr crosslink that makes the oxidase competent in the one-step reduction of oxygen without the release of partially reduced oxygen species (PROS). Such a protective mechanism is perhaps necessary since the physiological electron delivery by cytochrome \( c \) is a lot slower than the intramolecular electron transfer in the oxidase. In a very elegant biomimetic experiment, it was shown that under limiting electron supply, the presence of Fe\( a_{3} \), Cu_{b} and tyrosine were essential to the reduce oxygen without releasing partially
reduced oxygen species (PROS). The absence of any one of these resulted in a marked increase in the release of toxic PROS (21).

**In-vitro generation of the crosslink**

Cytochrome oxidases do not lend themselves well to the incorporation of active site metals *in vitro* and hence it is difficult to externally induce the covalent modification as has been reported for many other enzymes. However, a mutant described in this study, the LpM or ∆CuA mutant may be a suitable candidate for testing if the crosslink formation can be induced with exposure to H₂O₂ in the presence of a reductant.

In this work, we have described some of the conditions we found to be important for the formation of the post-translational cross-link modification between H284 and Y288 at the active site. We think that the active site metals, Fe₆₃ and Cu₉₁, in the presence of reductants and oxygen are sufficient to cause the formation of the crosslink. The reaction probably is mediated by a hydroxyl radical causing the formation of the tyrosyl radical which in turn leads to the oxidation of His-284 to form the covalent linkage.
5.5 Figures

Figure 5.1: This schematic shows heme $a_3$ and Cu$_B$ coordinated to their histidine-ligands. The unique covalent modification between one of the Cu$_B$ ligands and Tyr-288 is shown in the figure. Tyr-288 is at the end of the K-channel. The conserved motif for the crosslink in the A- and B-type oxidases is -Gly-His-Pro-X-Val-Tyr-. This figure was made using VMD.
Figure 5.2: Schematic of the steps involved in the MS/MS spectroscopy. Panel A shows the isolation of the peptide fragment of interest based on theoretical mass and Panel B shows how sequencing is done using a mass spectrometer.
Figure 5.3: This figure shows the trypsin cleavage sites in the subunit I of the $aa_3$-type oxidase from *Rhodobacter sphaeroides*. The tryptic peptide of interest N258-K307 is highlighted in bold. The H284 and Y288 residues involved in the crosslink are also shown.
Figure 5.4: Mass Spectrum and fragmentation of the cross-linked peptide from the wild-type *aai*-type oxidase from *Rhodobacter sphaeroides*. (A) Mass spectrum of the crosslinked tryptic peptide N258-K307. (B) CAD MS/MS results when analyzed with assumption of crosslink and (C) no crosslink. Panel B results show the His-Tyr crosslink is present in the wild-type.
Figure 5.5: A cartoon representation showing the effect of the different mutations on the oxidase. Panels A, B, C and D show the effects of H260N, ∆CuA, D132N/K362M and ∆CuB mutations on the enzyme.
Figure 5.6: Mass Spectrum and fragmentation of the cross-linked peptide from the electron-path mutant H260N from *Rhodobacter sphaeroides*. (A) Mass spectrum of the crosslinked tryptic peptide N258-K307. (B) CAD MS/MS results when analyzed with assumption of crosslink and (C) no crosslink. Panel B results show the His-Tyr crosslink is present in H260N.
Figure 5.7: Mass Spectrum and fragmentation of the cross-linked peptide from the ΔCuA mutant LpM from *Rhodobacter sphaeroides*. (A) Mass spectrum of the crosslinked tryptic peptide N258-G307. (B) CAD MS/MS results when analyzed with assumption of crosslink and (C) no crosslink. Panel C results show the His-Tyr crosslink is not present in LpM.
Figure 5.8: Mass Spectrum and fragmentation of the cross-linked peptide from the proton-channels double mutant D132N/K362M from *Rhodobacter sphaeroides*. (A) Mass spectrum of the crosslinked tryptic peptide N258-K307. (B) CAD MS/MS results when analyzed with assumption of crosslink and (C) no crosslink. Panel B results show the His-Tyr crosslink is present in D132N/K362M.
Figure 5.9: Mass Spectrum and fragmentation of the cross-linked peptide from the \( \Delta \text{Cu}_B \) mutant from *Rhodobacter sphaeroides*. (A) Mass spectrum of the crosslinked tryptic peptide N258-K307. (B) CAD MS/MS results when analyzed with assumption of crosslink and (C) no crosslink. Panel C results show the His-Tyr crosslink is not present in the \( \Delta \text{Cu}_B \) mutant.
**Figure 5.10:** This figure shows the active site of the oxidase before and after the post-translational modification. We propose that the formation of the crosslink is mediated by the hydroxyl radical. The heme $a_3$ structure has been truncated for clarity.
5.6 References


