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dissertation entitled

Search for Residues Critical to Proton Pumping in Cytochrome c Oxidase

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

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Date July 27/95

SEARCH FOR RESIDUES CRITICAL TO PROTON PUMPING IN CYTOCHROME *c* OXIDASE

By

John R. Fetter

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1995

ABSTRACT

SEARCH FOR RESIDUES CRITICAL TO PROTON PUMPING IN CYTOCHROME *c* OXIDASE

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Cytochrome *c* oxidase from *Rb. sphaeroides* is a good model for the highly homologous mammalian oxidase. A reconstitution and assay to look for proton pumping in the bacterial oxidase showed that it could pump protons with similar efficiency to that of the mammalian enzyme. Site-directed mutagenesis was used to find residues involved in a proton pumping pathway. Mutations were made in several conserved regions in Subunit I: in the II-III interior loop, Asp132Asn/Ala; in transmembrane helix VIII, Pro358Ala, Thr359Ala; in transmembrane helix X, Tyr422Phe/Ala; and in the exterior IX-X loop, His411Ala, Asp412Asn, Thr413Asn, and Tyr414Phe. All showed decreased electron transfer activity indicating that they could have a role in proton pumping. Only Asp132Asn/Ala showed inhibited electron transfer and complete inhibition of proton pumping, while retaining normal visible absorbance and resonance Raman characteristics. When reconstituted it showed inhibition instead of stimulation of electron transfer with release of the pH gradient or electrical gradient. Electron transfer activity of solubilized Asp132Asn showed the same response to pH as wild type. Addition of free fatty acids with long alkyl chains such as arachidonic acid to this mutant stimulated electron transfer activity up to six-fold, but the corresponding alcohols had no effect. Analogues of arachidonic acid showed less stimulation.

Stimulation by fatty acids was not seen on wild type or other mutants. It was concluded that the fatty acid may be replacing the missing carboxyl to improve proton access, or allowing protons to enter the enzyme through another path. Four more mutants were made in the IX-X loop: His411Gln, Asp412Ala, Thr413Ala, Tyr414Ala. Previously, His411 and Asp412 were shown to contribute to binding a manganese or magnesium. His411Gln retained native properties indicating that the Gln may act as a replacement ligand. Asp412Ala showed lower electron transfer activity indicating it may have lost the metal. Thr413Ala showed wild type characteristics. Tyr414Ala showed a spectral shift of the alpha band to 604 nm providing evidence that the shift to 610 nm in Tyr414Phe is caused by an aromatic interaction with heme *a*.

Dedicated to my wife

Suzanne

ACKNOWLEDGMENTS

I wish to thank the many scientists that I have had the opportunity to work with during my graduate training. First and foremost is Dr. Shelagh Ferguson-Miller who has provided much guidance and creativity in working on this project. I also would like to thank those who served on my committee Dr. Arnold Revzin, Dr. Loran Bieber, Dr. Rawle Hollingsworth, and Dr. Gerald Babcock. A special thanks to Jerry for all the chemical insights he has given to us biochemists at group meetings. I would next like to thank Jon Hosler and Thomas Nilsson for the scientific wisdom that they have passed on to me.

I am grateful to our many collaborators including Dr. Robert Gennis who allowed me to come to his lab and learn molecular biology, and especially to Jeff Thomas who took the time to teach me those techniques. Likewise I enjoyed my time in the lab of Dr. Peter Nicholls working with Dr. Martyn Sharpe learning the techniques used to measure membrane potentials. I would like to thank Jim Shapleigh and Arturo García-Horsman for making some of the oxidase mutants. I also greatly appreciated those who ran the resonance Raman in Jerry's lab: Einhardt Schmidt and Michelle Pressler.

I also enjoyed the time spent with my fellow graduate students: Wendy, Taha, Jie, Yuejun, Jung, Doug and also to the undergrads past and present: Barb, Kelly, Heather, Ann and Giancarlo.

I am glad to have met others during my stay in Michigan: Joe and his family, Dennis, Steve, and Anita and Fr. Bill and to Suzie's family Bruce, Mike, Liz, Justin, Jennifer, Kevin and Mom and Dad.

I am especially grateful to my parents for their patience and guidance while I was growing up. I am glad to have two kind sisters Brenda and Joyce along with two great brother-in-laws Doug and Keith.

A special thanks goes to my loving wife for her patience while I worked on this thesis, and the help that she gave me, especially her photocopying expertise.

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A.U.	absorbance units
bR	bacteriorhodopsin
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
DCCD	N,N'-dicyclohexylcarbodiimide
DEAE	diethylaminoethyl
diSC-3,5	3',3'-dipropylthiadicarbocyanine
EPR	electron paramagnetic resonance
ESEEM	electron spin echo envelope modulation
FPLC	fast protein liquid chromatography
FTIR	Fourier transform infrared spectroscopy
H^+/e^-	ratio of protons pumped per electron transferred from cytochrome *c* into cytochrome oxidase
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
His-tag	histidines that are genetically attached to an enzyme to allow purification by a nickel affinity column
Im	imidazole
Q	ubiquinone
RCR	respiratory control ratio
SDS	sodium dodecyl sulfate
TRIS	Tris(hydroxymethyl)aminomethane
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
ΔpH	transmembrane pH gradient
$\Delta\psi$	transmembrane electrical gradient

Chapter 1 – Background on cytochrome oxidase: a complex mitochondrial enzyme and many simpler bacterial forms

Introduction

Cytochrome oxidases participate along with other respiratory enzymes in the conversion of energy from exergonic electron transfer events into a transmembrane proton gradient. The respiratory enzymes occur in the inner mitochondrial membrane of eukaryotes, or in the inner membrane of prokaryotes. The proton gradient can be used directly to drive the transport of ions across the membrane or for mechanical work such as driving flagellar motion in bacteria. Another role of the proton gradient is to drive the conversion of ADP to ATP by the F_1F_0 ATP synthase. When the energy is stored in the form of ATP, it can easily diffuse throughout the cell to the site of numerous energy requiring reactions. Cytochrome oxidase, as the final electron acceptor, also has the unique role of catalyzing a reaction which provides the final low potential sink for electrons, by reducing oxygen to water. This reaction requires substrate protons that are derived from the mitochondrial matrix, or interior of the bacterium, and thus contribute to the formation of the gradient. At the same time oxidase uses the energy derived from the thermodynamically favorable conversion of O_2 to H_2O to drive proton pumping, further contributing to the gradient (For a textbook overview see Nicholls and Ferguson 1992).

Much of the research on oxidase has been done with the beef heart enzyme which contains thirteen subunits (Kadenbach *et al.*, 1983; Capaldi 1990b). Other oxidases such as yeast contain anywhere from 9 (Trawick *et al.*, 1992) to 12 (Taanman and Capaldi 1992) subunits and the plant enzyme

contains 8 (Peiffer *et al.*, 1990) subunits. The discovery that bacterial oxidases contain as few as three subunits that are highly homologous to the largest subunits of the other enzymes, provides a simpler enzyme to work with. Analysis of the aa3-type enzyme from *Rhodobacter sphaeroides* using EPR, visible absorbance and resonance Raman shows it to have the same metal centers as the eukaryotic enzyme. The enzyme also has high electron transfer activity, and normal proton pumping. These results give strong evidence that only the three subunit core is required for cytochrome *c* binding, electron transfer, and oxygen reduction (Hosler *et al.*, 1992). It has been possible to make deletions of the genes for the subunits of bacterial oxidases, and to reintroduce the genes on plasmids, making possible the creation of site-directed mutants. This has been done in *Rb. sphaeroides* (Cao *et al.*, 1991; Cao *et al.*, 1992; Shapleigh and Gennis 1992), and *E. coli* (Chepuri and Gennis 1990). Extensive site-directed mutagenesis and spectroscopic characterization has been done on subunit I allowing the definition of the active site of the enzyme in more detail than previously possible.

Only low resolution crystal structures have been published of the beef heart enzyme, and these do not provide information about the location of predicted transmembrane helices or the metal centers (Valpuesta *et al.*, 1990). However, recently detailed crystal structures have been determined of the four subunit *Paracoccus Denitrificans* aa3 (Personal communication: Hartmut Michel), and of the beef heart oxidase (Yoshikawa, personal communication).

Role of the three core subunits of the oxidase

Subunit I: the active site where oxygen is reduced; likely site of the proton pump This subunit contains three metal centers including heme *a* which accepts electrons from Cu_A in subunit II, and heme a_3 and Cu_B where

oxygen reduction occurs. In *Rb. sphaeroides* it has a molecular weight of 63 kDa (Hosler *et al.*, 1992), with hydropathy analysis and phoA/lacZ fusions predicting twelve transmembrane helices (Chepuri and Gennis 1990). The placement of the metal centers has become much clearer through mutagenesis of the proposed ligands, six totally conserved histidines, giving a more detailed model of the subunit (See Figure 1.1). Individual mutation of any four of the histidines 284, 333, 334, and 419 leaves heme *a* intact. Mutants of His102 and His421 lose the heme *a*, leading to the assignment of those two histidines as its ligands. Since mutation of His333 to asparagine leaves the heme *a*3 iron-his bond unaltered, it must be concluded to be a Cu_B ligand; the adjacent His334 must then also be a Cu_B ligand, since if it were the heme a3 ligand it would put the a3 and Cu_B in a sterically untenable position (Lemieux *et al.*, 1992; Minagawa *et al.*, 1992; Shapleigh *et al.*, 1992). Initial studies were unclear about whether His284 or His419 was the ligand to heme *a*3. However more recent results indicate that mutation of His284 to alanine leaves at least some heme *a*3 iron-his bond in a native state eliminating this residue as a heme *a*3 ligand and suggesting its role as a Cu_B ligand. In contrast, since all mutations of His 419 disrupt the heme *a*3, it is proposed that this residue is the iron ligand of heme *a*3 (Calhoun *et al.*, 1993). These assignments have now been confirmed by the crystal structure.

Other mutations have provided further information about the structure of subunit I and will be discussed in more detail in latter chapters of this thesis. Data show that the external loop between helices IX and X lays across the top of the binuclear center, since mutations in that region have some effects on both heme *a* and heme *a*3/Cu_B (Hosler *et al.*, 1994a; Hosler *et al.*, 1994b). Mutations in the highly conserved hydrophilic face of helix VIII greatly or completely inhibit the electron transfer activity indicating that the helix

Figure 1.1 Helical wheel model of the twelve predicted transmembrane helices of subunit I from the *Rb. sphaeroides* cytochrome *c* oxidase. The indicated ligands to the metal centers are all histidines with His102 and His421 acting as heme *a* ligands. His419 is the heme *a*3 ligand, and His333, His334 and His284 are shown as the Cu_B ligands (Calhoun *et al.*, 1993; Hosler *et al.*, 1993).

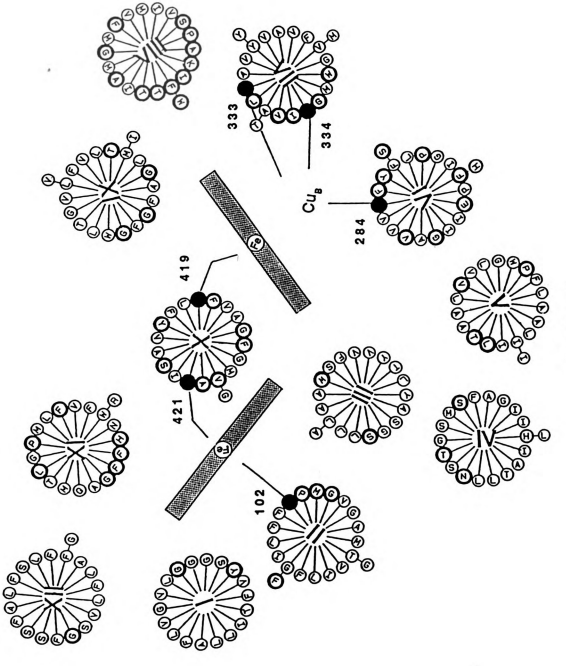


Figure 1.1

lies near the binuclear center (Hosler *et al.*, in press). A mutation of the highly conserved aspartic acid in the loop between helices II and III inhibits electron transfer and proton pumping but has no effect on the visible and resonance Raman spectra indicating an important role in proton translocation but from a position that is not adjacent to the binuclear center (Fetter *et al.*, 1995). This confirms the idea that subunit I may play an important role in proton pumping implying fairly direct coupling of proton translocation to the active site reactions.

The sum of the results of the mutational analysis leads to an ordered placement of at least 10 of the 12 helices of subunit I surrounding the metal centers, providing a useful semblance of a 3D model on which to base further predictions. The distances between metal centers match reasonably well those predicted from spectral analysis techniques (Trumpower and Gennis 1994). Thus this has become a generally accepted working model, giving considerable impetus to mechanistic thinking and experimental design. The accumulated results from much mutagenesis work on both the aa_3 oxidase from *Rb. sphaeroides* and the homologous bo_3 enzyme from *E. coli* have so far been consistent with this model (Hosler *et al.*, 1993).

Subunit II: the site of cytochrome *c* binding and electron entry

This subunit with a molecular weight of 33 kDa in *Rb. sphaeroides* (Hosler *et al.*, 1992) is proposed to contain 2 transmembrane helices and a soluble C-terminal domain based on hydropathy analysis and betagalactosidase/alkaline phosphatase (phoA/lacZ) gene fusion analysis (Chepuri and Gennis 1990). Cytochrome *c* is the soluble electron carrier that delivers electrons to the oxidase by first binding to a proposed site in the C-terminal domain. This subunit contains several conserved carboxyl side chains which appear to interact with conserved positively charged residues surrounding the exposed

heme edge of cytochrome *c* (Capaldi *et al.*, 1982). Reaction of the oxidase with carboxyl modifying reagents inhibits cytochrome *c* binding, while binding of cytochrome *c* (Millett *et al.*, 1983) or antibodies to subunit II protects against a number of carboxyl modifications (Taha and Ferguson-Miller 1992). There is also evidence for a second site of cytochrome *c* binding in subunit II which could contribute to the biphasic steady state kinetics (Ferguson-Miller *et al.*, 1978; Taha and Ferguson-Miller 1992). An alternative explanation for the biphasic kinetics does not require a second binding site: it is proposed that the enzyme has two conformations that can accept electrons, only one of which can pump protons (Brzezinski and Malmström 1986). More recent studies (Robinson 1995 Biochemistry, in press) lend credence to the former hypothesis.

Subunit II contains what was previously believed to be a mononuclear copper (Cu_A) in the C-terminal domain. Although the EPR signal from the Cu_A center is different from the blue copper proteins, the difference was proposed to be caused by ligation of the copper by two cysteines and two histidines (Stevens *et al.*, 1982), instead of the single cysteine of the blue coppers (Saraste 1990). Recent studies using S-band for EPR measurements have found similarity of the Cu_A site to a copper site in nitrous oxide reductase, with both giving a seven-line hyperfine splitting that can be explained by the presence of a binuclear copper (Kroneck *et al.*, 1989; Antholine *et al.*, 1992). Other evidence also predicts a binuclear Cu in oxidase including metal analysis showing 3 Cu/2 Fe (Bombelka *et al.*, 1986; Steffens *et al.*, 1987)and characterization of the binuclear copper in an isolated C-terminal domain of *P. denitrificans* (Lappalainen *et al.*, 1993). Again, recent crystallographic studies confirm the 2 Cu center.

There has been much controversy over the years as to whether the electrons from cytochrome *c* are transferred directly to Cu_A or to heme *a* or

both (Einarsdóttir 1995). Recent fast kinetic spectral studies suggest that electron transfer occurs most rapidly between cytochrome *c* and Cu_A with a forward rate constant of 10^5/s, followed by transfer from Cu_A to heme *a* with a forward rate constant of 7,000/s (Hill 1991; Hill 1994). It has been possible to measure values as high for electron transfer between cytochrome *c* and Cu_A using a photoactivatable ruthenium compound attached to cytochrome *c* (Pan *et al.*, 1993). Noting a conserved aromatic region in subunit II, possibly between Cu_A and heme *a*, it was proposed that the aromatics might assist the electron transfer between these centers (Steffens and Buse 1979). However, in reaction center, aromatics do not seem to be critical for fast electron transfer (Huber 1989). Initial site-directed mutagenesis efforts to remove one of the copper ligands indicate that when all of the Cu_A is lost but the other metal centers remain intact the enzyme retains 10% of the activity of the wild type (Yuejun Zhen and Shelagh Ferguson-Miller: personal communication). This result further supports the idea that under normal conditions the most kinetically efficient site of entry of electrons is through Cu_A.

Subunit III: a structurally important element This subunit does not contain any metal centers and in *Rb. sphaeroides* has a molecular weight of 30 kDa (Hosler *et al.*, 1993) and is predicted to contain 7 transmembrane helices from hydropathy analysis and gene fusion experiments (Chepuri and Gennis 1990). It had been considered for a possible role in proton pumping, since DCCD was found to bind to a glutamate in subunit III and inhibit proton pumping (Casey *et al.*, 1980; Prochaska *et al.*, 1981). However, subunit III-depleted oxidases are still capable of pumping protons (Thompson and Gregory 1985; Prochaska and Fink 1987) and mutagenesis of the glutamate to which DCCD binds in subunit III does not appear to inhibit proton pumping in intact *Paracoccus denitrificans* (Haltia *et al.*, 1991).

Other subunits in the eukaryotic enzymes

It has been shown that in yeast (Schatz and Mason 1974), *Neurospora crassa* (Macino and Morelli 1983) and in mammals (Anderson *et al.*, 1982), the three-subunit core of the oxidase is encoded in the mitochondria. The other subunits are imported into the mitochondria, being encoded by the nucleus (Capaldi 1990a). Evidence exists that the smaller subunits are involved in regulation of the enzyme (Kadenbach *et al.*, 1991). It has been shown that oxygen levels can effect the expression of subunit isoforms in *Dictyostelium discoideum* (Bisson and Schiavo 1986; Bisson and Schiavo 1988) and in yeast(Poyton *et al.*, 1988). Comparison of the reconstituted bovine and *P. denitrificans* enzymes showed that intraliposomal ATP could increase the Km for cytochrome c and ADP decrease the Km for the bovine enzyme while no effect of these nucleotides could be shown on the bacterial enzyme(Hüther and Kadenbach 1988). Phosphate (Büge and Kadenbach 1986; Malatesta *et al.*, 1987) and free fatty acids (Labonia *et al.*, 1988; Thiel and Kadenbach 1989) also have some modulatory effect on the bovine oxidase activity. Tissue specific expression of isoforms of some of the subunits has been shown for the mammalian oxidases and these appear to be species specific (Kadenbach *et al.*, 1991).

Overview of the oxidase reaction

Much of what is known about the mechanism of cytochrome *c* oxidase was deciphered before the recent mutationally-derived structural model through the use of various types of spectroscopy. The overall reaction for oxidase is:

$$8H^+_{in} + 4e^- + O_2 \longrightarrow 2H_2O + 4H^+_{out}$$

Thus for each electron entering the enzyme, one proton is taken up from the cytoplasm of bacteria or the matrix of mitochondria to permit reduction of oxygen to water. There are four electrons required for the complete reaction, and there is evidence to suggest that the last two electron transfers to the oxygen intermediates are coupled to the proton pump, with two protons being pumped at each of these reduction steps (Wikström 1989). Measured from the inside, $2H^+/e^-$ are taken up which contributes to the protonmotive force Δp (Δp contains an electrical component $\Delta\psi$ and a pH component ΔpH).

There has been some controversy about the actual number of protons being pumped. Measurements have been made in whole mitochondria of $2H^+/e^-$ being pumped (Lehninger *et al.*, 1981), while others made strong arguments for 1 H^+/e^- from measurements in whole mitochondria and artificially reconstituted oxidase vesicles (Wikström 1977; Casey *et al.*, 1983; Wikström 1984). Mitochondrial experiments are difficult because of the ion transporters that can affect the apparent stoichiometry. Reconstituted experiments are much cleaner, but it is possible that something could be lost in purification of the enzyme, and the apparent stoichiometry can be highly variable depending on the exact conditions including the quality of detergent and lipids used. From experiment and thermodynamic arguments it is now generally accepted that 1 H^+/e^- is pumped (Babcock and Wikström 1992). In the case of the reconstituted *Rb. sphaeroides* oxidase proton pumping appears slightly lower than the beef heart enzyme, with H^+/e^- somewhere between 0.5 and 0.8 (Fetter *et al.*, 1995). This is likely an artifact of the reconstitution process, possibly due to a requirement for different lipids to give optimum pumping.

There has been a question about whether the protons pumped to the intermembrane space of mitochondria contribute to the protonmotive force

since they may diffuse into the cytoplasm. Some recent studies of mitochondrial structure indicate that this space may be subdivided into separate intra-cristae and inter-membrane compartments which may not be in rapid equilibrium and thus could contribute more to formation of a pH gradient (Mannella *et al.*, 1994). It is also unclear whether protons that are pumped to the periplasm of bacteria contribute to the protonmotive force of most bacteria because small molecules from the periplasm can diffuse to the environment through pores in the outer membrane structure. Study of the alkalophiles may provide a clearer understanding of this question because they can make ATP even when the external pH is as high as 12 (Krulwich *et al.*, 1988). This is postulated to occur through some localized transfer of protons from the oxidase to the ATP synthase in which the pumped protons from the oxidase immediately return through ATP synthase, with this cycle possibly being compartmentalized (Westerhoff *et al.*, 1984; Ferguson 1985; Rottenberg 1985).

Chemistry of the oxygen reaction

Several types of experiments can be done to look for the intermediates in the oxygen reduction mechanism. Because of the speed of electron transfer events, many of the experiments require techniques which allow the analysis of fast kinetics on the usec or nsec time scale. An important experimental methodology is to inhibit the enzyme by binding CO to the high spin heme a_3 where O_2 normally binds. CO dissociates slowly in the dark (in seconds) so it is possible to mix the sample with O_2, and then initiate a fast dissociation with a nanosecond laser flash, allowing oxygen to react in its place (Gibson and Greenwood 1963; Greenwood and Gibson 1967). The two types of spectroscopy most often used in the analysis of the intermediates are uv-visible absorbance

(Oliveberg and Malmström 1991) which can monitor the absorption of the hemes and Cu_A, and resonance Raman (Varotsis *et al.*, 1993), which is much more sensitive to details of each of the heme environments. Visible absorbance can give better kinetic resolution showing four phases of the reaction, while the time-resolved resonance Raman provides spectral details which, through isotope experiments and comparison with model compounds, make clearer the true nature of the intermediates. Another method to detect intermediates uses optical absorption on whole mitochondria and a high protonmotive force to reverse the chemistry of the oxidase (Wikström 1989; Wikström and Morgan 1992). FTIR has also been helpful in resolving the changes at the binuclear center caused by mutation. In this technique CO is bound to the oxidase and then light is used to flash CO off of the Fe of heme $a3$, after which it binds to Cu_B (Calhoun *et al.*, 1993). These methods have provided pieces to the puzzle of the mechanism.

These data have been synthesized recently to provide a self-consistent model of the sequence of the oxygen intermediates and the kinetics of their interconversion (Babcock and Wikström 1992; Varotsis *et al.*, 1993; Einarsdóttir 1995). Figure 1.2 shows a summary of the chemistry at the binuclear center. Oxygen binds only after two electrons enter the enzyme probably by first interacting with the Cu_B (Woodruff *et al.*, 1991) and then moving to the heme a3 (2' and 2). Oxygen is then rapidly converted to the end bound peroxy form (3-5). Entry of the third electron to the binuclear center initiates the splitting of the oxygen to the ferryl (7). Finally the fourth electron

Figure 1.2 Pathway for the reduction of oxygen to water in cytochrome *c* oxidase with numbering as in Babcock and Wikström (1992). Only the changes occurring at the heme $a3/Cu_B$ are shown, although electron transfers are also occurring from the other centers Cu_A and heme *a*. The question mark indicates the possibility of additional intermediates. Only the substrate protons are indicated in the figure. Pumped protons are predicted to be moved in going from 3 to 7 and from 7 to 10 with two protons at each step (Wikström 1989; Varotsis *et al.*, 1993).

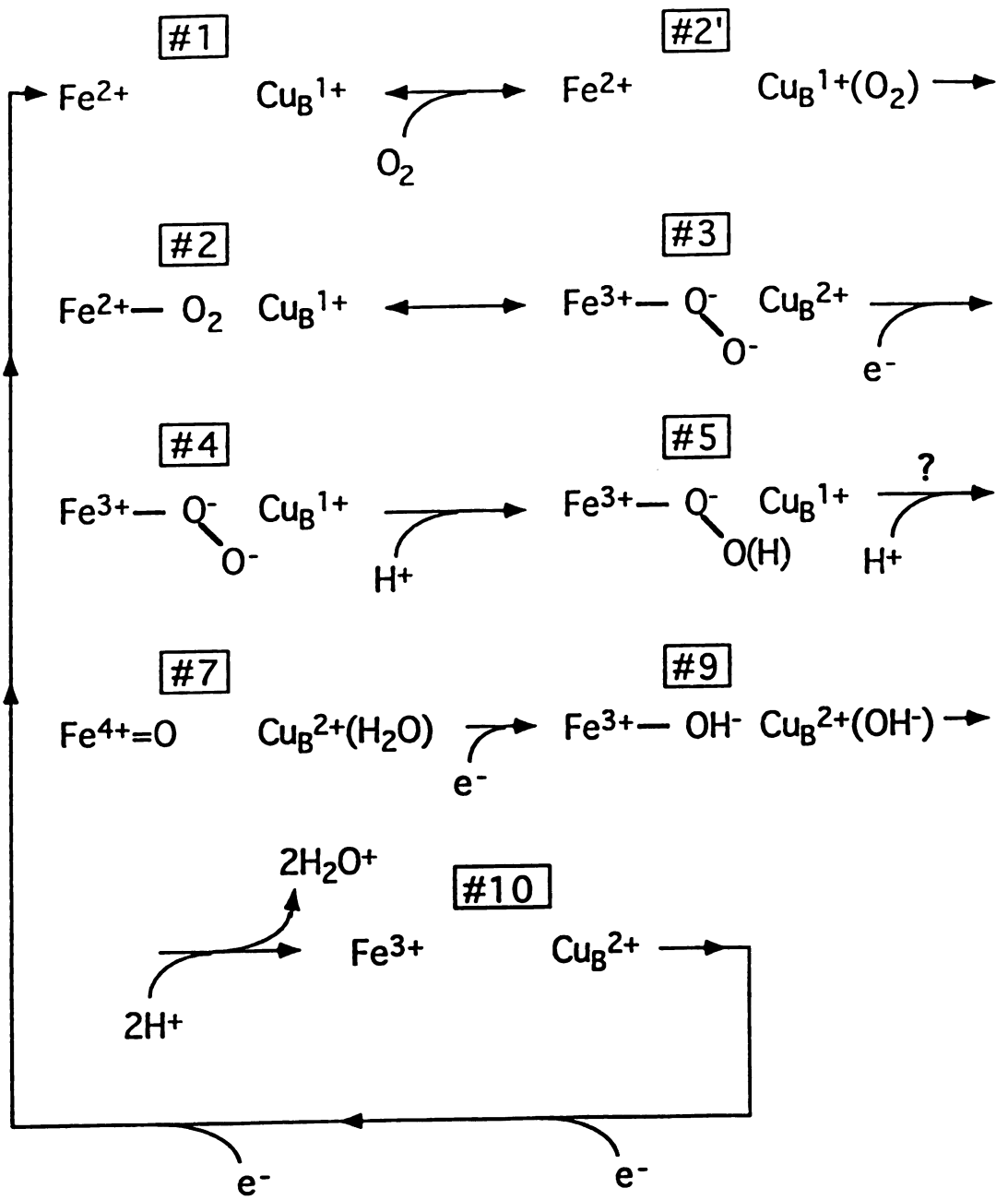

#1
#2'
Fe2+
CuB1+
Fe2+
CuB1+(O2)
O2
#2
#3
Fe2+— O2
CuB1+
Fe3+— O-
O-
CuB2+
e-
#4
#5
Fe3+— O-
O-
CuB1+
H+
Fe3+— O-
O(H)
CuB1+
?
H+
#7
#9
Fe4+=O
CuB2+(H2O)
e-
Fe3+— OH-
CuB2+(OH-)
2H2O+
#10
Fe3+
CuB2+
2H+
e-
e-

Figure 1.2

converts the ferryl to a ferric hydroxide (9). Time-resolved resonance Raman has identified modes which have been assigned for (2', 5, 7, and 9) (Varotsis *et al.*, 1993).

The least clear part of the mechanism is the peroxide form of the enzyme (3-5). Previously a mode at 358 cm^{-1} observed by resonance Raman was assigned as a protonated peroxy (5) by both Babcock and Kitagawa's groups (Ogura *et al.*, 1991; Varotsis *et al.*, 1993). Similarly optical absorption of intermediates formed during reversed electron transfer have assigned 607 nm as a peroxy and 580 nm as a ferryl (Wikström and Morgan 1992). Recently, the 607 nm form was characterized using resonance Raman but surprisingly gave a mode consistent with a ferryl intermediate (Proshlyakov *et al.*, 1994). Since only one group has been able to make this measurement so far, it requires further confirmation.

Many bacterial oxidases

Bacterial oxidases contain only three or four subunits, much simpler than those in eukaryotes, which contain up to thirteen (Capaldi 1990a; Capaldi 1990b). As discussed above, only three subunits appear to be intrinsically involved in the activity and thus the bacterial enzyme provides a good model system for studying the mechanism. The genes for most of these oxidases have been sequenced and comparisons among them that identify the conserved residues are offering insight into which residues are important. All of the oxidases so far discovered, except the *bd* oxidase in *E. coli.*, have homology to each other (Trumpower and Gennis 1994). Bacteria often contain multiple oxidases that differ in oxygen affinity and are induced under different conditions

Table 1.1 Variation in substrate specificity and heme type of the bacterial oxidases. The two columns categorize the oxidases by electron donor substrate. Rows indicate similar heme type, and show that the heme type does not relate to substrate specificity. Numbers in parenthesis listed after each bacterial type indicate references for that oxidase as follows: 1) (Hosler *et al.*, 1993; Trumpower and Gennis 1994) 2) (de Gier *et al.*, 1994) (van der Oost *et al.*, 1991) 3) (Mather *et al.*, 1993) 4) (Sone *et al.*, 1988) 5) (Sone *et al.*, 1994) 6) (Goldberg *et al.*, 1992) 7) (García-Horsman *et al.*, 1994b) 8) (Gray *et al.*, 1994) 9) (de Gier *et al.*, 1994) 10) (Preisig *et al.*, 1993) 11) (Lauraeus and Wikström 1993) 12) (Anemüller and Schäfer 1990) 13) (Matsushita *et al.*, 1990; Matsushita *et al.*, 1992) 14) (Trumpower and Gennis 1994) 15) (Ludwig 1992; de Gier *et al.*, 1994) 16) (Trumpower and Gennis 1994)

Table 1.1

Bacterial Oxidases

cytochrome *c* reductant*	quinol reductant
aa_3 *Rb. sphaeroides* (1) *P. denitrificans* (2)	aa_3 *B. subtilis* (11) *S. acidocaldarius* (12)
caa_3 *Thermus thermophilus* (3) *Bacillus PS3* (4) (also cao_3) (5)	
ba_3 *T. thermophilus* (6)	ba_3 (bo_3) *A. aceti* (13)
cbb_3 *Rb. sphaeroides* (7) *Rb. capsulatus* (8) *P. denitrificans* (9) *B. japonicum* (10)	bo_3 (bb_3) (oo_3) *E. coli* (14) – – – – – – – – – – – bb_3 (ba_3) *P. denitrificans* (15)
*bd*** *E. coli* (16)	

*all cytochrome *c* oxidases contain Cu_A except for the cbb_3
***bd* oxidase is not genetically related to the other oxidases

that may increase the chance of survival in variable environments (Rice and Hempfling 1978; Hosler *et al.*, 1992; García-Horsman *et al.*, 1994a).

Oxidases have been found to use either cytochrome *c* or quinol as substrate (See Table 1.1) A major difference even within these two categories is the type of hemes used with heme A or heme B almost always occurring in the low spin site, and heme A, B or O in the high spin site. The only known exception is that under overexpression conditions an *oo*3 with normal electron transfer activity can be produced in *E. coli* (Puustinen *et al.*, 1992). In general, variation of the heme does not appear to relate to any other characteristics of the oxidase. However, it has been suggested that high O_2 may be required for heme A synthesis (García-Horsman *et al.*, 1994a). Several of the thermophilic bacterial cytochrome *c* oxidases have the substrate, cytochrome *c* , covalently attached to subunit II (Mather *et al.*, 1993; Sone *et al.*, 1994). While almost all the cytochrome c oxidases have a Cu_A site, cytochrome *cbb*3 oxidase does not(García-Horsman *et al.*, 1994b; Gray *et al.*, 1994) . In this respect it resembles the quinol oxidases that lack the ligands for Cu_A. Thus in the quinol oxidases the electron transfer must go directly to heme *a* from oxidation of quinol. It is proposed that part of the quinone binding site occurs in subunit II of the *bo*3 (García-Horsman *et al.*, 1994a).

Cytochrome *bd* has no homology to the other oxidases. It contains two subunits, I and II, predicted to contain seven and eight transmembrane helices by hydropathy and gene fusions. It uses quinol as substrate and contains three metal centers, a low spin heme B, and two high spin sites, a second heme B, and the heme D. Initial electron transfer is into the low spin heme B while oxygen binding occurs to the heme D. The role of the second high spin heme D is suggested to correspond to that of Cu_B (Trumpower and Gennis 1994).

All the bacterial oxidases so far tested have shown proton pumping except cytochrome *bd* (Trumpower and Gennis 1994; van der Oost *et al.*, 1994). Although a model for proton pumping using ligand exchange around Cu_A had previously been proposed (Gelles *et al.*, 1987), it is clear from current results with bacterial enzymes that Cu_A is not required (Hosler *et al.*, 1993). While a different pumping mechanism might exist in the quinol oxidases that lack Cu_A or even in the multisubunit eukaryotic oxidases, the high degree of homology among these enzymes makes this unlikely (Trumpower and Gennis 1994).

Evolutionary relationships

Sequence comparisons among the oxidases has provided some predictions about evolutionary relationships (Saraste and Castresana 1994). A sequence alignment of the subunit I gene of *cbb*3 oxidase (FixN) shows 16% identity to the cytochrome *b* subunit of nitric oxide reductase (NorB) (van der Oost *et al.*, 1994). It is possible that the *cbb*3 oxidase and the NO reductase may have a common ancestor (van der Oost *et al.*, 1994). The *Paracoccus aa*3 oxidase subunit I gene (CtaDII) and the *E. coli* cytochrome *bo* oxidase subunit I gene (CyoB) show 35% identity to each other but only 11% identity to FixN and NorB (van der Oost *et al.*, 1994). Thus the *aa*3 and *bo* oxidases show a more distant relationship to the *cbb*3. The subunit I gene of *Rb. sphaeroides* (CoxI) that has high homology to the *Paracoccus aa*3 and to the *bo* oxidase of *E. coli* shows the greatest relationship to the bovine subunit I gene with 52% identity, providing evidence that the eukaryotic oxidases could have developed from these closely related bacterial oxidases (Hosler *et al.*, 1993).

The Cu_A domain in subunit II of some oxidases may have a common ancestor with the N_2O reductase since both show some similarity in the

region proposed to contain the Cu_A ligands (Buse and Steffens 1991; van der Oost *et al.*, 1991) and both have been shown to contain a binuclear copper (Kroneck *et al.*, 1989; Antholine *et al.*, 1992).. Because of the homology between the quinol oxidases and the cytochrome *c* oxidases it is proposed that the quinol oxidases have lost the Cu_A binding site (Castresana *et al.*, 1994; Saraste and Castresana 1994; van der Oost *et al.*, 1994). Further evidence of the close relationship between these two oxidases is given by the fact that the CuA ligands can be put into the quinol oxidase giving a binuclear copper with similar spectral characteristics as the native Cu_A domain (van der Oost *et al.*, 1992; Kelly *et al.*, 1993),

Mitochondria are proposed to have developed as organelles by prokaryotes being taken into an ancestral cell, setting up a symbiotic relationship (Gray 1989). Evidence for this comes from comparison of the sequences of ribosomal RNA genes from the nucleus, mitochondria, and bacteria. This analysis shows a closer relationship of the prokaryotes, especially *Rb. sphaeroides*, to mitochondria, than between mitochondria and the nucleus (Woese 1987). The nuclear encoded subunits of many of the respiratory complexes could have been added to the simpler bacterial core to allow greater regulation of the enzyme (Kadenbach *et al.*, 1991).

The close sequence, function, and spectral similarity between *Rb. sphaeroides* cytochrome aa_3 and the mitochondrial oxidase makes worthwhile the more time consuming processes involved in working with *Rb. sphaeroides* relative to *E. coli*. With *Rhodobacter* it is necessary to do site-directed mutagenesis in *E. coli* and then transfer the plasmid to *Rb. sphaeroides* by conjugation. It is also necessary to grow *Rb. sphaeroides* on a minimal media to prevent genetic rearrangement (personal communication: Timothy Donohue) which takes 2-3 days, compared to a single day on a rich media.

Although working with the oxidase from *Rb. sphaeroides* may not provide much information about the regulation of the eukaryotic enzyme, it does make it easier to define the details of the structure, the oxygen reduction chemistry, and the proton pumping reaction.

Chapter 2 – Finding amino acid residues important for proton pumping in cytochrome *c* oxidase

An interesting goal remains in bioenergetics, to determine the molecular mechanism by which cytochrome oxidases pump protons from the inside to the outside of bacteria or mitochondria. Three of the enzymes involved in the electron transport pathway contribute to the proton gradient, the NADH dehydrogenase (complex I), the *bc*1 complex (complex III), and the cytochrome oxidase (complex IV), while complex II, the succinate dehydrogenase, does not. Although it is known that the NADH dehydrogenase pumps protons, much less is known about its mechanism than for *bc*1 complex and cytochrome oxidase. While cytochrome oxidase and the *bc*1 have simpler enzymes in bacteria amenable to mutagenesis studies, the dehydrogenase still contains 14 subunits, making analysis more difficult (Weidner *et al.*, 1993; Yano *et al.*, 1994).

Difficulties in understanding proton pumping

Some problems exist in trying to better understand proton pumping by cytochrome oxidase at the molecular level. Since this enzyme is membrane bound, it has been difficult to get high quality crystals useful for x-ray diffraction data and the resulting structure which is critical to developing a clearly defined model. Recently, however, the 3-D crystal structure of the simpler bacterial oxidase from *Paracoccus denitrificans* has been obtained at 3 Å resolution (Michel personal communication), and a 2.8 Å resolution structure of the beef heart enzyme is at an early stage of development (Yoshikawa personal communication).

A second problem is that proton interaction sites and movements are more difficult to measure than those of other substrate molecules with enzymes, since protons are ubiquitous and difficult to detect. However, in bacteriorhodopsin it has been possible to monitor the protonation state of residues using FTIR difference spectroscopy (Rothschild 1992). While these experiments may be possible in cytochrome oxidase, they have not been done yet, and will probably be more difficult because of the larger size of oxidase relative to bacteriorhodopsin (125 kDa versus 27 kDa).

Proton pump versus Q loop

One way cytochrome oxidase differs from bc_1 is that oxidase does not contain soluble proton carriers. Instead the redox energy from electron transfer through the metal centers of the oxidase to the oxygen/water couple are used to drive the movement of protons through the protein and across the membrane, a proton pump mechanism (See Chapter 1 for a detailed discussion of the oxidase chemistry).

For the bc_1 complex, the mechanism involves what is referred to as a Q cycle in which the membrane soluble ubiquinol carries two electrons and two protons with the protons being picked up from the inside and released to the outside. This method of moving protons is more complex than it might appear. Bc_1 contains two B hemes, an Fe/sulphur cluster, a covalently bound cytochrome *c*, and a binding site for a soluble cytochrome *c*. Ubiquinone (Q) is reduced by either the NADH dehydrogenase or succinate dehydrogenase and picks up two protons from the matrix of mitochondria (or the cytoplasm of bacteria). Ubiquinol (QH_2) moves through the membrane to bind the bc_1 near the Fe/sulphur cluster towards the outside of the membrane (center P). Two protons are released and one electron travels through the Fe/S to the bound

cytochrome c_1 and the other electron travels through a high potential and then a low potential B heme where it reduces a temporarily bound ubiquinone (Q) to ubisemiquinone ($Q^{\cdot -}$) (center N). After the ubiquinone (Q) near the Fe/S exits the site, it will be one of many ubiquinones that is capable of binding near the low potential B heme site. Now a second ubiquinol (QH_2) binds near the Fe/S, releases its protons and transfers its electrons, one to cytochrome c_1 and the other through the B hemes. Thus the ubisemiquinone at the low potential B heme becomes fully reduced and protonated from the matrix to form ubiquinol (QH_2) and exits the site, becoming part of the ubiquinol pool which can move to the Fe/S site for that part of the reaction. The site where ubiquinol binds near the Fe/S is predicted to be in a hydrophobic environment, so that after electron transfer the protons must move through the protein to the outside of the mitochondria or bacteria (Beattie 1993). If the protons move through the protein, this part of the mechanism may be more like that of a proton pump. Electrons from cytochrome c_1 travel to the bound soluble cytochrome *c* which then departs from the bc_1 and carries the electrons to the cytochrome oxidase. Thus the net reaction becomes:

$$QH_2 + 2\ cytc_{ox} + 2H^+_{inside} \rightarrow Q + 2cytc_{red} + 4H^+_{outside}$$

This mechanism for bc_1 fits well with an aspect of Mitchell's model for the buildup of a proton gradient only using redox loops. Mitchell's chemiosmotic hypothesis proposed that it was the buildup of a proton gradient that could be used to drive the synthesis of ATP from ADP, instead of a direct chemical reaction that caused synthesis of ATP (Mitchell 1976). Another aspect of the original Mitchell hypothesis was that it was through such redox loops that electrons moved toward the negatively charged side of the membrane allowing protons to be picked up and moved to the positive side of the membrane (Mitchell 1976). Thus for cytochrome oxidase Mitchell proposed only the

uptake of substrate protons. Wikström showed otherwise with the discovery that oxidase actually pumped protons across the membrane and did not just consume them in the reduction of oxygen (Wikström 1977). Much controversy existed over this and many possible artifacts had to be ruled out before it was completely accepted that oxidase could pump protons (Mitchell *et al.*, 1985; Wikström and Casey 1985b).

Theoretical aspects of proton pumping

General requirements of a proton pump Most of the models for proton pumping are based on the concept that an entry path and an exit path for protons are required along with a directional driving force and some mechanism of gating, so that once protons are released toward the exit side a change occurs (gate closes) to prevent the protons from traveling back down the gradient (For an overview see (Gelles *et al.*, 1987)).

The simplest of these models shows four states, with two conformations and two protonation changes. A redox protein has two more states (reduced/oxidized) giving it eight possible states (2^3) which is referred to as the cubic model(Babcock and Wikström 1992). To have efficient pumping the protein can only change between certain states while others are generally disallowed, otherwise the proton pumping could become decoupled from electron transfer. Under certain conditions decoupling may occur, allowing slipping of the pump (Brand *et al.*, 1994).

General models for proton movement through proteins

Continuous chains of hydrogen-bonds as proton wires One of the early models for proton pumping was based on data from solid state chemistry in particular studies of proton transfers in ice (Nagle and Morowitz 1978). This model proposed a proton pump using a network of hydrogen-bonded serine

residues in a beta sheet structure. Two important concepts about the hydrogen-bond transfer of a proton between two residues were used. When a hydrogen bond is short, it is easier for a proton transfer to occur (hopping), but this makes it more difficult for the proton to reorient away from the bond (turning). In contrast when a hydrogen bond is long, it is more difficult for a proton transfer to occur, but it is easier to reorient the proton away from the bond.

For the model, an energy-driven conformational change in the protein causes a lengthening of the hydrogen bonds at the ends of the chain making it favorable for a reorientation of the protons to occur relative to the side chains with this propagating from the proton entry side to the proton exit side. As the protein relaxes back to the original conformation the hydrogen bonds at the ends of the chain are shortened making it more energetically favorable for the protons to be transferred. Thus a proton is pumped from one end of the chain to the other by sequential displacement of the side chain protons at which point the cycle can occur again. This theory was based on data from ice which showed this could occur at 10^5 per second. It was expected to be on a similar time scale in proteins, which with more thermal motion, would cause slower transfer of the hydrogens, but would also allow rapider proton reorientation. However, since a mixture of side chains in a protein instead of only serines might introduce irregularities, it was predicted to be slowed.

Proton movement through gramicidin Gramicidin is a small channel-forming antibiotic composed of an interwound dimer of peptides with alternating D and L amino acids. It is selective for the transport of protons and monovalent cations (Woolley and Wallace 1992). Proton conduction through a network of waters in gramicidin has shown maximal proton transfer rates of 10^9 per second at low pH (Akeson and Deamer 1991). This would be

slower at physiological pH with a lower concentration of protons. Calculations from this data to make a comparison to the CF_1F_0 ATP synthase (capable of 1200 H+/ channel^{-1}sec^{-1} during ATP synthesis (Althoff *et al.* 1989) indicate that at pH 5 the rate of proton transfer in gramicidin would drop off to 7 x 10^3/sec (Akeson and Deamer 1991). These authors also noted that under the conditions for the mitochondrial ATP synthase where proton uptake is from the cytosol at pH of 7.5 the rate would drop to 20/sec. The authors suggested three explanations for the low rate: 1) "a much wider channel mouth"; 2) "a much slower rate of ATP synthesis", or 3) $\Delta\psi$ driven hydrolysis of water to release protons into the entrance of the channel (Kasianowicz *et al.*, 1987).

It is interesting that Akeson and Deamer concluded that a pure water channel like that in gramicidin could not exist in CF_0. This is because the gramicidin water channel is not selective for protons and can easily transfer Na^+ and K^+ (although at a much slower rate) while the CF_0 does not even allow these ions to enter the channel (Lill *et al.*, 1987). It was concluded that this could explain why amino acid residues would be required during proton transfer: to hold the waters in place so that alkali ions could not push the waters through the channel as occurs in gramicidin (Levitt 1984), and to be selective for proton binding (NH_2 groups have a lower affinity for alkali cations than for protons (Williams 1988)).

Integral injector model as a driving force Other theoretical models have been proposed with one of the simpler ones to understand being the integral injector model (Nagle and Tristram-Nagle 1984). This model has a residue in the middle of the chain which is the driving force, or injector, for the pump, making it easier to visualize how this might be applied to bacteriorhodopsin, or oxidase. This residue releases a proton into a hydrogen bonded network pushing the other protons in the network along until one exits

into the solvent. Next a conformational change occurs with the injector residue turning 180^0 towards a hydrogen bonded network facing the uptake side of the membrane, making reprotonation from the exit side unfavorable. Reprotonation of the injector causes the protons to pass down the chain, with reprotonation finally occurring from the solvent. Immediate rotation of the injector towards the exit path would make protonation from the uptake path unfavorable, and allow the continuation of the cycle. This model can be adapted so that a redox change (oxidase), or light induced conformational changes (bacteriorhodopsin) could be the driving force of the injector's conformational change. Studies from ice predict that the injection of the proton toward the exit side would be faster than the uptake of the proton, making it kinetically favorable to have the injector nearer the uptake side of the membrane (Nagle and Tristram-Nagle 1984). This does not appear to be the case in bacteriorhodopsin where the retinal is in the middle of the membrane (Henderson *et al.*, 1990). Similarly oxidase models predict that the binuclear center sits towards the outside of the membrane (Hosler *et al.*, 1993), although exiting protons may travel through more protein on this side if as is predicted subunit II lies on top of the membrane (Hosler *et al.* 1994a; Hosler *et al.*, 1994b). (This also appears to be the case in Michel's recent crystal structure of oxidase.)

Other enzymes that transfer protons

To study the proton pumping mechanism, it is useful to look at what is known about proton transfers over a distance in other enzymes where some structural information is available. The most detailed information on proton transfer has been determined for bacteriorhodopsin (Krebs and Khorana 1993) and reaction center (Ermler *et al.* 1994b) that have crystal structures, and

have been characterized using site-directed mutagenesis. A crystal structure exists for the soluble portion of the enzyme F_1F_0 ATP synthase, the F_1 part that contains the binding site of ATP/ADP (Abrahams *et al.*, 1994). While no structure has been resolved for the F_0 which contains the proton pore, an NMR structure has been determined on subunit c of the F_0 (Girvin *et al.* 1995; Girvin *et al.* 1993). Also site-directed mutagenesis results have given some insights into the structure/function relationships in the F_0 part (Fillingame 1992). ATP synthase and bacteriorhodopsin are similar to oxidase in that they are capable of pumping protons, with the first using the ATP to ADP conversion as the driving force for the reaction. Bacteriorhodopsin uses a light-driven conformational change to drive the pumping. While reaction center is not a proton pump, it drives the uptake of protons from the aqueous media through the protein in response to the arrival of an electron at the quinone sites. Part of the proton uptake in the oxidase reaction is similar to that of reaction center in that the electrons reducing the oxygen drive the uptake of protons to convert O_2 to $2H_2O$. For the pumping process, the driving force and gating in oxidase may be more similar to that of bacteriorhodopsin and ATPase, involving an energy driven conformational change, in this case possibly coming from the changing redox state of the metal centers and the oxygen intermediates.

Reaction center While reaction center is not a true proton pump in that it does not move protons across a membrane, it does use a change in potential at the quinone site to drive the movement of protons through the protein. Reaction center from *R. viridis* was the first membrane protein for which a high resolution x-ray crystal structure was determined (Deisenhofer *et al.*, 1984; Deisenhofer *et al.*, 1985). It has also been crystallized from *Rhodobacter sphaeroides* (Allen *et al.*, 1988; Chang *et al.*, 1991). Recent

refinements of both structures have allowed better predictions about the location of bound waters (Ermler *et al.*, 1994a; Deisenhofer *et al.*, 1995). These structures have allowed more accurate thinking about the mechanism and testing of hypotheses by site-directed mutagenesis. Ten cofactors exist in the four subunit protein including four chlorophylls, two pheophytons, and two quinones, with these eight cofactors bound symmetrically in the L and M subunits. A carotenoid is bound asymmetrically near the chlorophylls and an iron is located between the quinones. Excitation of two of the chlorophylls (the special pair) causes an electron to travel through one set of the symmetrically positioned cofactors, referred to as the A branch. This electron rapidly travels through another chlorophyll in the A branch (Kirmaier and Holten 1993; Zinth and Kaiser 1993). The electron passes through a pheophyton, and to the quinone (Q_A), and then through the iron (removal of the iron by mutagenesis of its ligands shows only some inhibition of the activity, (Williams *et al.*, 1991)) to the other quinone (Q_B). A second electron follows the same path to fully reduce Q_B. It is not known whether Q_B picks up the first proton before or after the second electron transfer (For a review of the electron and proton transfers see Okamura and Feher 1992)).

Analysis of the crystal structure from *Rb. sphaeroides* has shown two chains of residues which could have a role in proton transfer to Q_B (Allen *et al.*, 1988). These residues include five arginines, four aspartic acids, a glutamate, four histidines, a serine, two threonines, and a tyrosine. Refinement of the crystal structure from *Rb. sphaeroides* has resolved a chain of fourteen waters connecting Q_B to the solvent outside of the protein, while the chain from *Rps. viridis* is significantly shortened and interrupted by a glutamic acid (Ermler *et al.*, 1994a).

Site-directed mutagenesis studies have shown several residues near Q_B to be required for proton transfer: GluL212, SerL223, and AspL213 (See Figure 2.1; L refers to the subunit) (Okamura and Feher 1992). Mutants of all three show greater than a 90% inhibition in turnover. Studies predict SerL223 to be involved in transferring the first proton while GluL212 seems to be necessary for the transfer of the second proton. AspL213 appears to be necessary for transfer of the first proton, and some evidence exists for its involvement in transfer of the second proton (Paddock *et al.*, 1994). Figure 2.1 shows how the AspL213 could be involved in either pathway of proton transfer. Further evidence of a role for GluL212 and AspL213 in proton transfer was provided by experiments in which the addition of high concentrations of weak acids increased the electron transfer rate of by presumably by improving proton access to Q_B (Takahashi and Wraight 1991). Mutating the amino acids implicated in the proton transfer to others with similar side chains did not affect activity: GluL212→Asp (Paddock *et al.*, 1990a) and SerL223→Thr (Paddock *et al.*, 1990b). It is interesting that mutation of some residues in the active site caused only a 50% inhibition in turnover, ruling out an absolute requirement of these residues for proton transfer: ArgL217, AspL210, and HisL190 (Okamura and Feher 1992).

The studies of reaction center seem to indicate an important role for hydrophilic residues in proton transfer, but further mutagenesis studies need to be done especially with the recent predictions of a pathway of waters in the *Rb. sphaeroides* structure (Ermler *et al.*, 1994). This should help determine if it is the waters that are critical in proton transfer, and if the mutants that affect the proton uptake are disrupting the path of the waters.

Figure 2.1 Model of the Q_B site in reaction center adapted from Okamura and Feher (1992). This shows the predicted path of the two protons transferred to Q_B driven by electron transfer to the cofactor. Boxed residues indicate those residues predicted to participate in the two paths based on studies of site-directed mutants at those sites. Water molecules are also predicted to be part of the second path.

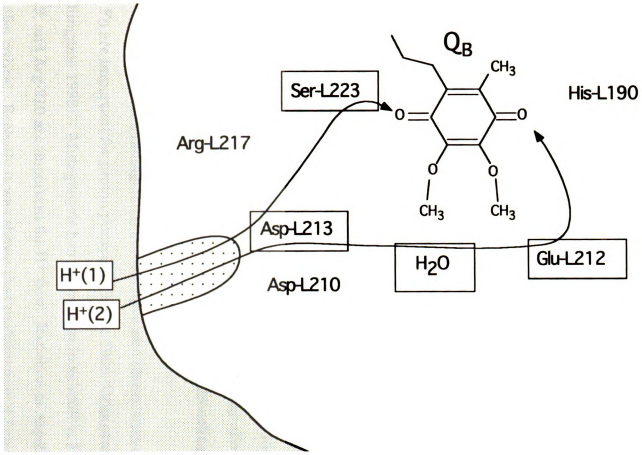

Q_B
CH_3
Ser-L223
His-L190
O
O
Arg-L217
O
O
CH_3
CH_3
Asp-L213
H_2O
Glu-L212
$H^+(1)$
Asp-L210
$H^+(2)$

Figure 2.1

F_1F_0 ATP synthase The ATP synthase is composed of two components: an F_0 (composed of $\alpha 3\beta 3\gamma\delta\varepsilon$ subunits) that is membrane embedded and through which protons flow towards the F_1; and the F_1 ($a_1b_2c_{10}$) which protrudes into the interior of bacteria or matrix of the mitochondria and contains the site for the conversion of ADP to ATP (Fillingame 1992). Under physiological conditions the proton gradient established by the electron transport chain drives the conversion of ADP to ATP, but the reaction can be run in reverse so that the enzyme functions as a proton pump. It is an important current goal in studying this enzyme to develop a detailed mechanism of the coupling between the proton flow and its conversion into the chemical energy of ATP. Experiments have shown there to be a single site for the nucleotide conversion on each of the three β subunits, and that at any one time each site is in a different stage of the catalysis (For a review see Pedersen and Amzel 1993). Recently, the F1 portion has been characterized crystallographically to atomic resolution. This has shown that the F_1 has 3 pairs of alternating α and β subunits organized in a circle with the γ subunit in the middle. It appears that the contact between the γ subunit and the surrounding α and β pairs is very hydrophobic and may allow rotation of the outer part around γ and δ which make up a stalk connecting to the F_0 (Eytan 1982; Abrahams *et al.*, 1994).

Site-directed mutagenesis studies have also shown that certain residues in F_0 are important for proton pumping (Senior 1988; Fillingame 1990; Fillingame 1992). Mutagenesis has shown that in subunit a, His-245, Glu-219, and Arg-210 are important for H^+ flow. Mutation of Asp-61 in subunit c is also critical. Recently it was shown that modification of Asp-61 in a single c subunit by DCCD could stop the proton flow (Hermolin and Fillingame 1989).

An NMR structure for subunit c shows it to be made of two antiparallel helices (Girvin *et al.* 1993). A previous study showed that a double mutant of Asp61Gly/Ala24Asp retains functionality (Miller *et al.* 1990). A recent more detailed NMR structure of purified subunit c agrees with this result showing that the Asp61 and Ala24 are in opposing helices, but are adjacent to each other (Girvin et al. 1995).

An FTIR study on the reconstituted F_0 showed a continuum from about 3000 to 1800 cm-1 indicating the presence of a chain of hydrogen-bonding residues with high proton polarizability (Bartl *et al.*, 1995). This continuum disappeared when the F_0 was either modified with DCCD or dehydrated showing the disappearance of the hydrogen-bonded network. Based on these results and the mutagenesis studies of other groups and the conservation of other residues, the following network was proposed: the carboxyl of Ala-79 c, Tyr-10 c, Glu-219 a, His-245 a, Asp-61 c, Lys-34 c, Arg-210 a, Arg-41 c, and possibly intervening waters. Mutagenesis studies on any residues not previously examined can test this hypothesis. It would also seem useful to apply the FTIR technique to mutants believed to be blocked in proton transfer to see if the mutation is having an effect on the hydrogen-bonded network or through some other structural effect on the enzyme.

Bacteriorhodopsin Bacteriorhodopsin has been highly characterized as to the mechanism of proton pumping. Its three dimensional structure has been determined from two dimensional crystalline arrays by electron diffraction and was found to confirm previous structural predictions based on site-directed mutagenesis in combination with biophysical studies (Henderson *et al.*, 1990). More detailed information is known about its mechanism of proton transfer than for the enzymes discussed above, and it has provided a model for consideration during this research on cytochrome oxidase. In the

case of bacteriorhodopsin, light induces the conformational change of all trans to 13-cis retinal which serves as the driving force for the pump.

Although no high resolution structure of bacteriorhodopsin existed until recently, a low resolution structure and biochemical data predicted the relative orientation of 7 transmembrane helices (Henderson and Unwin 1975; Khorana 1988). Khorana's group has extensively mutated amino acids which have side chains that could be involved in transferring protons to unprotonatable residues (Krebs and Khorana 1993). The mutants were assayed to see if light driven proton pumping was blocked. Two residues, Asp85 and Asp96 were absolutely required for proton pumping, while Asp212 had a significant effect (Mogi *et al.*, 1988). Although mutation of Tyr185 does not inhibit proton pumping, it slows the conversion from the O state to the bR state and may interact with Asp212 (Rothschild 1992). While it was concluded that mutation of individual serines and threonines did not have any dramatic affect on proton pumping, changing several of these residues, including Thr46 and Thr89, caused a reduction in the efficiency of pumping (Marti *et al.*, 1991; Verkhovskaya *et al.*, 1992). (These are included in a hydrogen-bonded network for a model suggested by Rothschild. See below.). Mutation of Arg82 slows proton pumping (Stern and Khorana 1989) and appears to be important for exit of the pumped protons (Otto *et al.*, 1990).

Finally, the advent of the higher resolution structure from 2D crystals showed that many of the conclusions already made were accurate and allowed more detailed predictions to be made (Henderson *et al.*, 1990). It also allowed the definition of two channels: a smaller, hydrophobic one containing Asp96 leading to the retinal, and a larger, hydrophilic one leading away from the retinal. It is predicted that water molecules could fill these channels, although the waters are not visible at this resolution. It is further predicted that the

hydrophilic residues and the waters could be involved in a hydrogen-bonded network similar to that suggested by Nagle and Morowitz (1978).

With the crystal structure and further analysis of the mutants, a more detailed mechanism has been proposed by Rothschild to account for protonation changes seen during the FTIR difference spectra along with kinetic measurements of proton uptake (Rothschild 1992). Figure 2.2 shows an overview of the photocycle, and Figure 2.3 shows the position of the residues that are involved in proton pumping. Initially in the state bR_{570} the protonated positively charged Schiff base formed by the attachment of the retinal to Lys 216 is stabilized by other negatively charged residues: Asp-85, Arg-82 and Asp-212. Light causes the conformational change of the retinal which goes from all-trans to the cis configuration at C-13 resulting in the change in state from bR_{570} to K_{630}. This disrupts an interaction of the positively charged Schiff base with the negatively charged residues, and disrupts the network of residues that were dissipating the charge of the Asp-212. In the L_{550} to M_{412} transition, a proton is transferred from the Schiff base to Asp-85 on the exit side of the proton pathway which allows a hydrogen bonded network to form from the proton uptake side through Asp-96, Thr-46, a water, Thr-89, a water, Tyr-185, Asp-212, to the Schiff base (see Figure 2.3). Protonation of the Asp-85 releases its interaction with Arg-82, so that a water will release a proton to the exterior leaving a hydroxyl to stabilize the Arg-82. From M_{412} to N_{550}, the proton on Asp-96 moves forward into the hydrogen-bonded network with each proton shifting by one residue so that the final proton reprotonates the Schiff base. From N_{550} to O_{640}, reprotonation of the Asp-96 occurs and the retinal converts from the 13-cis back to the all trans

Figure 2.2 Photocycle of bacteriorhodopsin. Each of the intermediates is designated by a letter name and the subscripted absorption maxima in nanometers. Light causes the conformational change from bR_{568} to K_{625}. This drives the pumping of a proton in going from L_{550} to M_{412}, and the uptake of a proton in going from N_{520} to O_{640}.

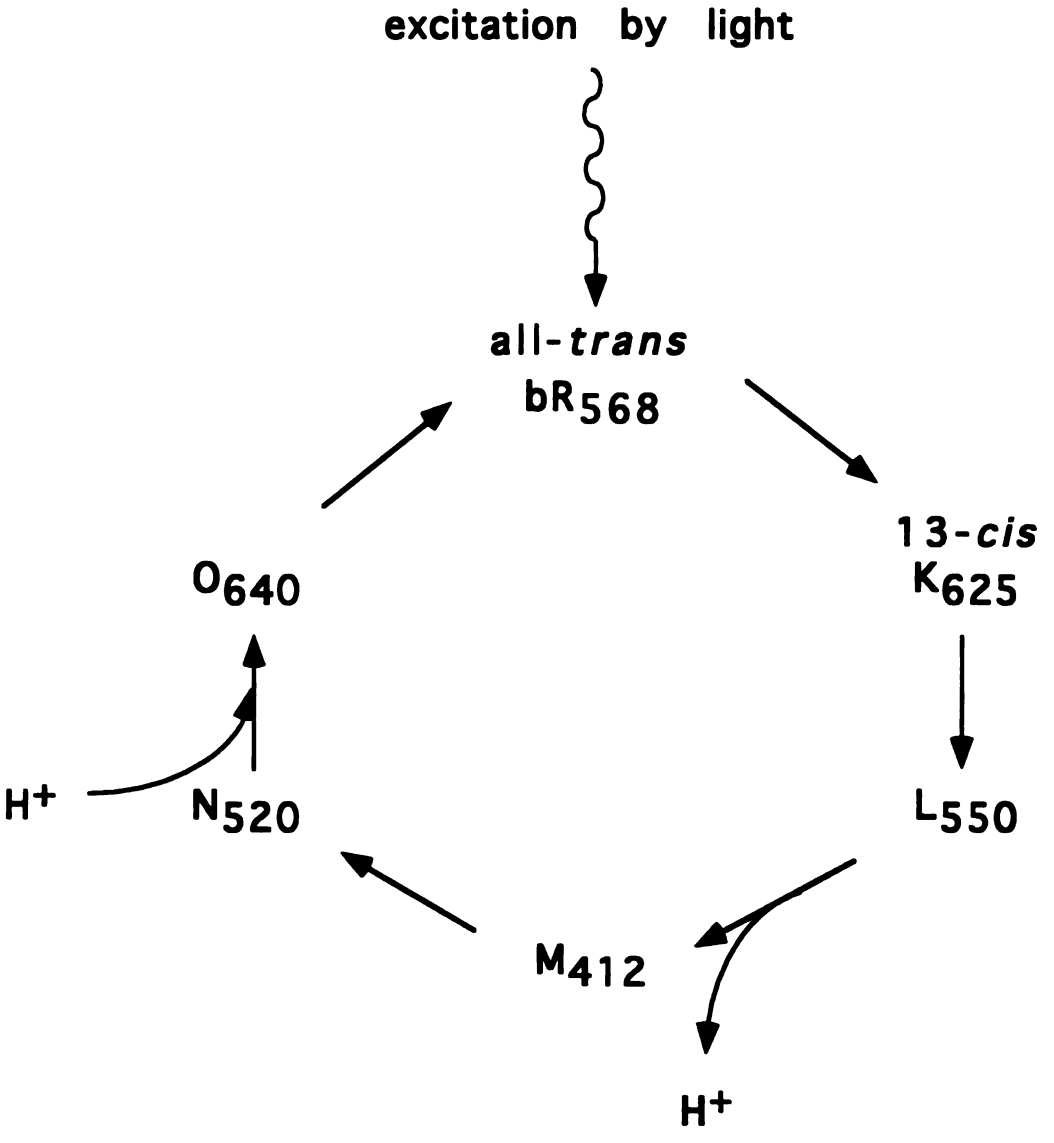

excitation by light
all-trans
bR568
13-cis
K625
L550
M412
H+
N520
O640

Figure 2.2

Figure 2.3 Model of the predicted hydrogen-bonded network that develops in bacteriorhodopsin during the M_{412} intermediate (Rothschild 1992). This path indicates that the protons are in a rapid equilibrium along this network like that shown recently using FTIR (Olejnik *et al.*, 1992). At the same time the negative charge of the oxygen would be dispersed throughout the network. The large arrow indicates the proton transfer predicted to occur from Asp212 to the Schiff base in the transition from M_{412} to N_{550}. Simultaneously with this transfer, a proton at the other end of the network from Asp96 would be removed.

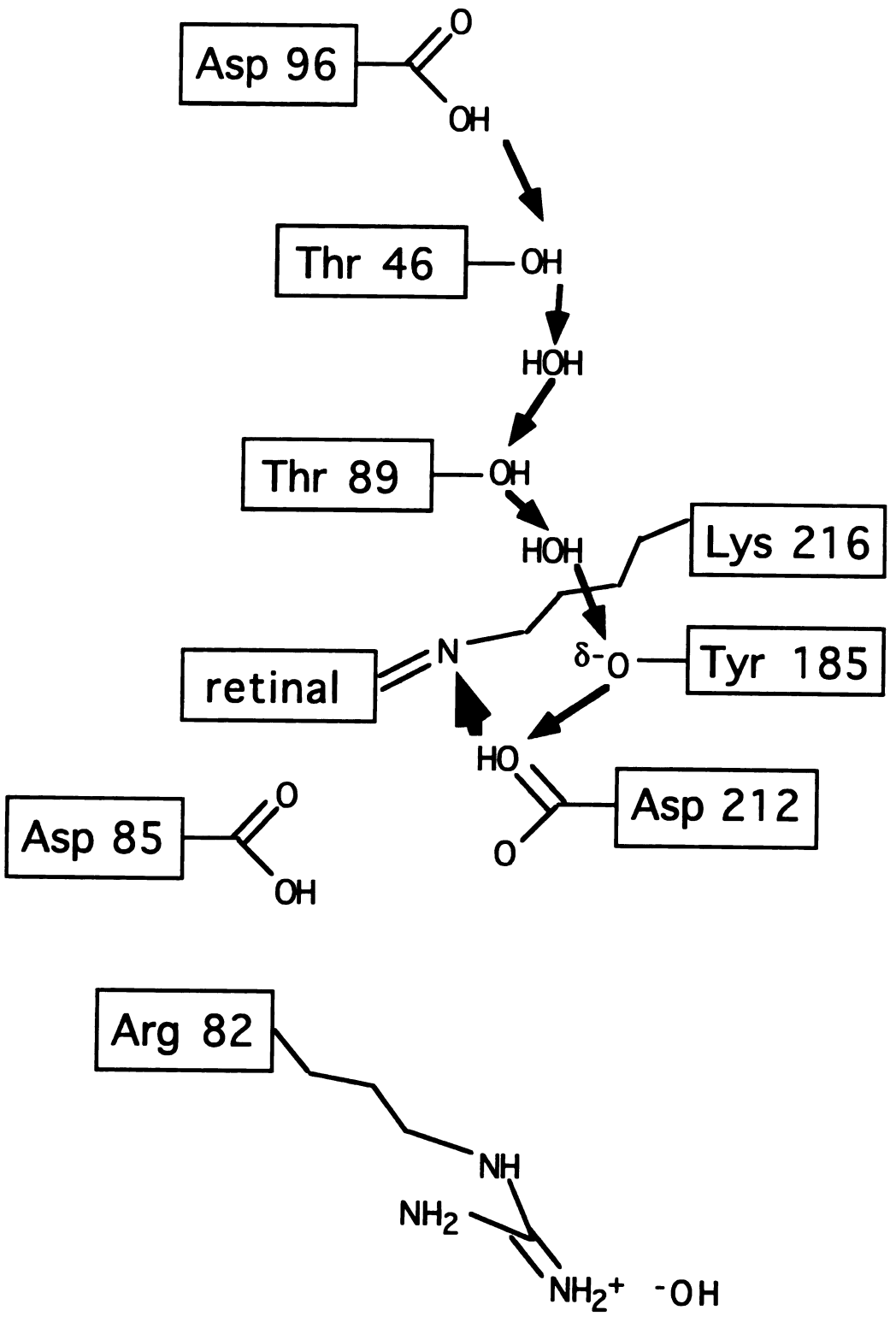

Asp 96
O
OH
Thr 46
OH
HOH
Thr 89
OH
HOH
Lys 216
retinal
N
δ-O
Tyr 185
HO
Asp 212
O
Asp 85
O
OH
Arg 82
NH
NH2
NH2+
-OH

Figure 2.3

configuration. Finally, from O_{640} to bR570, Asp-85 releases a proton to the hydroxyl that is interacting with Arg-82. This frees the Arg-82 to again interact with the other residues surrounding the Schiff base.

No direct structural evidence for the hydrogen-bonded network was available when this model was proposed. The hydrogen-bonded networks were proposed based on theory, model systems such as gramicidin (both were discussed above), and FTIR experiments of Zundel showing the possibility of the formation of hydrogen-bonded networks in which a proton can have high polarizability allowing for extremely fast proton movement (picoseconds) (Zundel 1986; Zundel 1988). Recently FTIR difference spectra were taken of bacteriorhodopsin to compare K_{630}, L_{550}, and M_{412} to bR_{570} (Olejnik *et al.*, 1992; Zundel 1994). This showed that in fact a continuum develops in the FTIR with formation of the L_{550} intermediate indicating a hydrogen-bonded network, and that in M_{412}, the network is broken but that some strong hydrogen bonds still exist. This would seem to require a modification to the model proposed by Rothschild in which the network would form during the M_{412} and Asp-96 is deprotonated during L_{550} (Rothschild 1992). However Asp-96 does not deprotonate under low temperature experiments like that used to show the FTIR continuum, but only at room temperature (Braiman *et al.*, 1991). Thus the hydrogen-bonded network may form earlier, during L_{550}, at low temperature, but later, during M_{412} at room temperature.

Models which have been proposed for proton pumping in cytochrome *c* oxidase

Since the discovery that cytochrome *c* oxidase could act as a proton pump, many creative models for proton pumping have been envisioned. Some of these appear less likely considering what is now known about the oxidase. A

model was proposed for using the change in redox state at heme *a* to change the strength of a hydrogen bond to the heme formyl group (Babcock and Callahan 1983). This model would not be possible in all oxidases considering that it is now known that heme B, which has no formyl group, can participate in the low spin site (García-Horsman *et al.*, 1994a)

Woodruff ligand shuttle model A model for proton pumping has recently been proposed using what is referred to as a ligand shuttle in which a protonatable residue could switch between Cu_B and the distal side of heme a_3, causing displacement of the proximal histidine ligand (Casey 1986; Woodruff 1993). Upon association of the distal ligand with heme a3, it would lose a proton to be pumped. The distal ligand would then be displaced from the heme and the proximal histidine would rebind. The model then requires reprotonation of the distal ligand by the proximal histidine, after which the distal residue would move back to the copper, and reprotonation of the proximal histidine would occur. The loss of the proximal ligand under some conditions offers an interesting mechanism for control of electron transfer rates between hemes a and a3, since detachment of the proximal histidine would force a through space jump significantly slower than the through bond transfer when the ligand is attached. However, the model presents some difficulties as a proton pumping mechanism. One difficulty is imagining a proton path through or around the heme. It also seems that the protons would be pumped only in the binding and exchange of oxygen between Cu_B and heme a_3. Once the oxygen reduction begins at a_3 it would be less likely for ligand exchange to occur, although not impossible if one of the intermediates moved to the Cu_B. With the use of a histidine to carry two protons at a time, a two proton exchange occurring once during the oxygen binding and a second time during the chemistry might account for the stoichiometry of $1H/e^-$ of pumped

protons. This would still require an explanation for why the pumped protons are only seen during the final intermediates of the oxygen chemistry (Wikström 1989).

Rousseau model requiring proximal ligand exchange of a tyrosine Another model recently proposed involves that of ligand exchange between the proximal histidine and a proximal tyrosine based on the known selectivity of the ferric form of hemoglobin for tyrosinate over histidine (Rousseau *et al.*, 1993). Both residues would transfer one proton each with two exchanges of the tyrosinate for the histidine occurring at the ferric and ferryl steps in the chemistry. This model has recently been tested by the mutation of the Tyr422 which is in a possible location for this exchange. Mutants at this residue were still capable of pumping protons (See chapter 4).

Chan model of ligand exchange at CuA or CuB A very complete model for pumping was proposed based on changes in the ligands around Cu_A (Gelles *et al.*, 1987; Chan and Li 1990). This model would not be possible in the quinol oxidases or the *cbb*3 oxidase which do not contain Cu_A but are able to pump protons, although a version of this model applied to the Cu_B site would still be possible (Larsen *et al.*, 1992). In the model for Cu_B, reduction of Cu^{2+} to Cu^{1+} would cause a ligand exchange, which would cause a change from a strained square planar to a tetrahedral configuration, and the movement of a proton from the incoming ligand to the departing ligand. Next, as the electron left the Cu, the site would convert back to a Cu^{2+} acting as the gate, making it electrostatically unfavorable for the proton to reverse its movement. Finally, the proton would be released to the side of the forming gradient and the ligands could exchange back to their original location for another cycle. Larsen *et al.* proposed that their model could only pump 0.75 H+/e- and only 0.5 during the peroxy to ferryl and ferryl to hydroxy transitions when pumping is believed

to occur (Wikström 1989), making it seem unlikely as the only pumping mechanism, but the authors concluded that it could be part of the pump along with Cu_A.

Indirect coupling of electron transfer and proton pumping combined with a peroxide pump Other models have been proposed involving redox Bohr effects in which the pumping at a site away from the electron transfers could be coupled through the protein (Chance *et al.* 1977; Brzezinski and Malmström 1986). These models are more complicated to study making it more difficult for them to be ruled out or confirmed. Other models have been proposed involving hydroxide and water as ligands at the binuclear center which might be involved in pumping protons (Mitchell 1987; Mitchell 1988).

A model using peroxide similar to Mitchell's model and proposing some inclusion of redox Bohr effects has recently been proposed (Papa *et al.*, 1994). In this model oxygen would bind at Cu_B and be temporarily converted to a peroxide, after which the protons from the peroxide would be pumped across the membrane by a Bohr effect due to redox change at heme *a* leaving a bound oxygen. Then this oxygen would bind to the heme a3 where it would begin going through its characteristic reduction. A second oxygen could also bind to the Cu_B and again be involved in pumping two more protons before it was released. Although the chemical basis for this mechanism is questionable, the lack of ability to monitor Cu_B during the oxygen chemistry makes it difficult at the present time to completely rule out a mechanism such as this. As noted by the author, it is interesting that there are believed to be Cu^{2+}-peroxide complexes that form during the chemistry of copper monooxygenases (Klinman and Brenner 1988). One difficulty with this mechanism might be

making it energetically favorable for the protons to be pumped from the peroxide intermediate once it is formed (Morgan *et al.*, 1994).

Histidine Cycle One of the most well-developed models recently proposed uses His284, a Cu_B ligand, as a key residue in the proton pump (See Figure 2.4) (Morgan *et al.*, 1994; Wikström *et al.*, 1994). Their idea to use a histidine is based on evidence showing that the *cbb*3 oxidase can pump protons (Raitio and Wikström 1994). If this is the case, then considering that very few residues besides the histidines are conserved in this distantly related oxidase, it would appear necessary to use them for pumping, assuming a conserved pumping mechanism. The lack of conserved residues in the *cbb*3 also tends to rule out mechanisms where the pump is coupled at a distance from the site of electron transfers and oxygen chemistry, since such linkage would likely require other conserved residues. They discuss five postulates for the model summarized as follows : 1) Histidine can carry two protons to keep the $2H^+/1e^-$ stoichiometry (Wikström 1989). 2) Oxygen intermediates have a role but do not carry the pumped protons. 3) Imidazolium ($ImH2^+$) acts to increase the redox potential of the peroxy and ferryl intermediates, thus promoting electron transfer. 4) Substrate protons bind to oxygen during intermediate formation, and thus promote the release of the pumped protons from the histidine. 5) The protons which bind the oxygen to form water and those that are pumped involve separate and independent pathways.

Figure 2.4 shows a summarized version of the model. Notice the alternating access of the proton paths allowed by the oxygen chemistry: (1) pumped → (2) substrate → (3) pumped → (4) substrate. Pumped protons bind to the imidazolate (Im^-) when oxygen binds and forms the peroxy intermediate (1). The imidazolium (ImH_2^+) stabilizes and is stabilized by the oxygen

Figure 2.4 Condensed version of the histidine cycle showing the changes that would occur at the binuclear center to drive proton pumping. For the complete chemistry see Morgan *et al.* (1994). The pathway on the left is for substrate protons and the one on the right is for pumped protons. The diagram does not indicate when the protons enter from outside the oxidase since presumably a hydrogen-bonded network exists, but indicates when the protons act at the active site. In #1, the sequence of entry into or at least near the active site is electron, proton , electron, proton, oxygen binding, electron, proton, with one of those protons not shown, but located in a position to act as a substrate proton in #2 (This is done to keep the neutrality of the site.). Two substrate protons are required at two steps in going from #2 to #3, and again in going from #4 to #1.

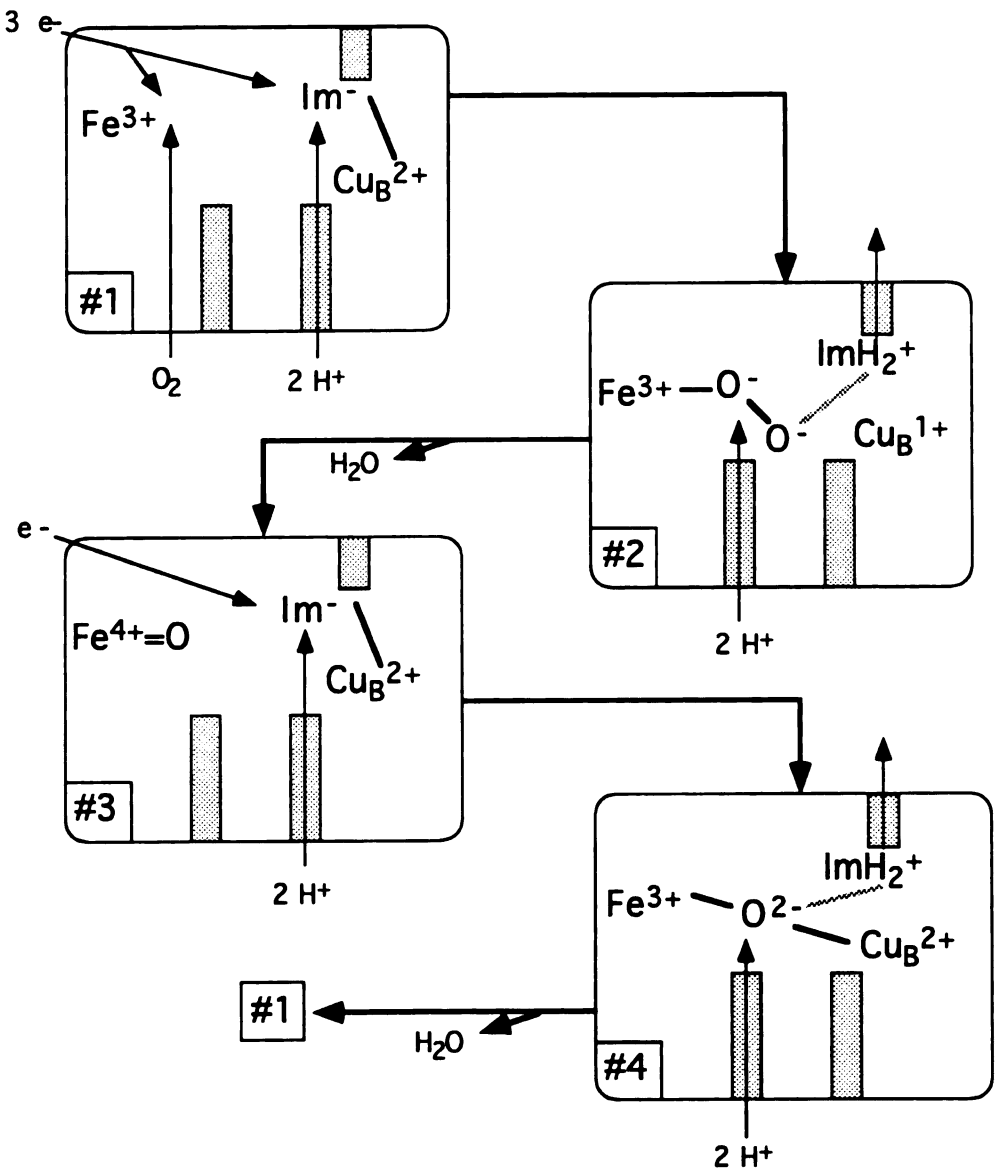

3 e-
Fe3+
Im-
CuB2+
#1
O2
2 H+
ImH2+
Fe3+ —O-
O-
CuB1+
#2
2 H+
H2O
e -
Fe4+=O
Im-
CuB2+
#3
2 H+
ImH2+
Fe3+ — O2-
CuB2+
#1
H2O
#4
2 H+

Figure 2.4

intermediate (2), increasing the redox potential, and allowing the electrons to convert the peroxy (2) to the ferryl (3) while simultaneously the substrate protons are taken up causing the release of the imidazolium (ImH_2^+) protons to the exit path. This same general mechanism works again in (3) to (4) when the ferryl is converted to a hydroxy intermediate again stabilizing ImH_2^+; with the uptake of substrate protons the final two protons are pumped. It is a complete mechanism that thoroughly takes into account the data that is known about the oxidase chemistry. The challenge is in trying to test this hypothesis since modification of any of the histidines that are involved results in an inactive oxidase (Hosler *et al.*, 1993). The main difficulty would seem to be the intermediates (2) and (4) in which the doubly protonated histidine interacts with the negatively charged peroxide without protonating it. It would be useful to find model compounds or to do theoretical calculations that could provide an explanation for how this could occur.

Conclusions

The data from the various enzymes provides some information about the flow of protons in proteins. Evidence is increasing for some type of hydrogen-bonded network, but is not conclusive. One problem is the idea of having a network seems to imply that a break anywhere in the chain, perhaps caused by a single mutation would disrupt this network. That does not seem to be the case in bacteriorhodopsin in that very few residues have a significant effect on proton pumping. It has been pointed out that waters tend to order well in hydrophobic environments (Zundel and Brzezinski 1992). Perhaps when some residues that are important in transferring protons are mutated, a water is capable of replacing the residue. Of course one way to address this would be to have higher quality crystal structures of bacteriorhodopsin. Reaction center

may be useful in this regard in that already various mutant crystal structures have been determined, although they are not to a refinement that allows the prediction of water sites (Chirino *et al.*, 1994). If mutants could be made to the recently seen path in the *Rb. sphaeroides* structure and high resolution structures determined, it would help to answer this question. It would also be useful to have greater application of the experimental FTIR technique developed by Zundel to show hydrogen-bonded networks in proteins. It will be especially interesting to see if networks visualized by this method can be disrupted by mutations. Our group is planning to test this on some of the *Rb sphaeroides* mutants.

As to the driving force and gating mechanism for the pumping enzymes, this seems to be most clearly worked out in the case of bacteriorhodopsin. In the case of the cytochrome oxidases this is still a challenging question. Many models have been proposed and some have been ruled out based on the result that the relatively unconserved *cbb*3 oxidase pumps protons and the evidence that pumping is associated with the final two electron transfers to the oxygen intermediates. Since these two results appear to rule out many of proton pumping models, it would be useful to have verification of these two findings by different techniques or systems. Presently, however, the histidine ligand exchange at Cu_B proposed by Wikström provides the best fit for the existing data. It will be challenging to develop experiments to test this model.

Finding residues in oxidase involved in proton pumping

Similar to the research on bacteriorhodopsin, the search for proton pumping residues in cytochrome oxidase has followed a strategy of mutating amino acid residues with side chains that could be involved in a hydrogen-bonded network. A big advantage, however, has been the more than 80 species

for which the oxidase genes have been sequenced. Thus the comparison of these showed residues which were both highly conserved and with side chains that could be involved in proton transfer (See Figure 2.5). While other conserved residues could play a role by hydrogen bonding to the carbonyl or amide groups, the involvement of these in stabilizing the helical structure makes this less likely. Also considered were the possibility of the involvement of prolines which, through providing flexibility to the helix or their capability of cis/trans isomerization, might have a role in a proton relay system (Brandl and Deber 1986). The focus is presently on subunit I since it contains the binuclear center where the largest energy release occurs during reduction of oxygen and where direct coupling could be most readily accomplished. Since this reaction occurs at the heme a3/Cu_B center, most of the focus is near this site. Subunit II could play a role through an indirect coupling mechanism or as part of the proton exit pathway.

The focus of this study was on three of the most highly conserved regions in subunit I, and a tyrosine on the proximal side of heme a3. On the proton loading side of the enzyme in a loop between helices II and III, a conserved aspartate (Asp132) was mutated. In the amphipathic transmembrane helix VIII, several residues were mutated, Thr352, Pro358, Thr359, Lys362. On the proton exit side of the enzyme in the loop between helices IX and X, another set of conserved residues were mutated, His411, Asp412, Thr413, and Tyr414. In helix X, which provides the ligands for both heme *a* and *a*3, Tyr422 was mutated to test a model for proton pumping recently proposed (Rousseau *et al.*, 1993). These mutants were then

characterized as to their visible absorbance spectra, carbon monoxide binding to heme a3, steady state electron transfer activity, proton pumping activity, and resonance Raman spectra to provide predictions about their role in the oxidase pumping mechanism.

Figure 2.5 Topological model of subunit I of the *aa*3-type oxidase from *Rb. sphaeroides*. It is based on hydropathy analysis and the phoA/lacZ fusion analysis done in the homologous cytochrome *bo* from *E. coli* (Chepuri and Gennis 1990). Only protonatable residues that might play a role in proton pumping through their side chain are indicated. Those residues that are totally or almost totally conserved (>90%) from more than 80 species of oxidase are indicated by squares. Residues that are highly conserved (>80%), but less so than the first set are indicated by circles. The six histidines that act as the ligands to heme *a*, heme *a*3, and Cu_B are indicated by hexagons. Residues that were studied for this thesis are indicated in bold. These are located in the II-II loop, helix VIII, the IX-X loop, and the Tyr422 in helix X.

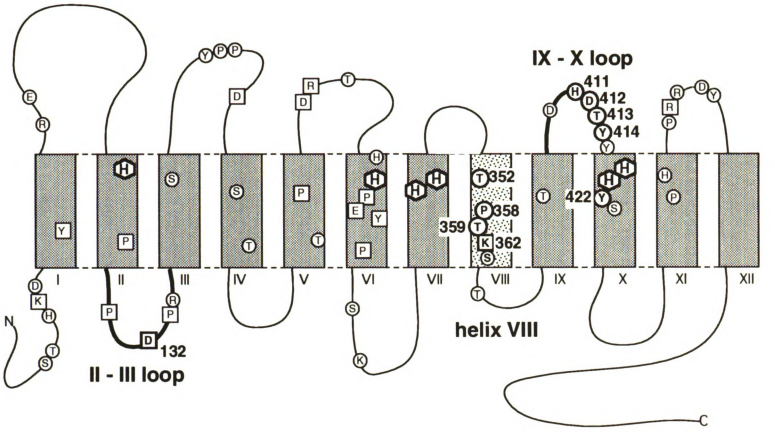

IX - X loop
411
412
413
414
352
358
359
362
422
helix VIII
II - III loop
132
N
C
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII

Figure 2.5

Chapter 3 – Development and refinement of a reconstitution and proton pumping assay for cytochrome *c* oxidase from *Rhodobacter sphaeroides*

Introduction

In order to characterize mutants that may be inhibited in pumping protons, it was necessary to set up an assay to measure this. Several methods have been previously used. The first consideration was whether to use whole bacterial cells, or to reconstitute the purified enzyme into artificial vesicles. Whole mitochondria from beef heart have been used to measure proton pumping (Wikström 1984; Wikström and Casey 1985a) and spheroplasts, whole bacteria with a disrupted outer membrane, are used to assay proton pumping of cytochrome *bo* oxidase from *E. coli* (Verkhovskaya *et al.*, 1992). Reconstituted cytochrome *c* oxidase from the beef heart enzyme has also been used to measure proton pumping (Krab and Wikström 1978; Casey *et al.*, 1984). In the case of *Rhodobacter sphaeroides*, an additional cytochrome *c* oxidase (*cbb*3) is known to be expressed along with the *aa*3 enzyme (García-Horsman *et al.*, 1994b). Therefore there would be no easy way to distinguish which oxidase was pumping protons in spheroplasts from *Rb. sphaeroides*. Thus it was necessary to develop a reconstitution procedure for the purified *aa*3 cytochrome *c* oxidase from *Rb. sphaeroides*. The method of cholate dialysis was used in which soybean phospholipids are sonicated to clarity in the presence of the detergent cholate. After the addition of enzyme, dialysis is used to remove the detergent, allowing the oxidase to incorporate into bilayer vesicles. This method results in an oriented oxidase insertion with the cytochrome *c* binding site facing outward (Eytan 1982).

A second consideration in assaying for proton pumping is the method of initiating the oxidase turnover that drives proton pumping. One method is to

assay the enzyme with an oxygen pulse. First the enzyme is fully reduced with reductants such as ascorbate, TMPD and cytochrome *c* so that all oxygen is removed, followed by the addition of oxygen saturated buffer that initiates the pumping (Casey 1986). A second method, and the one developed for the experiments in our lab, is to start with the enzyme in a solution containing oxygen, and to initiate the reaction with the addition of cytochrome *c* (Casey *et al.*, 1979; Casey *et al.*, 1984; Casey 1986). A major advantage is that there is no necessity for working under anaerobic conditions, and thus it can be used more easily when measuring pH change by spectral methods.

A final consideration is the method used to measure the pH changes, either with a high sensitivity pH electrode, or spectrophotometrically using a pH sensitive dye (Casey *et al.*, 1979). The pH electrode had been previously used in this lab (Gregory 1988), but it is susceptible to electrical interference and rapid aging (loss of sensitivity) making it difficult to use for the small pH changes of proton pumping. The spectral method using phenol red was chosen for this reason and because of its additional potential for further development for rapid kinetic measurements in the future.

It is known that many variables including preparation variation of the beef heart oxidase can influence the reconstitution (Casey *et al.*, 1979), so that it was necessary to find optimum conditions with the bacterial enzyme to give reproducibility. It had previously been published that the *Rb. sphaeroides* enzyme lacking subunit III could not pump protons (Gennis *et al.*, 1982), but considering the high degree of homology to the mammalian enzyme it seemed likely that this oxidase should have the same proton pumping activity. In fact, the highly homologous two subunit enzyme from *Paracoccus denitrificans* has been shown to pump protons (Solioz *et al.*, 1982). With the development of an improved purification in our lab (Hosler *et al.*, 1992), it was important to

establish whether this enzyme could pump protons. If it could not, it would not serve as a good model system for studying the energy transduction mechanism of cytochrome oxidase. After many attempts at reconstitution and fine tuning of the proton pumping assay, I was finally able to detect proton pumping from the *Rb. sphaeroides* enzyme (Hosler *et al.*, 1992). Many further refinements of the reconstitution and the assay were necessary before enough reproducibility was achieved to allow the reliable comparison of the proton pumping activity from different mutants.

Experimental procedures

Materials Horse heart cytochrome *c* (Sigma type VI) was purified by carboxymethyl cellulose chromatography (Brautigan *et al.*, 1978), rapidly frozen in liquid nitrogen, and stored at -80 °C. To prepare reduced cytochrome *c* for proton pumping, dithionite was added to fully reduce the purified cytochrome *c*, and then the reducing agent was removed by gel filtration on a 1.0 x 21-cm Sephadex G-10 column, using 1 mM HEPES-KOH, pH 7.4, 44.6 mM KCl. Stock solutions of valinomycin (2 mM) and CCCP (1 uM and 10 mM) (both from Sigma) were made by dissolution in ethanol and stored at -20 °C. Lauryl β-D maltoside was purchased from Anatrace Inc., Toledo OH. Acid standard (0.1 M HCl) was obtained from Aldrich.

Purification of asolectin Asolectin from Associated Concentrates (now out of business) was purified to remove fatty acids and other oxidative products, or with inconsistent results, unpurified asolectin was used from Sigma (L-α-phosphatidylcholine, type II-S)(Unpurified Sigma type IV-S may also be usable but has not been tested for this oxidase). Purification of the phospholipids was done by an adaptation of the procedure in Sone *et al.* (1977) as follows. Asolectin (25 g) was stirred in 500 mL of dry acetone containing

500 mg of betahydroxytoluene (BHT); the cloudy supernatant was removed by vacuum filtration using Type 5 Whatman Filter Paper, leaving dry phospholipids. These were dissolved in 100 mL of dry diethylether. Then 900 mL of dry acetone was titrated into the diethylether solution at 4 ^{o}C to form a precipitate. This was collected by filtration through Type 5 Whatman filter paper. This was repeated starting at dissolution in the diethylether, after which the phospholipids were dissolved in diethylether with 200 mgs of alpha tocopherol and centrifuged at 8000 x g for 10 min. The supernatant was concentrated using a rotovap, and then allowed to further dry under vacuum in a desiccator overnight. The lipids were stored under argon in small tubes at -80 ^{o}C, and each tube was only used once.

Purification of cholate Cholate from Serva was recrystallized based on the procedure of Kagawa and Racker (1971) as follows. Cholate (200 g) was dissolved in a minimal volume of 80% ethanol at 60 ^{o}C (approximately 700 mL). Charcoal was heated for at least 2 hr at 120 ^{o}C to activate, and then added to the ethanol and stirred for 1/2 hr. This was filtered several times through Type 5 Whatman filter paper. The filtrate was stored on ice to allow recrystallization to occur. The recrystallization was repeated a second time. Cholic acid was dissolved using KOH to make a final stock concentration of 20% which should be colorless.

Growth of bacteria and oxidase purification *Rhodobacter sphaeroides* strain CY91 was grown at 30 ^{o}C with 25 ug/mL of kanamycin (the antibiotic resistance comes from the deletion of the *b*562 gene which leads to overproduction of the oxidase), and then purified following the procedure in Hosler *et al.* (1992), except that the hydroxyapatite purification step was excluded. During the early stages of development of the reconstitution and

proton pumping assay, it appeared that the hydroxyapatite step gave samples that did not reconstitute as well.

Reconstitution The reconstitution was done generally as indicated in Hosler *et al.* (1992). Glassware was rinsed with ethanol and then thoroughly with water to remove any traces of dish washing detergent. Asolectin phospholipids at 40 mg/mL, 2% cholate, and 75 mM HEPES-KOH, pH 7.2-7.4 (a lower pH seemed to give better results) were sonicated with a Heat Systems-Ultrasonics sonicator, model W-255, using a microtip, alternating between a power setting of 5 for 30 s and then off for 30 s at 0° C under argon until clarity occurred. This sample was centrifuged for 15 min at 12,000 x g to remove titanium particles and any unsuspended phospholipids. Cytochrome *c* oxidase in 0.2% lauryl maltoside was diluted with 300 mM KCl, 0.2% lauryl maltoside, after which 6.7 mg of cholate was added per nmol of enzyme to give a final oxidase concentration of 1.8 uM (cholate was now 4%) and allowed to incubate for 2 hr at 0 °C. (Note that for beef heart oxidase twice the concentration of enzyme was used.) The asolectin suspension was added to the enzyme to give a final concentration of 0.65 uM oxidase. This sample was added to Spectrapor dialysis tubing (number 25225/204, 12-14,000 M_r cutoff) and the tubing was closed using a floatable plastic clamp on one end and a weighted plastic clamp on the other. Dialysis was performed with medium stirring speed on a Sybron Thermolyne, Nuova II stir plate using the following protocol: 6 hr in 100 volumes of 75 mM HEPES-KOH, pH 7.4, 14 mM KCl, 0.1% cholate; 12 hr in 100 volumes of 75 mM HEPES-KOH, pH 7.4, 14 mM KCl; 12 hr in 100 volumes of 50 mM HEPES-KOH, pH 7.4, 24 mM KCl, 15 mM sucrose; 12 hr in 500 volumes of 1 mM HEPES-KOH, pH 7.4, 44.6 mM KCl, 43.4 mM sucrose.

Orientation assay Reconstituted oxidase was tested to determine the percentage that was in the outward facing orientation, which exposes the site on oxidase where cytochrome *c* binds, allowing binding of this substrate and electron transfer to the oxidase. Spectra were taken on a computerized Perkin Elmer Lambda 4B spectrophotometer using 0.5 cm cuvettes at 24 ^{o}C. To 400 uL of 50 mM potassium phosphate at pH 7.0, 80 uL of vesicles were added. Cytochrome *c* was added to a concentration of 35 uM along with ascorbate to 24 mM to reduce the oxidase with an outwardly facing cytochrome *c* binding site. This was allowed to incubate for several minutes to be sure complete reduction had occurred after which a spectrum was taken from 540 nm to 660 nm. A lipid soluble reductant, TMPD, was then added to reduce inwardly facing oxidase, allowed to incubate for several minutes, and a second spectrum was collected. To be sure that complete reduction had occurred, a pinch of the reductant dithionite was added to allow complete reduction of the enzyme (in some cases lauryl maltoside was added to 0.5% to dissolve the vesicles, with the same results), and this spectrum was determined. The same experiment was also performed using blank vesicles (containing no oxidase), and spectra were collected after the same additions. These spectra were used as a background which were subtracted from the spectra taken on the oxidase containing vesicles, using the Perkin Elmer UVDM software package. To determine the oxidase concentration an extinction coefficient of 27 cm-1 mM-1 was used for the peak at 605 nm with a baseline from 580 to 640 nm.

Proton pumping assay Proton pumping was measured at 22 ^{o}C at a measuring wavelength of 556.8 with a reference wavelength of 504.7 nm and a 3 nm slit width on an Aminco DW-2 dual wavelength spectrophotometer using the pH sensitive dye phenol red in a stirred cell. HEPES at 100 mM, 50 uM phenol red, at pH 7.4 was used to set a baseline on the spectrophotometer.

The assay buffer was 50 uM $NaHCO_3$-KOH, 45 mM KCl, 44 mM sucrose, 50 uM phenol red, pH 7.4 at a volume of 2.35 mL. After addition of 150 uL of reconstituted oxidase, containing 0.1 nmol of enzyme, to the assay buffer, 3.2 uM valinomycin and 0.4 nM CCCP were added to allow equilibration. Then the pH was adjusted to 7.4 with 0.1 M KOH using the baseline previously set. An addition of cytochrome *c* was used to initiate proton pumping measured as acidification of the external medium. After the addition of uncoupler, 5 uM CCCP, a second addition of cytochrome *c* was added to allow quantitation of the net alkalinization caused by consumption of substrate protons inside the vesicles. Additions of 1 nmol H^+ standard, before and after the addition of the 5 uM CCCP, allowed calibration of the size of the acidification and the size of the alkalinization. To determine the H^+/e^- ratio, the extent of acidification was determined by extrapolating the alkalinization rate, due to proton leakage back into the vesicles, back to zero time-the time of cytochrome *c* addition. Electron input was based on 1 e^- per cytochrome *c* with the cytochrome *c* concentration being determined at 550 nM using an extinction coefficient of 28 $mM^{-1}cm^{-1}$.

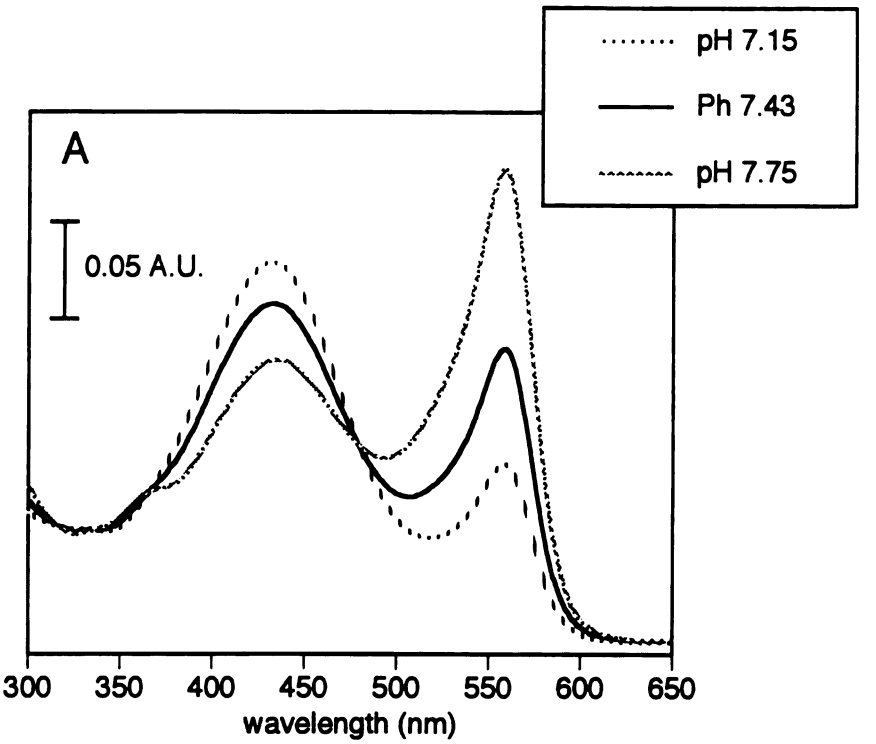
Results

Isosbestic points of phenol red and cytochrome *c* The proton pumping assay developed for the *Rhodobacter sphaeroides* enzyme is similar to that previously used for the reconstituted cytochrome oxidase from beef heart (Casey *et al.*, 1979). However, a number of refinements were required to obtain reproducible pumping with the bacterial enzyme. Figure 3.1 A shows the spectra of phenol red in the buffer used for proton pumping at three pH's 7.15, 7.43, and 7.75. Part B shows the overlapping spectra of reduced and oxidized cytochrome *c* at a concentration 50 times higher than what is

normally used in the pumping assay. It can be seen that the region around 560 nm shows the maximal change in absorbance with pH. Other experiments, not shown, indicate that the largest change with a particular quantity of acid or base occur near pH 7.4. Thus this pH provides maximum sensitivity and was used throughout the reconstitution and in the proton pumping assay. A potential problem is that the reductant used to initiate turnover, cytochrome *c*, also has an absorbance within this region. The isosbestic points can be seen to be at 503.8, 525.3, 541.1, and 556 nm. Thus the two isosbestic points for cytochrome *c* are used, one at 556 and the other at 503.8 nm, so that as the cytochrome *c* goes from reduced to oxidized no change will interfere with the spectra of the phenol red . The actual values for the isosbestic points were recalculated for the different calibration of the Aminco, giving 556.8 and 504.7 on that instrument. A small spectral change will occur with the addition of cytochrome *c*, but this is corrected for by adjusting the pH of the cytochrome *c* sample until no change is observed when it is added to blank vesicles (dialyzed through the same protocol as the oxidase-containing vesicles). This adjusts for both the initial absorbance change upon addition of cytochrome *c* and for any differences in the pH of the cytochrome *c* relative to the vesicles.

Controlling the pH for the proton pumping assay Since small pH changes are being measured, almost no buffering is used in the assay. Initial experiments were done using 50 uM HEPES at pH 7.4, with additional buffering from the aliquot of vesicles providing another 50 uM HEPES. This buffer is fairly unstable to pH as CO_2 is continuously absorbed giving a

Figure 3.1 Spectral components in proton pumping assay. A) Determination of phenol red and cytochrome c isosbestic points. Spectra were taken using the proton pumping buffer, 50 uM NaHCO3-KOH, 45 mM KCl, 44 mM sucrose, 50 uM phenol red, pH 7.4. Additions (0.5 uL) of 0.02 M NaOH were added to give pH's at 7.0, 7.15, 7.32, 7.43, 7.75, and 8.15. Only those at 7.15, 7.43, and 7.75 are shown. B) Oxidized cytochrome c (50 uL) was added to 3 mL of 100 mM potassium phosphate buffer, pH 7.0 and the spectra was scanned between 300-650 nm on a computerized Perkin Elmer Lambda 4B. Dithionite was used to reduce the cytochrome *c*, after which a second scan was taken. Only the spectra from 450 to 650 are shown. This can be used to identify the four isosbestic points at 503.8, 525.3, 541.1, and 556. This can be converted to values for the Aminco DW2a by adding 0.8 nm.


pH 7.15
Ph 7.43
pH 7.75
A
0.05 A.U.
300
350
400
450
500
550
600
650
wavelength (nm)

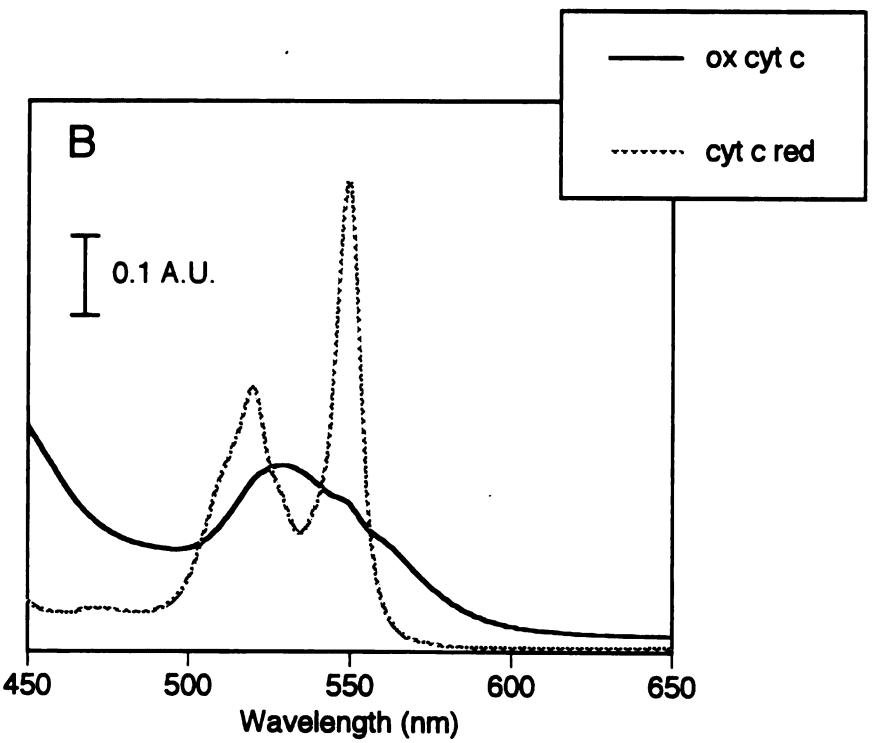

ox cyt c
cyt c red
B
0.1 A.U.
450
500
550
600
650
Wavelength (nm)

Figure 3.1

baseline drift. It was found that using a 50 uM $NaHCO_3$ buffer stabilized the pH to some extent during the time required for the assay.

Another development that greatly improved the reproducibility of the assay can be seen in Figure 3.2, namely, the adjustment of the pH to 7.4 for each run before starting the pumping assay. Originally it was assumed that from one assay to the next the pH would be stable at 7.4. There were, however, gradual changes in the pH of the buffer and differences between vesicles, causing the starting conditions to vary. There were clearly differences between protein samples used in the reconstitution of different mutants, even though reconstituted under identical conditions. The nature and extent of these differences was revealed by carefully monitoring the starting pH and the response of the vesicles to various ionophores. During the assay it is necessary to have valinomycin present to equilibrate K^+ across the membrane and prevent build up of a membrane potential. The immediate creation of a membrane potential could cause the protons to be pulled back into the vesicles too quickly for a pH change to be measured (Nicholls and He 1993). A very low concentration of CCCP was also found to be beneficial in the beginning of the assay since it allows more rapid and complete equilibration of the vesicle interior with the medium, so protons are not being pumped against an existing gradient.

The presence of an existing gradient is revealed by the fact that after the addition of valinomycin and the low concentration of CCCP the pH rises significantly (approximately 0.1-0.2 pH units) and then gradually levels off. There is variation in the final pH after equilibration, depending on which set of vesicles are used and even with the same vesicles over time due to CO_2 absorption.

Figure 3.2 Equilibration of vesicles in proton pumping buffer and adjustment of pH to preset baseline (The full scale shown is equal to 0.5 A. U.). Top) Initially a concentrated buffer containing phenol red is used to adjust the baseline of the spectrophotometer to pH 7.4. Bottom) The reconstituted vesicles are added to the indicated proton pumping buffer in a stirred cell. Their starting pH ranges from 7.1-7.2. To prepare the vesicles for the proton pumping assay , valinomycin, and a low concentration of CCCP are added and cause the vesicles to alkalinize. NaOH (0.02 M) is added to adjust the pH to the previously set baseline at 7.4. The full scale shown is equal to 0.5 A.U.

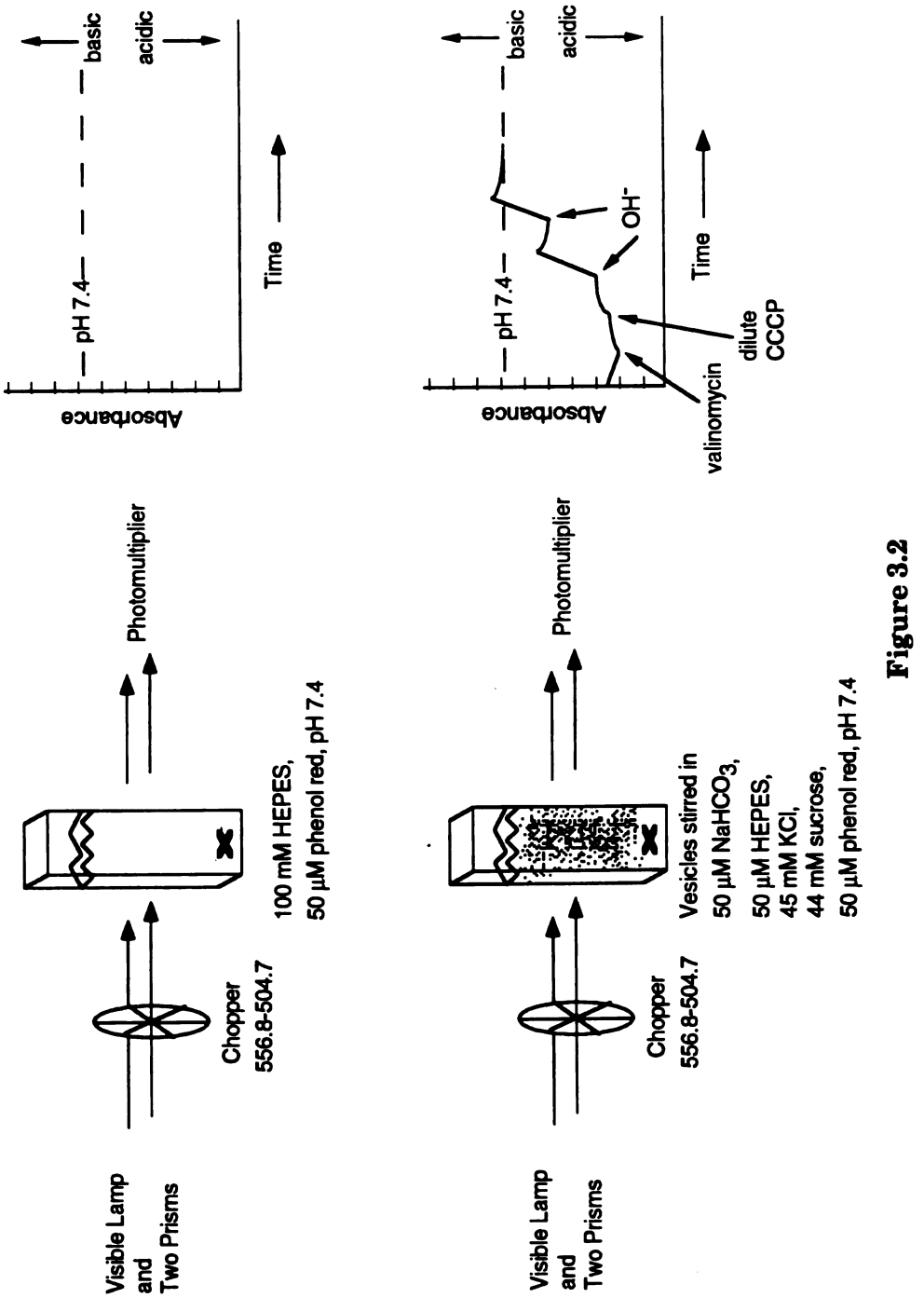

Absorbance
pH 7.4
basic
acidic
Time
Photomultiplier
Visible Lamp
and
Two Prisms
Chopper
556.8-504.7
100 mM HEPES,
50 µM phenol red, pH 7.4
Absorbance
pH 7.4
basic
acidic
OH-
Time
valinomycin
dilute
CCCP
Photomultiplier
Visible Lamp
and
Two Prisms
Chopper
556.8-504.7
Vesicles stirred in
50 µM NaHCO3,
50 µM HEPES,
45 mM KCl,
44 mM sucrose,
50 µM phenol red, pH 7.4

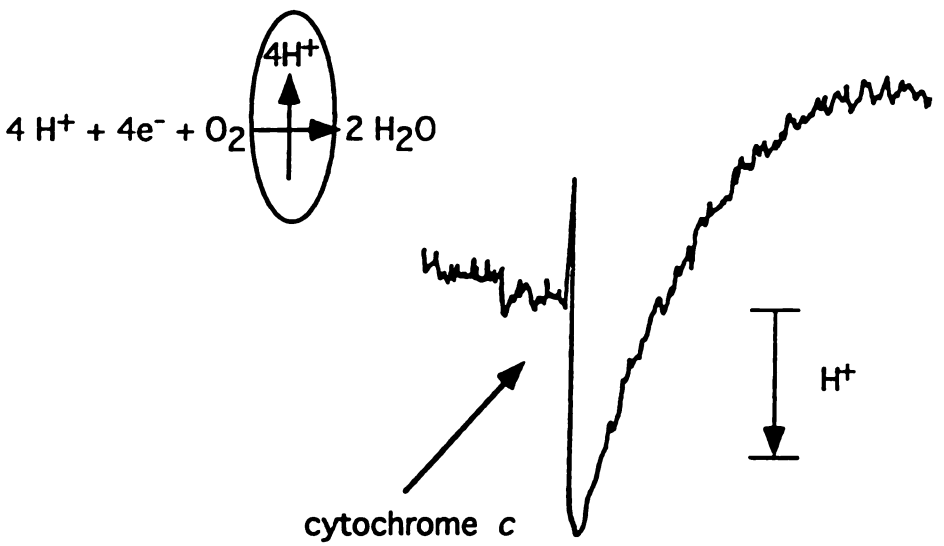
Figure 3.2

This variable introduced some error into the measurements, which could be reduced by adjusting the pH to 7.4 as noted above and diagrammed in Figure 3.2. A concentrated buffer at pH 7.4 containing phenol red is used to set the baseline of the spectrophotometer. As shown in the bottom part of the figure, the addition of the valinomycin and dilute CCCP causes the pH of the vesicle suspension to alkalinize to different extents with different vesicles. Then base is added to bring the pH to the baseline set on the spectrophotometer. This two step procedure is used with each experiment to give greater precision and reproducibility.

Proton pumping of the wild type cytochrome *c* oxidase from *Rb. sphaeroides* Figure 3.3 shows proton pumping data for the wild type enzyme. Addition of cytochrome *c* causes the protons to be pumped from inside of the vesicles to the outside, which gives the initial rapid spectral decrease of the phenol red indicative of acidification. Next, the direction of spectral change reverses with an exponentially decaying in rate. This alkalinization proceeds past the baseline due to the net consumption of protons in the conversion of O_2 to H_2O in the interior of the vesicles. The size of this change can be calibrated by the addition of an acid standard. A control experiment is done by adding concentrated CCCP to the vesicles bringing its concentration to 5 uM. This allows rapid equilibration of protons across the vesicle membrane. Subsequent addition of cytochrome *c* causes only alkalinization. This is also calibrated against an acid standard and can then be compared to the known amount of cytochrome *c* that is added during the experiment. Knowing that one proton per electron is consumed during this reaction provides a control for any artifactual pH change caused by the cytochrome *c* alone.

Figure 3.3 Proton pumping of the wild type enzyme from *Rhodobacter sphaeroides*. Reconstituted oxidase was assayed using phenol red to spectrophotometrically detect pH changes at 556.8 minus 504.7 nm (See Experimental Procedures). A) Addition of 2.1 nmol of cytochrome *c* caused a rapid acidification indicative of proton pumping. B) After the addition of concentrated CCCP (5 uM), addition of cytochrome *c* produced a rapid alkalinization indicative of substrate proton consumption. Addition of 0.5 nmol of acid standard before and after the addition of the concentrated CCCP was used to calibrate the size of the pH changes. The assay was normally performed on an 0.02 A.U. full scale and gave ΔA.U. values of 0.002 to 0.004.

A) Reconstituted wild type *Rb. sphaeroides* oxidase shows proton pumping.



B) After the addition of uncoupler (CCCP), only the alkalinization is seen.

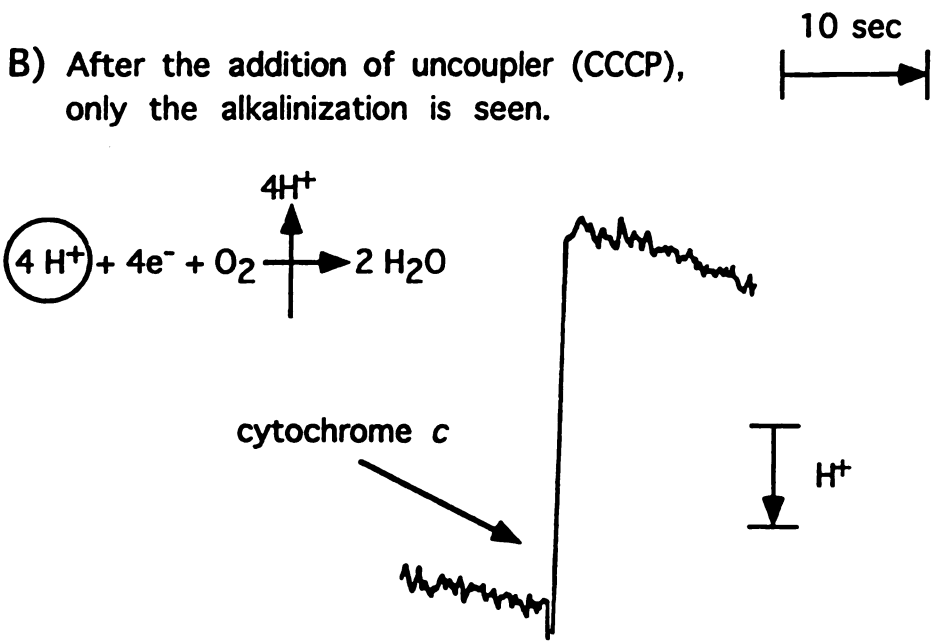


Figure 3.3

As discussed in Hosler *et al.* (1992), various controls were done to assure that proton pumping was being measured. Sequential additions of cytochrome *c* gave the same size of pH changes, indicating a quantitatively repeatable reaction. Addition of azide or cyanide to the system causes elimination of the proton pumping reaction, indicating that it is caused by the turnover of the enzyme. Finally, when the kinetics of the cytochrome *c* oxidation are monitored, complete oxidation is observed as measured by decreased absorbance at 550 nm with vesicles containing oxidase, but no oxidation occurred with blank vesicles.

Determination of oxidase orientation after reconstitution

Previously it had been shown that when beef heart oxidase was reconstituted by this type of procedure, 80% or more was oriented with the cytochrome *c* binding site facing outward (Wrigglesworth and Nicholls 1979; Gregory 1988). It is necessary for the oxidase to be in this orientation to measure proton pumping and thus, if mutants are found that do not appear to pump protons, it is essential to establish that they are correctly oriented. To determine the orientation, the reconstituted oxidase is reduced first with water-soluble reductants cytochrome *c* and ascorbate. Under these conditions only oxidase with its cytochrome *c* binding site facing outward is reduced. A hydrophobic reducing agent, TMPD, which can penetrate the vesicles, is then added to reduce the inwardly facing enzyme. Figure 3.4 shows the spectra of the alpha band at 605 nm of the reconstituted wild type cytochrome *c* oxidase.

Figure 3.4 Determination of percentage of reconstituted wild type cytochrome c oxidase with its cytochrome c binding site facing outward. Cytochrome c oxidase vesicles were assayed as indicated in the Experimental Procedures by first reducing the outward facing enzyme with cytochrome c and ascorbate. Inward facing oxidase was assayed by the further addition of TMPD. The size of the peak at 605 nm from the hemes of the enzyme indicates the concentration of oxidase. By dividing the peak intensity of the first spectrum by the peak intensity of the second spectrum, the percentage of outward facing oxidase is determined.

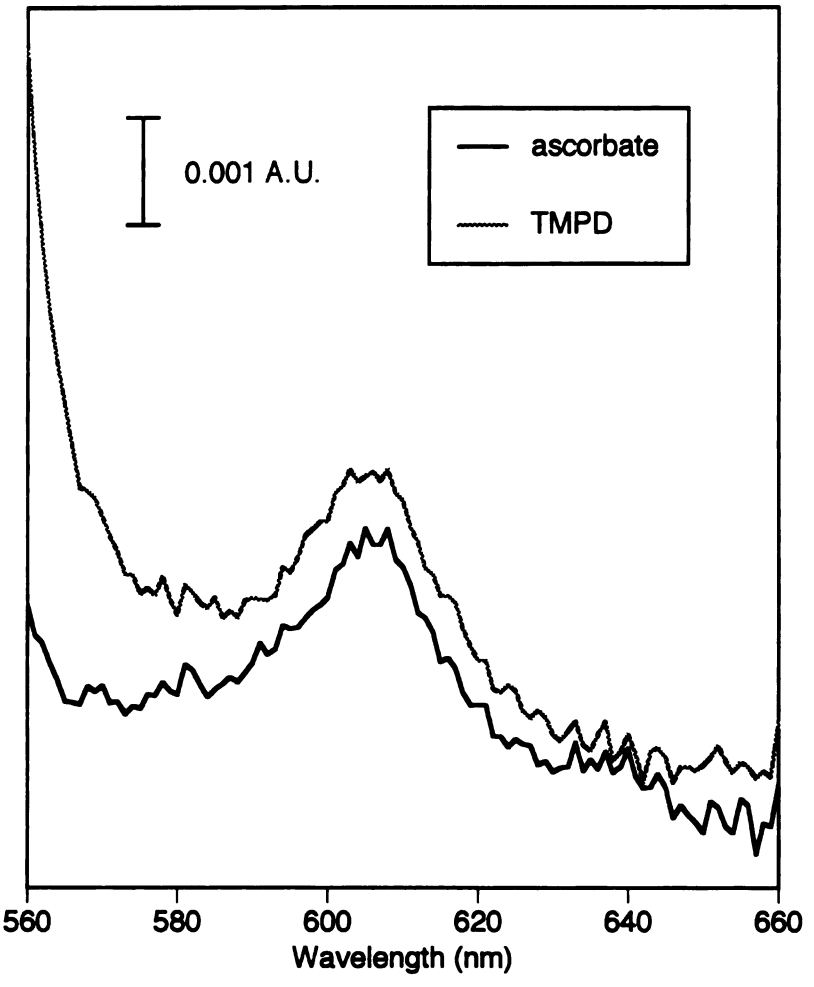


Figure 3.4

It can be seen that there is little change in the reduction of the oxidase in going from the cytochrome *c*/ascorbate spectrum to the spectrum with the added TMPD, except that the spectra are offset. Addition of the powerful reducing agent dithionite and then the detergent lauryl maltoside to dissolve the vesicles showed the same level of reduction, indicating that the ascorbate and TMPD were effective in fully reducing the enzyme. Calculations from these spectra show that 100% is in the outward orientation. The concentration of oxidase can also be calculated from these spectra and shows that 60% of the enzyme originally used was still present after several days of reconstitution. Thus the reconstitution of the bacterial enzyme gives as high a percentage of correct orientation as is seen with the thoroughly characterized beef heart enzyme.

Respiratory control of the reconstituted oxidase Another important criterion for determining the success of a reconstitution is the measurement of "respiratory control" in the reconstituted enzyme. If the enzyme is properly reconstituted, stimulation of activity with excess reducing agents (ascorbate, TMPD, and cytochrome *c*) will cause a build up in the protonmotive force across the membrane which will inhibit the oxidase. Addition of valinomycin and CCCP will release this inhibition and allow oxygen consumption to proceed. Dividing the released rate by the initial inhibited rate gives a respiratory control ratio (RCR). After each reconstitution the respiratory control is determined. A previous report compared several batches of reconstituted beef heart enzyme and showed that by looking at the ratio of the release of inhibition with valinomycin and CCCP to that with valinomycin alone, a correlation was found with the efficiency of proton pumping (Wilson and Prochaska 1990). This was not found in the studies on the *Rb. sphaeroides* wild type or mutant oxidases, but other variables in this system may have

been more influential. A significant RCR is needed for pumping, but values from as low as 3 to as high as 8 did not correlate with the efficiency of proton pumping.

Discussion

Contrary to previous results (Gennis *et al.*, 1982), the oxidase from *Rb. sphaeroides* is capable of pumping protons. It seems to reconstitute with a normal orientation in the membrane and to be capable of showing good respiratory control. Why this was not observed in the previous measurements probably relates to the fact that the reconstitution procedure must be independently established for different enzyme sources. Reconstitution of membrane proteins was developed by Racker (Kagawa and Racker 1971; Hinkle *et al.*, 1972; Eytan 1982) and has been useful to assaying many proteins in a more native environment while still free from potential artifacts caused by the presence of other enzymes and transporters in the native membrane. Many of the problems that I encountered during the last several years are only occasionally discussed in the literature. Clearly most of the details for reconstituting a particular enzyme need to be worked out on a case by case basis and many subtleties about any procedure remain unpublished. In fact, few studies have been done to establish in any systematic way what the important variables are in reconstituting membrane proteins. It seems worth discussing potential problems of the reconstitution procedure, and thus the variables which may be worth modifying to improve the procedure. Figure 3.5 lists the major ones that I have noticed during my experiments listed from most to least important. Those at the top of the list caused more obvious changes in the quality of the reconstitution, while some of the other variables

Figure 3.5 Summary of some variables to consider during the reconstitution of the *Rhodobacter sphaeroides* enzyme. These are listed in order of what were considered to be most important (three check marks) to least important (one check mark). For those listed with one or two check marks, the effect of changing these were difficult to quantitate because of the subtle changes that often occurred relative to other variability in the assay.

Some Important Considerations for the Reconstitution and Proton Pumping Assay

✔✔✔ purity of enzyme

✔✔✔ purity of cholate

✔✔✔ purity of phospholipid

✔✔✔ type of syringe used to deliver acid standard during assay

✔✔ pretreatment of enzyme with cholate before reconstitution

✔✔ standardization of proton pumping buffer pH for start of assay

✔✔ ratio of enzyme/lipid during reconstitution

✔ pH of HEPES/cholate buffer used in reconstitution

✔ concentration of enzyme sample during reconstitution

Figure 3.5

were more difficult to quantify, but even small improvements were helpful. Standardization of the proton pumping buffer pH was previously discussed in the Results section in conjunction with Figure 3.2; the other considerations will be discussed now.

Enzyme purity Of course one of the biggest effects on the quality of the reconstitution was the purity of the enzyme. It might not seem that purity of the oxidase should affect the reconstitution. However, attempts to use the oxidase after isolating inner membranes, doing a NaBr wash, and a single DEAE anion exchange purification, but leaving out the second anion exchange, did not give good reconstitution and proton pumping. The spectrally visible contaminants still left at this stage are *b* and *c* cytochromes. The enzyme may also be in an aggregated state and have higher levels of associated bacterial lipid. During the reconstitution, these contaminants may coreconstitute with the oxidase and make it more difficult for proper vesicles to form. Possibly, it is a problem for multiple membrane proteins to fit into the narrower curvature of the vesicles relative to the native membranes. Other contaminants that are not visible spectrally might also cause a problem, such as F_0 from the ATPase which would uncouple the vesicles.

Detergent purity Similarly the purity of the cholate was critical for a good reconstitution. Although what would appear to be highly pure cholate is available (>99%), it is still not of a quality suitable for reconstitutions. This can easily be seen by making an aqueous solution of the detergent which will form a clear yellow solution. It is necessary to do a recrystallization to remove the remaining contaminants. Problems have also been seen in this lab when using the unpurified cholate for the preparation of beef heart cytochrome oxidase following the procedure of Suarez *et al.* (1984).

Phospholipid purity Purity and composition of the phospholipids was also important. Asolectin, which is soya bean phospholipid, is a mixture of predominantly phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. Unfortunately, these ratios are not constant between different brands and batches. One batch of asolectin that was useful in the reconstitutions was put through an organic extraction to remove oxidation products and fatty acids. This may be necessary if the fatty acid content of a particular batch is high since these will uncouple the membrane (Sharpe *et al.*, 1994). Another batch of type II-S lipids from Sigma was found to give good results without being put through the purification, although a more recent batch of the same type does not work well. One conclusion from these results is that with the increased availability of individual phospholipids at lower prices (from Avante for example), development of their use in a reconstitution of oxidase may provide more consistent results.

Accuracy of standardization One of the most critical elements of the experiment was found to be the type of syringe that is used to deliver acid standard during the assay. Before this was discovered, analysis of most assays showed that the alkalinization after the CCCP had about a 20% shortfall from the expected size of the change. This was thought to be an artifact created by the low buffering capacity and the slowly decreasing pH caused by CO_2 absorption from the atmosphere. It was finally discovered that the problem was the use of a Hamilton syringe that tended to inject slightly larger amounts of acid than expected, which then produced an error in the calculations. It was shown that when the syringe was emptied and rinsed several times in the assay buffer, changes in pH occurred that were several times the entire capacity of the syringe, indicating that the metal of the syringe may be binding excess acid. A positive displacement syringe from SMI

using siliconized glass capillaries gave smaller injections of acid, eliminating the shortfall from the alkalinization. Further confirmation that this was now an accurate standard was found when a 10 uL Gilson gave the same results.

Presolubilization of the oxidase with cholate As mentioned in Fetter *et al.* (1995), it was found useful to solubilize the enzyme with cholate detergent before doing the reconstitution. The bacterial oxidase is purified in lauryl maltoside detergent which has a lower CMC than the cholate and is thus more difficult to remove by dialysis. The bacterial enzyme also retains phospholipids. Although phospholipids solubilized in cholate are added to the enzyme at the start of the reconstitution, it was found useful to saturate the enzyme with even more cholate before mixing with the sonicated cholate/phospholipid mixture. Addition of this higher concentration of cholate may help displace the lauryl maltoside and the bacterial phospholipids which could interfere with association of the oxidase with added phospholipids. Higher cholate may also improve the dispersion of the oxidase. This procedure resulted in improved respiratory control values and higher efficiency in proton pumping.

Enzyme to lipid ratio It has previously been shown that varying the enzyme to lipid ratio has an effect on the quality of the reconstitution and proton pumping (Casey *et al.*, 1979). That group found that for reconstitution of the beef heart enzyme there was variability in the optimum ratio between different preparations of the enzyme. In the case of the bacterial enzyme better reconstitutions were found by increasing the amount of lipid relative to the beef heart oxidase. By purifying each growth of wild type and the mutants to the same extent, much of the variation was eliminated so that the same ratio of enzyme to lipid could be used for each sample reconstituted. Presolubilization of the enzyme in cholate also helped to eliminate some

variation, since the amount of lauryl maltoside and lipids bound to the enzyme could have varied between different purifications.

pH of the HEPES/cholate buffer The reconstitution buffer contains a high concentration of HEPES buffer. This becomes incorporated into the interior of the vesicles and helps prevent a build up of the pH gradient during turnover, with higher concentrations of internal buffer allowing a higher apparent pumping efficiency (Proteau *et al.*, 1983). In many of the initial reconstitutions, a stock solution of HEPES was made up to a high concentration and the pH adjusted to 7.4. The pH of the cholate stock solution is similarly adjusted. These solutions are diluted along with the phospholipids to make up the reconstitution buffer. It was found that by not adjusting the pH of the stock solution of HEPES the initial pH was lowered to about 7.2. Since the first dialysis buffer is at pH 7.4 the pH of the reconstitution mixture should rapidly equilibrate back to 7.4. Yet, for some reason the reconstitution started at lower pH seemed to give better proton pumping results.

Concentration of the enzyme in the reconstitution Another problem is the concentration of the enzyme sample used for the reconstitution, since each purified sample is at a different concentration as it comes off the column. To reduce the variable of adding different volumes of enzyme, each sample of oxidase was diluted with the final elution buffer from the anion exchange column to the same concentration. It was difficult to discern a clear effect of controlling this variable, but it was one less complication to worry about.

This discussion has covered considerations which I found to be important in doing the reconstitution. To answer the question of how oxidase pumps protons, a reproducible assay is needed. A better understanding of the reconstitution process would provide information about how to better control it.

In general, reconstitution of membrane proteins may become a more important issue in the future, as more receptors and channels are cloned and purified and an understanding of their activity is sought. This knowledge would also be useful to the pharmacologist trying to optimize drug delivery using artificial vesicles.

Chapter 4 – Possible proton relay pathways in cytochrome *c* oxidase

Introduction

Cytochrome *c* oxidase, a key enzyme in aerobic energy metabolism, reduces oxygen to water, yielding substantial energy which drives the formation of a proton gradient; however, the mechanism of coupling between oxygen reduction and proton translocation remains obscure.

Recognition of the strong homology between mitochondrial and bacterial enzymes (Saraste 1990; Gennis 1991) has stimulated the application of molecular genetic tools to the analysis of the oxidase mechanism. The genes for cytochrome *c* oxidase from *Rhodobacter sphaeroides* have been cloned, sequenced, deleted and reintroduced into the bacterium, and sequence comparisons reveal a high degree of homology with the three mitochondrially encoded subunits of mammalian oxidase (Cao *et al.*, 1991; Cao *et al.*, 1992; Hosler *et al.*, 1992; Shapleigh and Gennis 1992; Hosler *et al.*, 1993). Extensive site-directed mutagenesis of the largest subunit, COX I, has permitted the assignment of the ligands for the three redox active metal centers, heme *a*, heme a_3, and Cu_B (Lemieux *et al.*, 1992; Minagawa *et al.*, 1992; Shapleigh *et al.*, 1992; Hosler *et al.*, 1993), suggesting that all three metal centers are located in COXI toward the outer side of the membrane, while substrate and pumped protons come from the inside (Wikström 1984). Thus some kind of proton channel or relay system is required to convey protons to the site of oxygen reduction, the heme a_3-Cu_B center, and beyond. It is reasonable to look for residues involved in proton pumping near this center, although indirect coupling and involvement of other subunits are also possible.

Figure 4.1 A model of the active site region of cytochrome *c* oxidase indicating residues analyzed by mutagenesis (filled boxes, white letters). Tyr422 is not shown, but would be located near the two histidines on helix X. This model shows six of the twelve predicted transmembrane helices of subunit I. These contain five of the proposed histidine ligands of heme *a*, heme *a*3 and Cu_B. Helix VI, not shown, contains a third Cu_B ligand, H284, which is indicated. The dotted arrows indicate the hypothetical, tested pathways for protons through the protein. Two possible pathways are indicated for substrate (H_S^+) and pumped (H_P^+) protons as suggested in Morgan *et al.* (1994). Other highly conserved residues are indicated with bold circles.

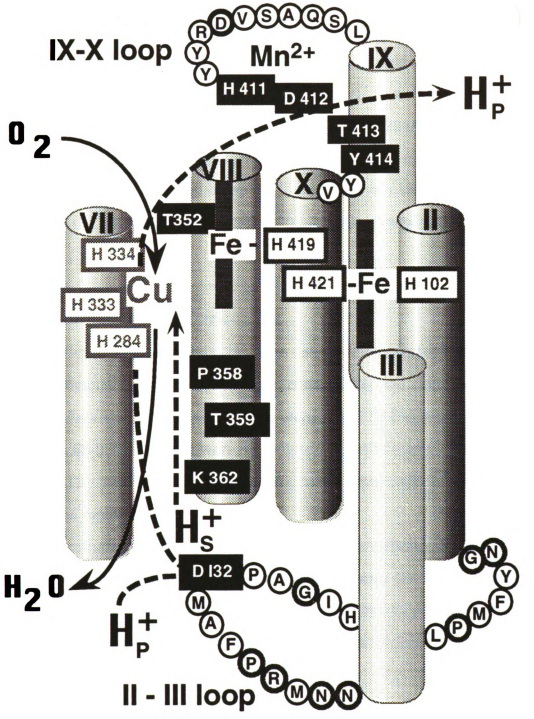

IX-X loop
R D V S A Q S L
Y
Y
Mn2+
IX
H 411
D 412
H+P
T 413
Y 414
O2
VIII
X
V
Y
VII
T352
II
H 334
Fe
H 419
H 421
Fe
H 102
H 333
Cu
H 284
III
P 358
T 359
K 362
H+S
D I32
P A G I H
G N Y
H2O
M
A
F
P
R
M N N
L P M F
H+P
II - III loop

Figure 4.1

This chapter covers the results of analysis of mutant forms of *Rb. sphaeroides* cytochrome *c* oxidase with respect to their proton and electron transfer activity. Four regions in COX I were targeted, each of which contains highly conserved residues capable of participating in a proton relay system (see Figure 4.1). The results strongly suggest a critical role in proton pumping for an aspartate in the loop between helices II and III as well as possible involvement of helix VIII and the IX-X loop region. Tyr422 in helix X was predicted to be involved in proton pumping through ligand exchange with His419, the proximal ligand of heme a3 (Rousseau *et al.*, 1993). Mutation of this tyrosine did not affect proton pumping ruling out a requirement for the residue.

Experimental procedures

Site-directed mutagenesis was done using various restriction fragments of *coxI* as previously described (Shapleigh *et al.*, 1992) (see also chapter 6). The Asp132Asn/Ala mutants were made using a 450 bp fragment coding for the residues 68-226, and both were sequenced to check for any secondary mutations. The mutants in helix VIII were made using a 450 bp fragment coding for the residues 225-378.

Determination of CO spectra, the pyridine hemochrome assay, resonance Raman spectroscopy were done as previously described (Hosler *et al.*, 1992). Proton pumping was determined as indicated in chapter 3. Other methods and experimental conditions are described in the figure legends.

Results

Figure 4.1 indicates the predicted locations of the residues subject to mutagenesis in this study. (Tyr422Ala/Phe) in helix X; (His411Ala, Asp412Asn, Thr413Asn, Tyr414Phe) in the IX-X loop; (Thr352Ala, Pro358Ala, Thr359Ala, Lys362Met) in helix VIII; and (Asp132Asn/Ala) in the II-III loop. All were purified as in Hosler *et al.* (1992) and characterized as described below. Lys362Met was inactive and Thr352Ala had very low activity and could not be purified sufficiently to permit proton pumping measurements.

Electron transfer, spectral, and structural properties As shown in Table 4.1, all the purified mutants have lower activity than the 1700 s-1 turnover (molecular activity) of the wild type. Excluding Asp132Asn/Ala, activities range from 300 sec-1 to 1300 sec-1. The Asp132Asn mutant has a lower turnover, only 70 sec-1, raising the possibility that this activity could be from a contaminating oxidase, such as cytochrome *cbb*3 (García-Horsman *et al.*, 1994b; Gray *et al.*, 1994). However, visible spectra (Figure 4.2) show the enzyme to be highly pure of the *cbb*3 oxidase and analysis by SDS gel (data not shown) gives the same purity and subunit composition as wild type. This mutant is also oxidized to the same extent as wild type in the native membrane, as is the case for other active mutants; whereas, inactive mutants usually show almost complete reduction. The Asp132Ala mutant shows essentially identical characteristics to Asp132Asn.

Most of the mutants studied here have spectral characteristics indicative of a native binuclear center, and in so far as any alteration in the visible and Raman spectra is observed, it does not correlate with the loss of the ability to pump protons. Specifically, Tyr414Phe shows a 5 nm α band red shift and His411Ala shows a 0.8

Table 4.1 Comparison of mutant and wild type oxidases: activity before and after reconstitution, respiratory control ratios, and proton pumping efficiency. Proton pumping values were calculated using data similar to that in Figure 4.10. Activity of the purified enzyme was measured polarographically in 50 mM KPi, pH 6.5, 0.056% lauryl maltoside, 2 mg cholate-solubilized soybean phospholipid, at 25 °C, with 30 uM cytochrome *c* kept fully reduced with 2.8 mM ascorbate and 1.1 mM TMPD. Activity in the vesicles was measured using the reaction conditions as in Figure 4.7., assuming no loss of the oxidase during the reconstitution procedure. Respiratory control ratios are calculated by dividing the activity after valinomycin plus CCCP addition by the initial activity. H^+/e^- ratios are calculated as described in Figure 4.10. Activities and H^+/e^- are the average from two or more independent reconstitutions.

Table 4.1

Enzyme	Activity Purified (s^{-1})	Activity Vesicles (s^{-1})			RCR	H^+/e^-
		initial	+val	+val +CCCP		
b. h.	600	30	100	200	6	0.7-0.8
w. t.	1700	200	400	900	5	0.6-0.8
D132N	70	80	50	70	0.9	0
D132A	50	50	20	20	0.4	0
P358A	600	100	200	400	4	0.5-0.7
T359A	500	50	100	200	4	0.7-0.8
H411A	800	100	300	400	4	0.5-0.6
D412N	300	100	300	400	4	0.5-0.6
T413N	1300	200	400	700	3	0.5-0.6
Y414F	700	100	200	500	4	0.5-0.7
Y422F	800	90	100	300	4	0.5-0.6
Y422A	600	80	100	300	4	0.5-0.6

nm α band blue shift (Hosler *et al.*, 1994b), but neither is altered in proton pumping efficiency. In contrast, the severe loss of proton pumping activity in the Asp132 mutants is accompanied by no alteration in the visible spectra nor in the Raman spectra, as shown for Asp132Asn (Figure 4.2; for wild type spectra see Hosler *et al.*, 1992). However, after bubbling the reduced enzyme with 1 mL of CO which gives 100% conversion of wild type cytochrome oxidase to a CO bound form (Hosler *et al.*, 1992), the Asp132 mutants were only 70-85% saturated (Figure 4.3). A similar slightly lower CO binding (84%) is observed when His411 is converted to alanine (Hosler *et al.*, 1994b), a mutant with high activity and normal proton pumping. Pyridine hemochromagen analysis was done on Asp132Asn, in which the heme A is extracted and can be quantitated by its visible absorbance spectra (Berry and Trumpower 1987). Figure 4.4 shows the spectra of the extracted heme A. Using an extinction coefficient of 25 mM-1 cm-1, the concentration of the heme was determined to be 16.7 uM. The actual concentration of the enzyme is 6.8 uM (at two heme A's per enzyme), indicating no significant loss of the heme, suggesting only minor disturbance of the heme pocket. Similarly, analysis of His411Ala (Hosler *et al.*, 1994b) shows no loss of heme A. Whether the lower CO binding represents an altered affinity for CO or a heterogeneous population is not clear. Also note that recently Asp132Asn was rapidly purified using a genetically attached histidine tail and a nickel affinity column (for the procedure see Mitchell and Gennis, in press). This sample showed 100% CO binding, and still had the same characteristics including a lack of measurable proton pumping.

Figure 4.2 Visible and resonance Raman spectra indicating a native active site of purified Asp132Asn-cytochrome *c* oxidase. A. Visible spectrum: 0.8 μM in 100 mM KP_i, 0.2% lauryl maltoside, pH 7.0 reduced by dithionite. B. Resonance Raman spectra: 35 μM oxidase in 100 mM potassium phosphate, reduced with dithionite, excitation at 441.6 nm (Shapleigh *et al.*, 1992). The following modes are indicated: 214 cm^{-1}, Fe-N_{his} stretch of heme *a*3; 365 cm^{-1}, ring bending of heme *a*3; 1611 cm^{-1}, formyl stretch of heme *a*; 1624 cm^{-1}, vinyl stretch of heme *a*; 1662 cm^{-1}, formyl stretch of heme *a*3.

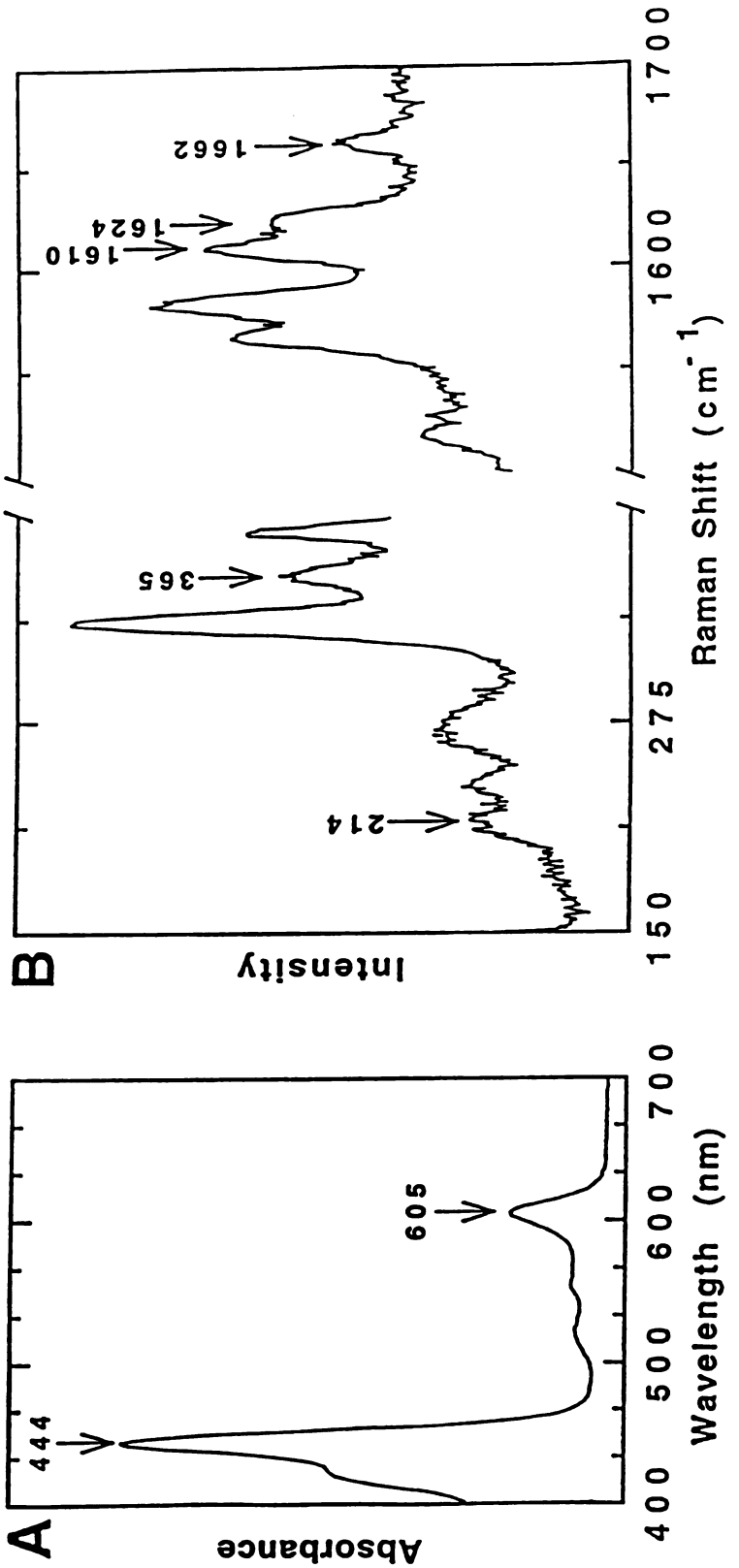

A
Absorbance
444
605
400
500
600
700
Wavelength (nm)
B
Intensity
214
365
1610
1624
1662
150
275
1600
1700
Raman Shift (cm-1)

Figure 4.2

Figure 4.3 Asp132Asn shows lower CO binding than wild type. Visible spectra were collected on 0.78 uM wild type and on 0.76 uM Asp132Asn in 100 mM potassium phosphate buffer, 0.2% lauryl maltoside, pH 7.0, at 22 ^{o}C, using a computerized Perkin Elmer Lambda 4B. Samples were reduced with dithionite, and a spectrum was taken. Then 1 mL of CO was slowly bubbled through the solution to allow the oxidase to bind CO, and a second spectrum was taken. Shown are the difference spectra of the CO bound form minus the reduced form of the enzyme. The trough at 447 nm indicates the remaining reduced form relative to the CO binding. The size of this trough for the mutant relative to the wild type indicates the percentage of CO binding. With less CO binding, the size of the trough will decrease as can be seen for the Asp132Asn. For this particular experiment the CO binding was 70% of that seen in the wild type oxidase.

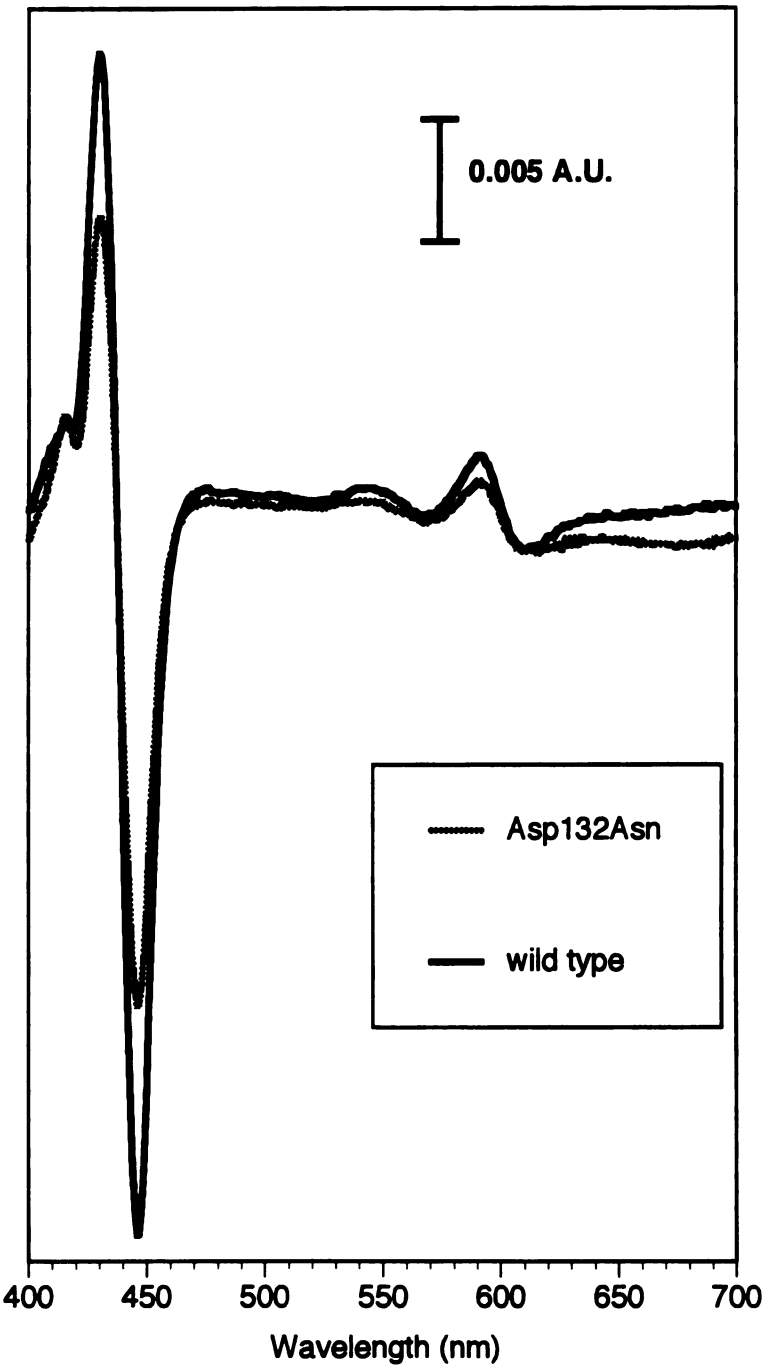

0.005 A.U.
Asp132Asn
wild type
400
450
500
550
600
650
700
Wavelength (nm)

Figure 4.3

Figure 4.3 Asp132Asn shows lower CO binding than wild type. Visible spectra were collected on 0.78 uM wild type and on 0.76 uM Asp132Asn in 100 mM potassium phosphate buffer, 0.2% lauryl maltoside, pH 7.0, at 22 ^{o}C, using a computerized Perkin Elmer Lambda 4B. Samples were reduced with dithionite, and a spectrum was taken. Then 1 mL of CO was slowly bubbled through the solution to allow the oxidase to bind CO, and a second spectrum was taken. Shown are the difference spectra of the CO bound form minus the reduced form of the enzyme. The trough at 447 nm indicates the remaining reduced form relative to the CO binding. The size of this trough for the mutant relative to the wild type indicates the percentage of CO binding. With less CO binding, the size of the trough will decrease as can be seen for the Asp132Asn. For this particular experiment the CO binding was 70% of that seen in the wild type oxidase.

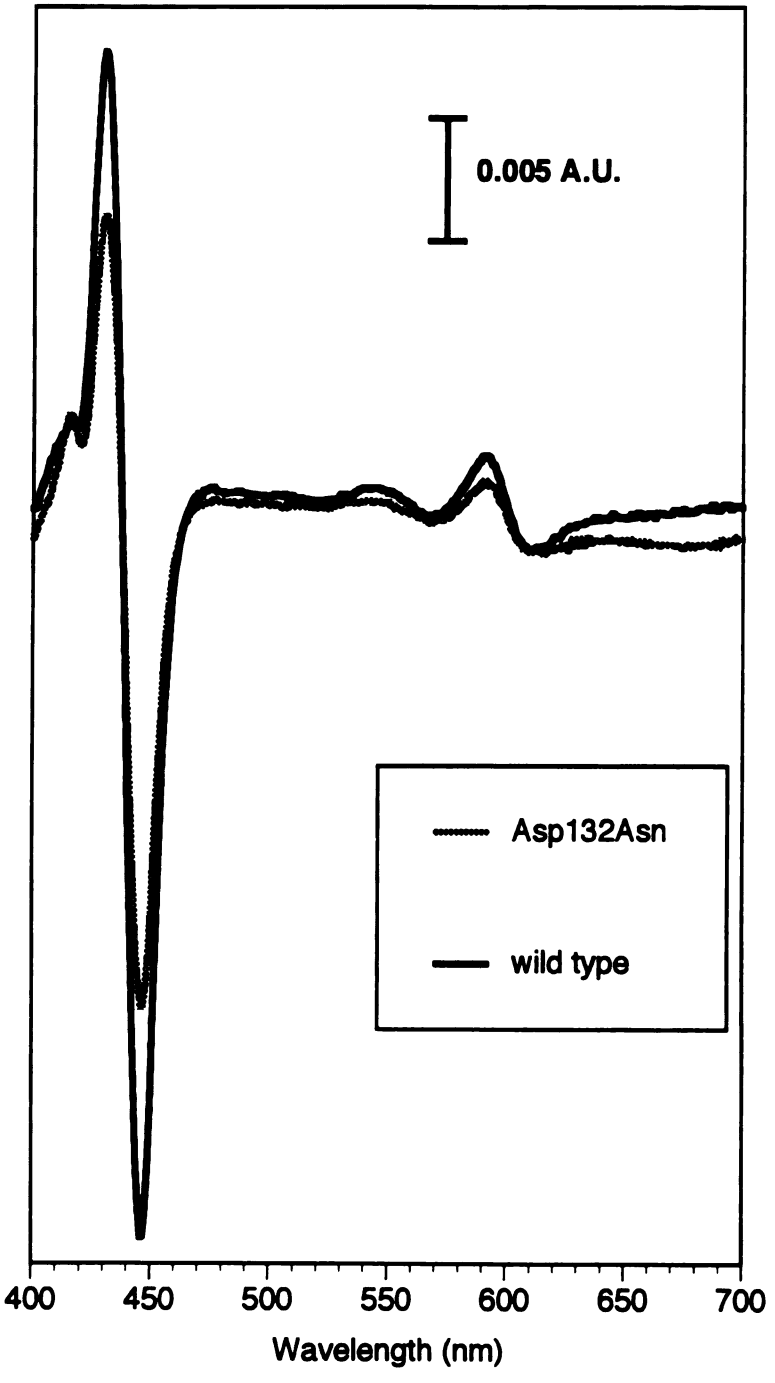

0.005 A.U.
Asp132Asn
wild type
400
450
500
550
600
650
700
Wavelength (nm)

Figure 4.3

Figure 4.4 Assay of the heme A content of Asp132Asn using pyridine hemochromagen. Protein samples were denatured and the heme extracted by dissolving them in 100 mM NaOH, 20% pyridine. This solution was oxidized using potassium ferricyanide, and spectra were collected. After reducing the enzyme with dithionite, spectra were again collected. Displayed is the reduced minus oxidized spectra. Using an extinction coefficient of 25 mM^{-1} cm^{-1}, the concentration of heme A was determined to be 16.7 uM. The concentration of the oxidase before the extraction was 6.8 uM.

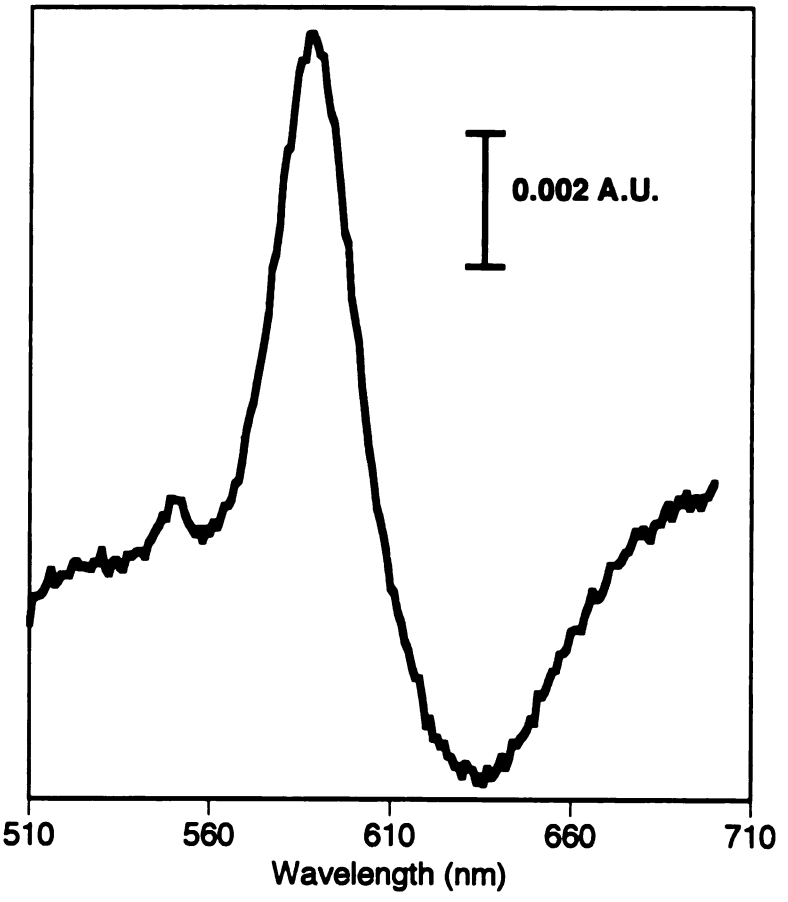


Figure 4.4

Another test of the stability of Asp132Asn was done by assaying the detergent solubilized enzyme for the release of peroxide. Normally, the pocket of the binuclear center prevents any escape of intermediates in the O_2 reduction reaction (Chance *et al.*, 1979). Since the pumping is proposed to be coupled to the peroxy to ferryl and ferryl to hydroxy transitions (Wikström 1989), if the mutant enzyme is blocked in proton pumping, the possibility exists that the enzyme might release hydrogen peroxide during steady state turnover. There is some evidence that hydrogen peroxide could be released, in that it can be added to the wild type enzyme to undergo a transition at the active site (Proshlyakov *et al.*, 1994), indicating that under some conditions the reverse might be possible. Catalase which converts hydrogen peroxide to water and oxygen was used to assay for the formation of hydrogen peroxide during the steady state assay (see Figure 4.5). Addition of catalase after three minutes from the start of the assay did not generate any measurable amount of oxygen. Control experiments showed that hydrogen peroxide was stable in the reaction mixture. This experiment should have detected a release of hydrogen peroxide during 20% of the turnovers of the oxidase. A lower rate of conversion might not be detected, but the evidence suggests that the mutant is intact enough that no peroxide is released and that the mutant completes the catalytic cycle of converting oxygen to water greater than 80% of the time.

Reconstitution and respiratory control The activities of the mutants assayed before and after reconstitution are listed in Table 4.1. In most cases activity measured for the reconstituted vesicles is significantly lower than that of the purified enzyme due to different assay conditions:

Figure 4.5 Assaying Asp132Asn mutant with catalase fails to show production of H_2O_2. Assays were determined on an oxygen polarograph at 25 ^{0}C in 50 mM potassium phosphate, 0.056% lauryl maltoside pH 6.5 with 5.6 mM ascorbate and 0.28 mM TMPD and 30 uM cytochrome *c*. Solubilized Asp132Asn was added to a stirred cell. Catalase (cat) was added (244 units) after 3 min to test for the presence of H_2O_2 (catalase converts 2 H_2O_2 to 2 H_2O plus O_2). No significant amount of H_2O_2 was released from the mutant (100% conversion of O_2 to H_2O_2 would yield 215 nmol). Control experiments in which 44 nmol and 88 nmol of H_2O_2 were added initially, showed measurable levels remaining after a 3 min incubation during turnover of the mutant.

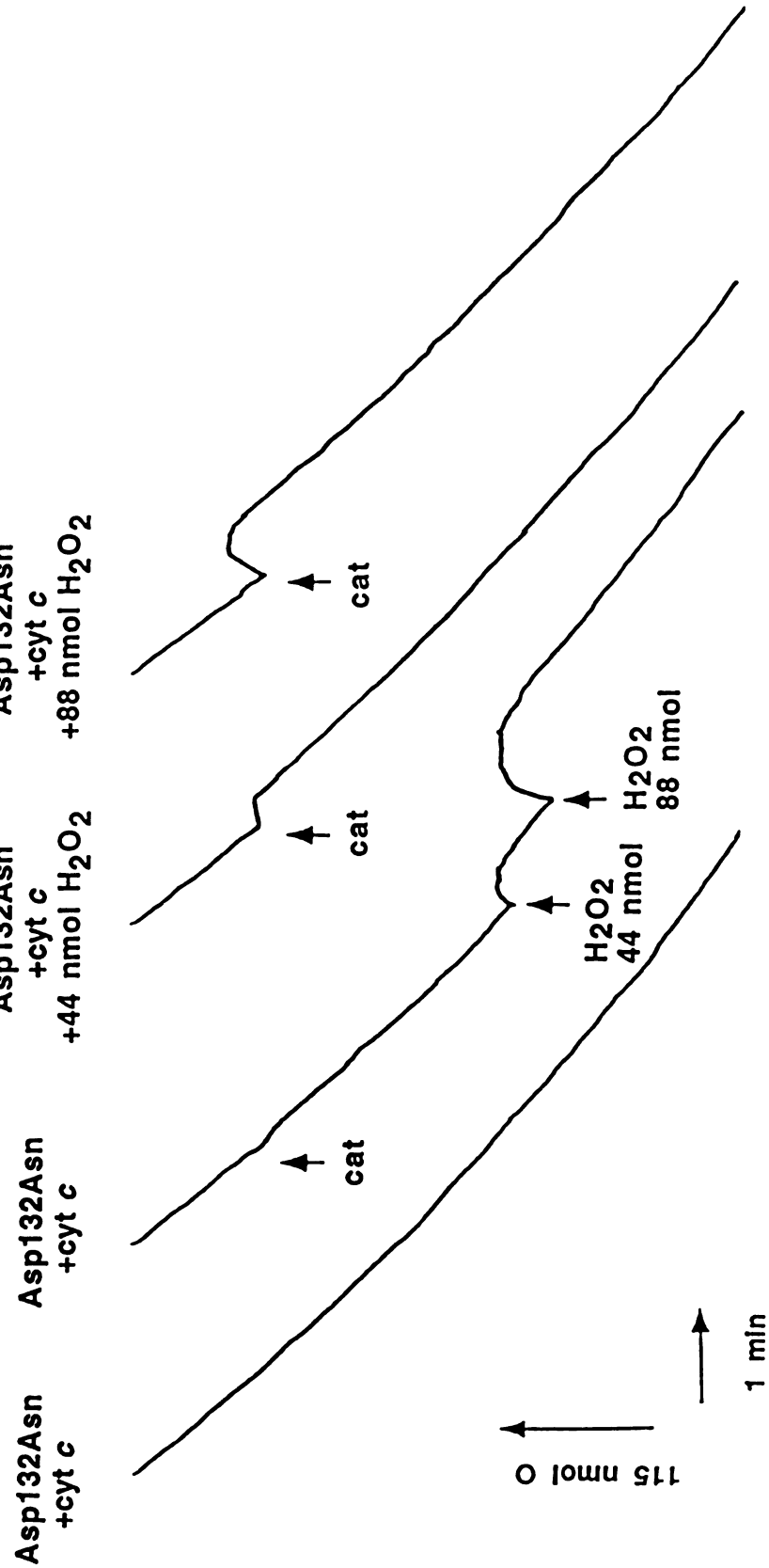

Asp132Asn
+cyt c
Asp132Asn
+cyt c
cat
Asp132Asn
+cyt c
+44 nmol H_2O_2
cat
H_2O_2
44 nmol
Asp132Asn
+cyt c
+88 nmol H_2O_2
cat
H_2O_2
88 nmol
115 nmol O
1 min

Figure 4.5

Figure 4.6 Determination of percentage of reconstituted Asp132Asn with its cytochrome c binding site facing outward. Cytochrome c oxidase vesicles were assayed as indicated in the Experimental Procedures of Chapter 3 by first reducing the outward facing enzyme with cytochrome c and ascorbate. Inward facing oxidase was assayed by the further addition of TMPD. Addition of dithionite after each assay showed that complete reduction of the oxidase had already occurred with the TMPD addition. The size of the peak at 605 nm from the hemes of the enzyme indicates the concentration of oxidase. By dividing the peak intensity of the first spectrum by the peak intensity of the second spectrum, the percentage of outward facing oxidase is determined. In this case it was determined to be 90% (This Asp132Asn contained a bound His-tag which was used to purify the sample (Mitchell and Gennis, in press)).

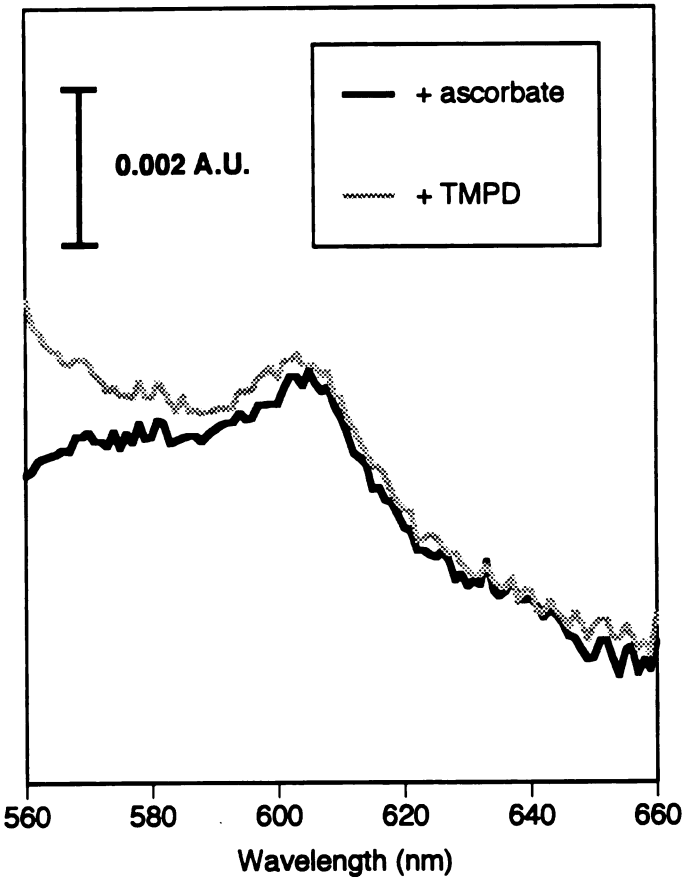


Figure 4.6

Table 4.2 Reconstituted orientation assay of wild type, beef heart, and several mutants. These samples were assayed as indicated in Figure 4.6. Correct orientation indicates the percentage of the oxidase with its cytochrome *c* binding site facing outward from the vesicles. The percent remaining indicates the amount of oxidase remaining after the reconstitution as compared to the known amount of oxidase before the reconstitution. The concentration in the vesicles were determined from the same data used to determine the orientation, by using the absorbance at 605 nm after the addition of the TMPD, and an extinction coefficient at (605-580 nm) of 27 mM^{-1} cm^{-1}.

Table 4.2

	% correct orientation	% remaining
reconstituted oxidase		
wild type	79	79
beef heart	74	94
Asp132Asn	53	45
Asp132Asn(His-tag)	90	59
Asp132Ala	89	80
Tyr422Phe	92	59
Tyr422Ala	79	59

including lower TMPD levels, higher pH, and some degree of inverted orientation of the enzyme; and also due to some loss of the enzyme during reconstitution. In contrast, the mutants Asp132Asn/Ala and Asp412Asn show no loss of activity suggesting that they are stabilized by reconstitution.

Orientation was determined for the wild type, beef heart, Asp132Ala/Asn, and Tyr422Ala/Phe (Wrigglesworth and Nicholls 1979; and Chapter 3). Figure 4.6 shows the spectra for Asp132Asn reduced with ascorbate to determine amount of oxidase with its cytochrome *c* binding site facing outward, and with TMPD to determine the oxidase with this binding site facing the interior of the vesicles. There is little significant difference indicating that most oxidase is in the correct orientation, and ruling this out as a possible problem for measuring proton pumping. Table 4.2 shows the orientation results for this mutant, the wild type, beef heart, and the Tyr422 mutant. Beef heart only shows 70% in the correct orientation, but the *Rb. sphaeroides* enzymes are incorporated correctly 90-100% of the time. There does appear to be some loss of the enzyme during the reconstitution, which may be the major cause for the reduced activity seen after the reconstitution (See Table 4.1).

Addition of cytochrome *c* to the reconstituted oxidase does not cause much increase in oxygen consumption if the vesicles are intact and the oxidase is inserted properly, because a proton gradient is immediately established which inhibits electron transfer activity. Addition of a protonophore (CCCP) and an ionophore (valinomycin) releases the proton and electrical gradient and stimulates the O_2 consumption rate of the native reconstituted enzyme.

Figure 4.7 Effects of valinomycin and CCCP on the oxygen consumption of reconstituted wild type and mutant Asp132Asn cytochrome oxidase. Rates of oxygen consumption were measured polarographically at 25 oC in 10 mM HEPES, 41 mM KCl, 38 mM sucrose, pH 7.4, with 5.6 mM ascorbate and 0.28 mM TMPD and 30 μM cytochrome *c*. After adding 5 uL of wild type vesicles or 100 uL of Asp132Asn (D132N) vesicles to 1.8 mL of medium in a stirred cell, controlled activity was measured. The effect is shown for addition of A. valinomycin (4.4 μM) followed by CCCP (5.6 μM) or B: CCCP followed by valinomycin.

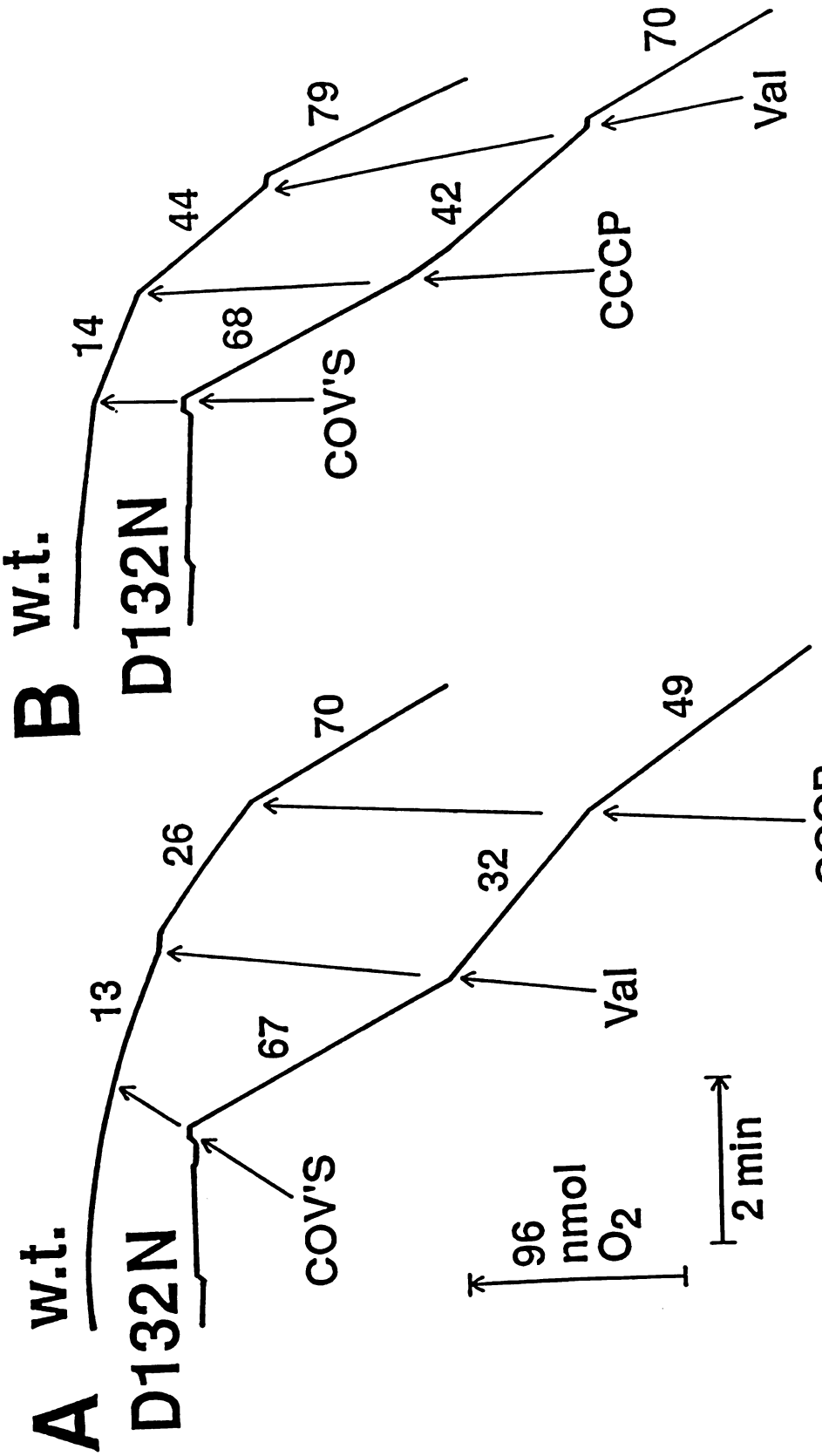

A
W.t.
D132N
COV'S
13
67
26
70
Val
32
49
CCCP
96 nmol O2
2 min
B
W.t.
D132N
14
68
COV'S
44
42
CCCP
79
70
Val

Figure 4.7

Figure 4.8 A comparison of the effect of pH on the activity of purified D132N and wild type cytochrome *c* oxidase. Activities were assayed polarographically using 0.003 μM wild type or 0.06 μM D132N in 50 mM potassium phosphate for pH 6.5 to 7.5 and in 50 mM TRIS for pH 7.5 to 9, including 0.056% lauryl maltoside, no phospholipids, but using the concentrations of cytochrome c, ascorbate, and TMPD as indicated in Table 4.1. Relative activities were calculated as a percent of the highest turnover measured: for wild type (1000 s^{-1}) (•); for D132N (70 s^{-1}) (❑).

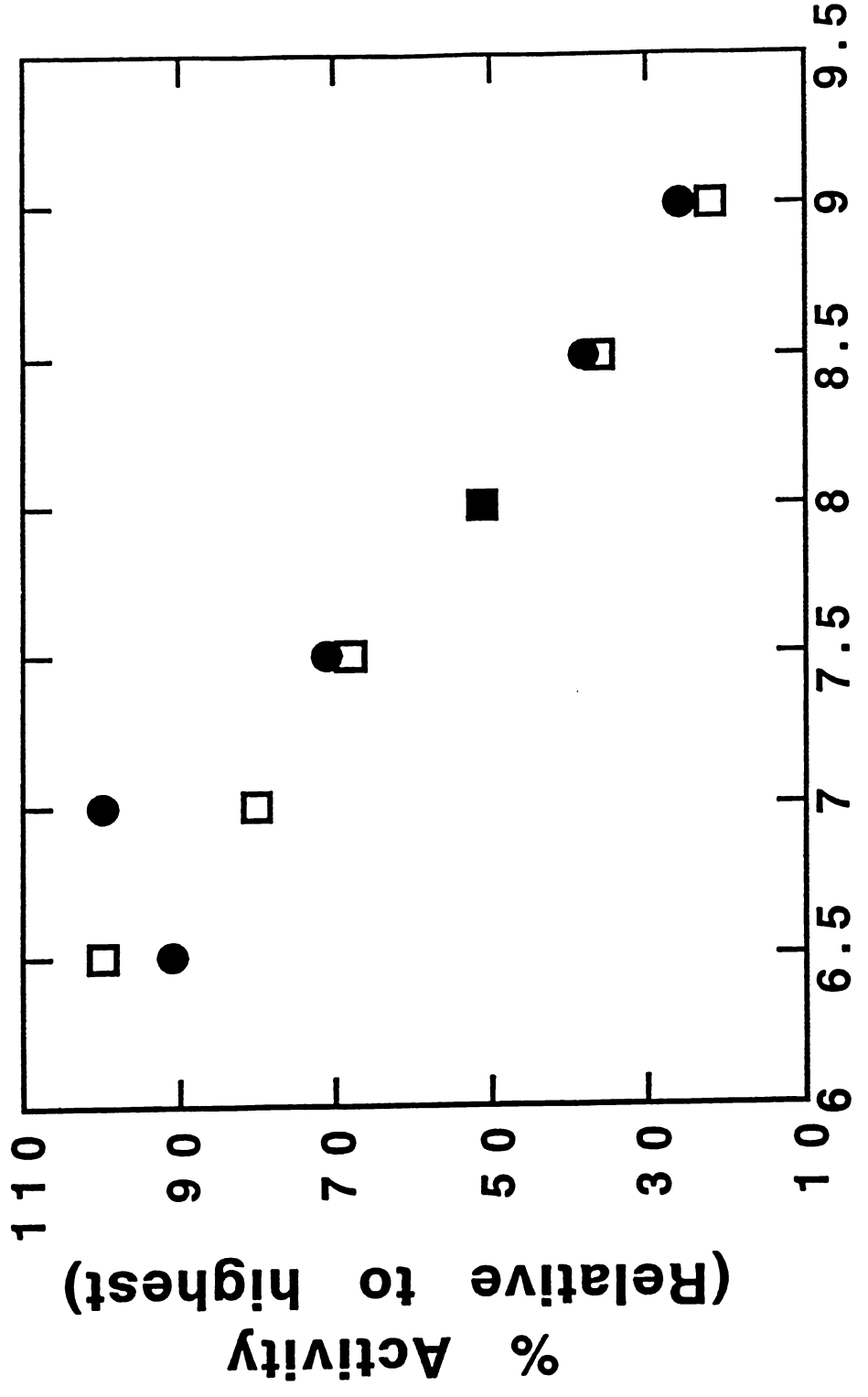

% Activity
(Relative to highest)
110
90
70
50
30
10
6
6.5
7
7.5
8
8.5
9
9.5
pH

Figure 4.8

Significant stimulation shows intactness of the vesicles and good incorporation of the oxidase (Table 4.1). All of the vesicles except those of the Asp132 mutants show expected increased activity. In contrast, the Asp132 mutants show decreased activity in response to either valinomycin or CCCP (See Figure 4.7) or nigericin (data not shown). Nevertheless, the fact that the reconstituted Asp132 mutants do respond to a change in the proton or electrical gradient is evidence that the oxidase inserts properly and that an intact membrane is maintained. Indeed, disruption of the vesicles with detergent overcomes the inhibition, presumably by completely eliminating membrane gradients. Furthermore no inhibition is observed with the purified enzyme at similar concentrations of lipid, valinomycin, CCCP, or nigericin.

A direct effect of altered internal or external pH on the activity of the mutant was also considered. However, no difference is observed in the pH response of purified wild type and Asp132Asn (Figure 4.8); both activities increase with decreasing pH as previously reported for the beef heart enzyme (Gregory and Ferguson-Miller 1988).

Proton pumping Proton pumping of the Tyr414Phe/Ala mutants was determined to test the hypothesis that it may be a critical residue for proton pumping (see Figure 4.9 for the proton pumping of Tyr422Phe). Both mutants showed normal proton pumping in which the addition of cytochrome *c* caused a rapid acidification. After the addition of the uncoupler CCCP, a second addition of cytochrome c gave only the net alkalinization showing substrate consumption of 1 H^+/e^-. Proton pumping was calculated to be 0.5-0.6 H^+/e^-, which was the same range as that shown by the wild type for the same set of experiments.

Figure 4.9 Tyr422Phe shows normal proton pumping. Proton pumping measurements were made as indicated in Figure 4.10 using a spectrophotometric assay by following the pH change of phenol red. To 2.35 mL of proton pumping buffer, 150 uL of reconstituted vesicles were added containing Tyr422Phe (0.1 nmol). Equilibration was done as in Figure 4.10. Addition of 2.24 nmol of cytochrome *c* caused the decrease in absorbance indicating the acidic pH change of proton pumping. After the addition of CCCP only the alkalinization is seen. For this particular experiment 0.5-0.6 H^+/e^- were pumped. An H^+/e^- of 0.5-0.6 was also obtained for the wild type oxidase during this set of experiments.

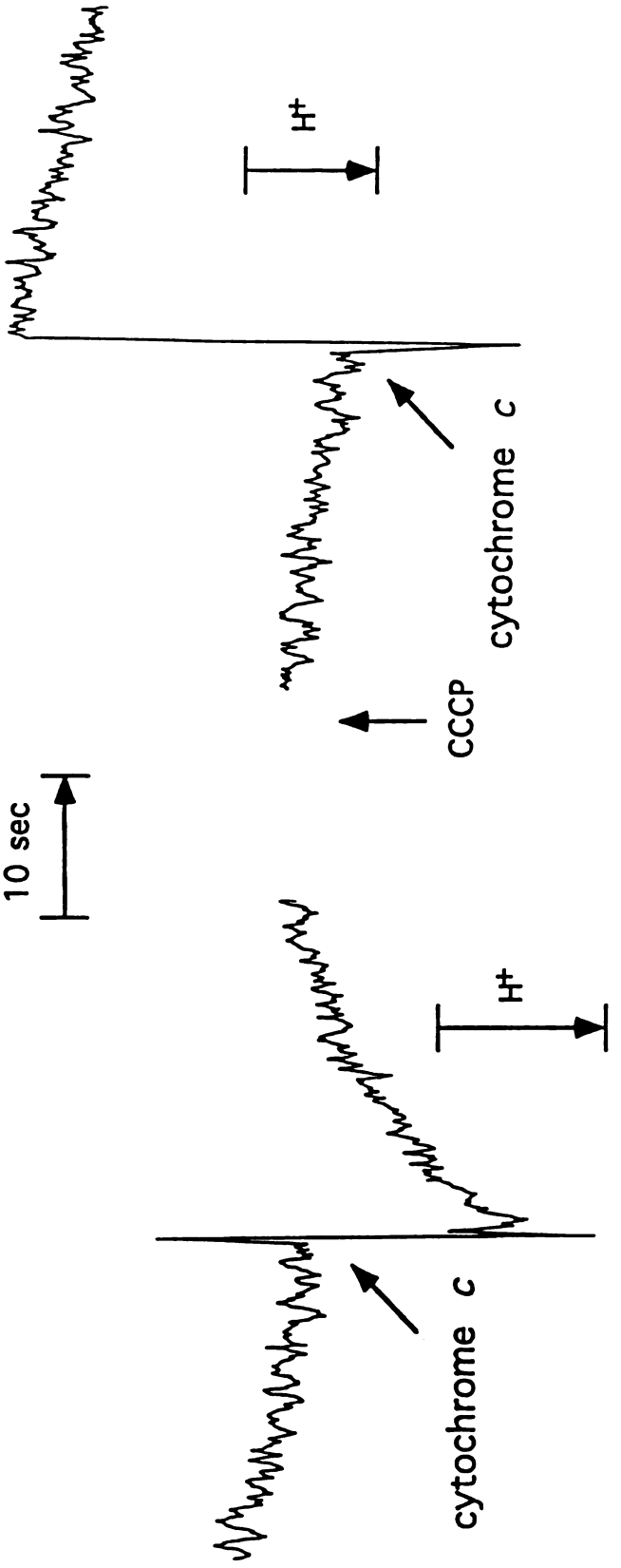


Figure 4.9

Figure 4.10 Spectral analysis of proton pumping and cytochrome c oxidation by reconstituted wild type and mutant cytochrome *c* oxidases. Both activities were assayed at 22 ^{0}C in 2.5 mL of 50 μM $NaHCO_3$, 45 mM KCl, 44 mM sucrose, with or without 50 μM phenol red, pH 7.4, with 0.08 nmol of cytochrome *c* oxidase reconstituted into soybean phospholipid vesicles. Following addition of the vesicles to a stirred cell in an Aminco DW2a spectrophotometer, valinomycin (3.2 μM final concentration) and dilute CCCP (0.4 nM final concentration) were used to equilibrate the vesicles. The upper tracings show the oxidation of reduced cytochrome *c* at 550 minus 505 nm in the absence of phenol red. Extravesicular pH changes are recorded in the lower tracings, as measured at 556.8 minus 504.7 nm by phenol red. Rapid acidification occurred upon addition of 2.1 nmol cytochrome *c* (final concentration 0.8 μM) to wild type (A) or Thr413Asn (C), but not in the case of Asp132Asn (B). In the presence of concentrated CCCP (5 uM), addition of cytochrome *c* produced the expected alkalization. Addition of 0.5 nmol HCl standard before CCCP addition (H^+_0), and after (H^+_i) caused the changes in absorbance indicated and were used to calculate the protons extruded and the protons consumed, respectively. The H^+/e^- ratio can be calculated from the protons extruded (H^+), knowing the amount of cytochrome *c* added (e^-).

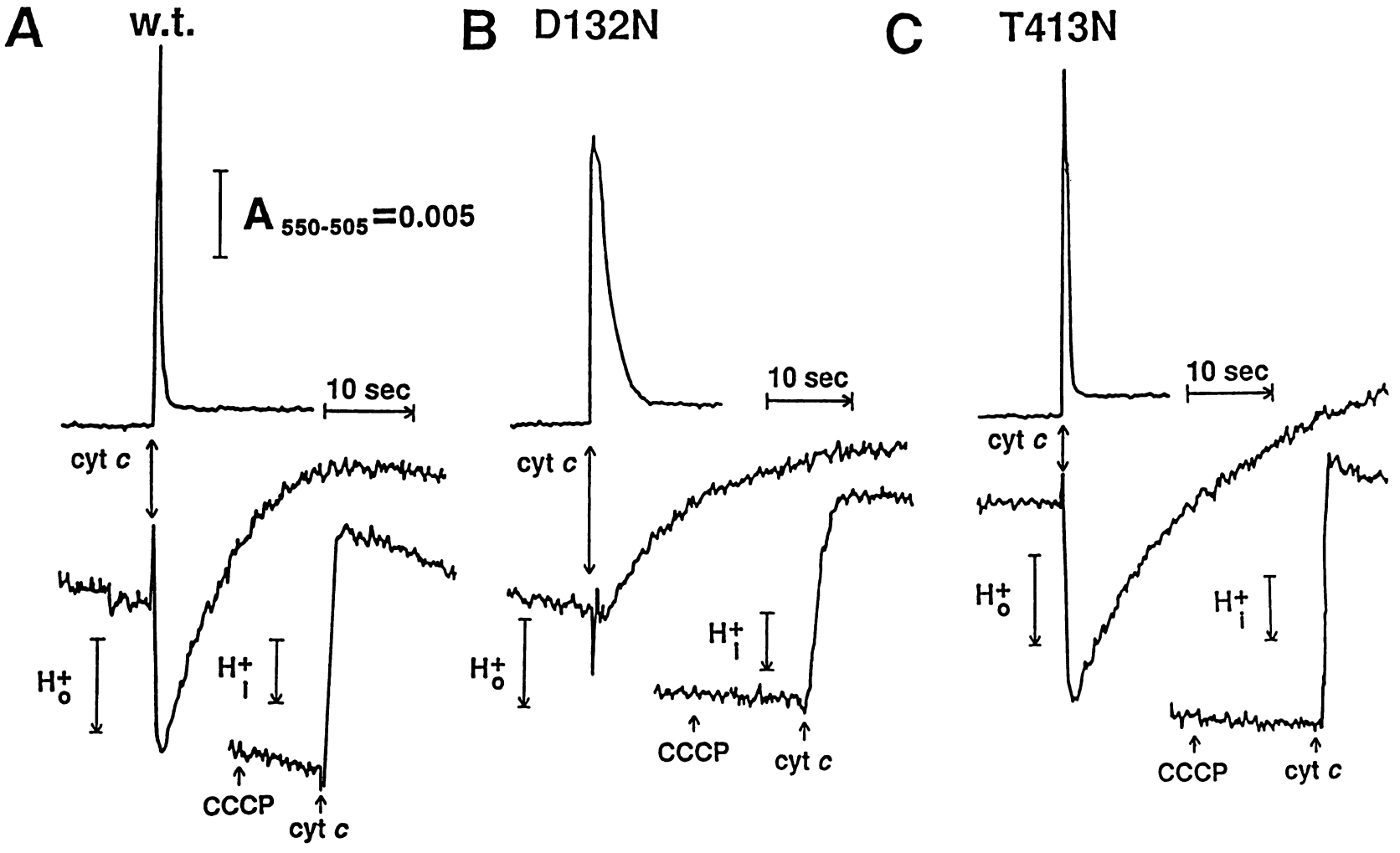

A
w.t.
$A_{550-505}$=0.005
10 sec
cyt c
H^+_o
H^+_i
CCCP
cyt c
B
D132N
10 sec
cyt c
H^+_o
H^+_i
CCCP
cyt c
C
T413N
10 sec
cyt c
H^+_o
H^+_i
CCCP
cyt c

Figure 4.10

The upper portions of Figure 4.10 are recordings of the change in oxidation state of cytochrome *c* after addition to the oxidase-containing vesicles; the lower portions show the concomitant changes in pH in the extravesicular solution. Shown are wild type, Asp132Asn, which does not pump protons, and Thr413Asn, another example of a mutant that has normal proton pumping efficiency. Reduced cytochrome *c* is rapidly and completely oxidized in all cases, though with a half-time about three-fold greater for Asp132Asn than for wild type or Thr413Ala. This three-fold difference in rate is observed at 0.8 μM cytochrome *c* and is in marked contrast to the >20 fold difference in maximal velocity observed at 30 μM cytochrome *c* with the purified enzyme. These results suggest that the mutation has a profound effect on the internal rate of electron transfer that would only be limiting at saturating cytochrome *c*.

The proton concentration changes shown in the lower portion of Figure 4.10A are typical for wild type enzyme. Acidification is followed by alkalinization due to proton leakage back into the vesicles in response to internal proton consumption. A similar result is obtained with Thr413Ala (Figure 4.10C), but Asp132Asn (Figure 4.10B) shows no acidification, only alkalinization. Asp132Ala showed similar results to Asp132Asn. In all three cases shown, addition of the uncoupler CCCP allows proton equilibration across the membrane and only net rapid alkalinization is seen. Stimulation of the rate of alkalinization after addition of CCCP is evidence that the vesicles were maintaining a proton gradient. Even Asp132Asn showed stimulation under these assay conditions where valinomycin had already been added, consistent with the oxygen consumption assay (Figure 4.7). The other mutants (Table 4.1) showed proton pumping behavior similar to wild type and Thr413Asn giving normal H^+/e^- ratios.

Discussion

General Considerations Bacteriorhodopsin provides the only protein model for a proton pumping mechanism that is backed by extensive structural and mechanistic data. Several aspartic acids and an arginine have been identified as part of a proton pumping pathway for which the driving force is a light-induced conformational change of retinal (Henderson *et al.*, 1990; Krebs and Khorana 1993). It is suggested that the path for protons also involves water molecules within the protein to complete a proton relay system (Henderson *et al.*, 1990) similar to that proposed by Nagle and Morowitz(Nagle and Morowitz 1978).

To test whether the proton pumping model provided by bacteriorhodopsin is applicable to cytochrome oxidase, it is important to identify mutants in which proton pumping is inhibited. A complication in interpreting any effects on proton pumping is the dual role of protons in the oxidase reaction: four protons are consumed from the inside of the membrane for each pair of electrons transferred, two protons in the reduction of oxygen to water and two in the pumping reaction. Different proton paths may exist for substrate and pumped protons, since substrate protons must directly access the oxygen intermediates at the binuclear center while pumped protons may not.

If there is only one path for protons to the binuclear center, then blocking this path would be expected to inhibit electron transfer and proton transport equally, unless protons were used preferentially for reduction rather than for pumping. Even if two separate paths exist, inhibition of the proton pumping pathway could slow electron transfer in so far as there is tight coupling between the two processes. .

Although theoretical and mechanistic arguments can be made for obligatory coupling (Brand *et al.*, 1994), there is evidence for decoupling or slipping in cytochrome *c* oxidase (Murphy 1989). Reaction of oxidase with DCCD, or removal of subunit III, reduces the number of protons pumped per electron transferred (Azzi *et al.*, 1984; Prochaska and Fink 1987; Gregory and Ferguson-Miller 1988). Other evidence suggests that proton pumping by the fully reduced enzyme is only 50% efficient (Oliveberg *et al.*, 1991; Babcock and Wikström 1992). The proton to electron ratio may also vary depending on the rate of electron input to cytochrome oxidase in whole mitochondria and reconstituted vesicles (Capitanio *et al.*, 1991; Papa *et al.*, 1991).

Rationale for mutant selection Site-directed mutagenesis in cytochrome *aa*3 was focused on residues that could participate in a proton transfer network and were highly conserved. Likely candidates are amino acids with side chains having available protons. While any amino acid except proline might play some role in a network through its backbone carbonyl or amide, these groups are normally occupied in hydrogen bonds to stabilize the α-helix. In the case of proline, its amide nitrogen is involved in forming the cyclic structure of the side chain. Thus the carbonyl of the amino acid preceding the proline by four residues in the chain, will be free to accept another hydrogen-bond (Piela *et al.*, 1987). It has also been proposed that prolines in transmembrane helices could be important for regulating ion transport by increasing the flexibility of the helix through cis/trans isomerization (Brandl and Deber 1986). While in the lac permease these residues do not appear to be critical (Consler *et al.*, 1991), in bacteriorhodopsin mutation of the prolines has an effect on the rates of deprotonation and reprotonation of the schiff base (Zhang *et al.*, 1993).

According to the bacteriorhodopsin model, acidic residues should be prime candidates for a proton relay system. Only one conserved acidic residue, E286, is predicted to exist in the membrane domain of cytochrome oxidases. While the E286N mutant of cytochrome *aa*3 is inactive, the same mutation in the homologous cytochrome *bo* is active and pumps protons (Thomas *et al.*, 1993); therefore a critical role in proton pumping is unlikely.

Helix X Tyrosine 422 is predicted to be in transmembrane helix X which also contains one of the heme *a* ligands, His421, and the single heme *a*3 ligand, His419. Experiments have indicated that the His419 ligand dissociates during photolysis of CO (Woodruff *et al.*, 1991). This led to the development of a detailed mechanistic model of proton pumping in which the Tyr422 and the His419 could carry protons, and through an alternating exchange of the ligands, drive proton pumping (Rousseau *et al.*, 1993) (also see Chapter 2). If oxidase follows this mechanism, then mutation of the tyrosine should have a severe effect on the activity of the enzyme. However, the mutation of the residue to phenylalanine or alanine still leaves significant electron transfer and does not inhibit proton pumping. Thus it is not required for proton pumping in the way that is suggested. Since electron transfer is inhibited there is still the possibility of it having some role in the proton pumping mechanism, but it is not critical for the pump.

IX-X Loop Among the mutants analyzed in this extra-membrane region, Thr413Asn is most like wild type with only slightly lower electron transfer activity and normal pumping, arguing against an important mechanistic role. Modification of Asp412 to Asn, on the other hand, causes strong inhibition of electron transfer, but minimal alteration of spectral properties (Hosler *et al.*, 1994b) and no change in protons per electron pumped. Tyr414Phe and His411Ala have shifted α bands (Hosler *et al.*, 1994b) and

inhibited electron transfer but again unaltered pumping efficiency. Thus there is no positive evidence to implicate residues 411, 412, and 414 in proton pumping, but a role in this process cannot be ruled out since their overall activity is diminished. It should be noted that studies indicate that residues 411 and 412 participate in a Mn/Mg binding site (Espe *et al.*, 1995; Hosler *et al.*, 1995); loss of the metal after mutation of these residues could account for the loss of function (Hosler *et al.*, 1995). Indeed, a role for this non-redox active metal in proton pumping can be envisaged.

Helix VIII Because Lys362Met was inactive and Thr352Ala was difficult to purify and had low activity, neither could be tested for proton pumping. Both these residues have been proposed for a role in proton transfer to the binuclear center (Hállen and Nilsson 1992; Hosler *et al.*, 1993). Their activity characteristics and altered spectral properties support the idea of helix VIII being proximal to the binuclear center, but do not establish its role. Mutants Thr359Ala and Pro358Ala show reduced activity, about 30% of wild type, but no significant change in spectral properties or proton pumping efficiency, as measured by H^+/e^-. Since the native spectral characteristics of these mutants indicate no general disruption of protein structure, the observed loss of activity could be due to a limitation of proton access to the active site. Indeed, if two pathways for substrate and pumped protons exist, as indicated in Figure 4.1, inhibition of a substrate proton pathway might be expected to give these results. Similarly, inhibition of a tightly coupled pathway for pumped protons would equally limit electron transfer. Thus these results are consistent with a central role for helix VIII in proton transfer.

II-III loop Conversion of Asp132 in the II-III loop to Asn or Ala caused a dramatic inhibition of electron transfer and these were the only mutations that eliminated proton pumping while retaining the ability to reduce oxygen at

a significant rate. Visible absorbance spectra show that neither mutation has a significant effect on hemes *a* or *a*3. The absence of changes in the vibrational modes of either heme *a* or heme *a*3 in resonance Raman analysis of Asp132Asn is further strong evidence of native heme environments. Only the CO binding appears to be slightly altered. Heterogeneity of the sample or a minor structural change in the binuclear center (possibly in the vicinity of spectrally silent Cu_B) may have occurred such that the CO binding equilibrium is altered. Although CO binding would suggest a quantitatively similar structural perturbation in His411Ala (Hosler *et al.*, 1994b), it remains competent in proton pumping.

Lack of evidence of a significant structural change at the binuclear center favors the idea that electron transfer activity in the Asp132 mutants is limited by either availability of substrate protons to the active site (single pathway model) or by the obligatory coupling of proton pumping to electron transfer (two pathway model). Either scenario, along with the predicted location of the II-III loop, would implicate Asp132 as a critical residue in a proton relay system from the intracellular side of the membrane. Similar conclusions have been reached from studies of mutants in the homologous residue in cytochrome *bo* of *E. coli* (Thomas *et al.*, 1993). However, in that study, proton pumping was carried out in spheroplasts where the detailed response to pH and electrical gradients could not be easily examined.

When incorporated into phospholipid vesicles, mutants of Asp132 show an unusual response to addition of ionophores or protonophores: their activity is inhibited rather than stimulated by release of either the proton or electrical gradient. A possible explanation for this response is that the mutation has changed the pH sensitivity of the mutant. Although the pH inside and outside of the vesicles should be the same after reconstitution, when valinomycin is

added before beginning the proton pumping assay, external alkalinization is observed. This implies a potassium gradient (higher internal [K^+]), but is also consistent with the observation of Madden and Redelmeier (1994) that there is a higher pH inside than outside of the vesicles (greater than 8.6, versus 7.4). If Asp132Asn were less active than wild type at low pH, then addition of valinomycin (or CCCP or nigericin) might inhibit the enzyme activity. However, the pH dependence of the activity of the purified D132N is essentially identical to wild type, arguing against a simple pH effect. Thus it seems more likely that release of a gradient of pH or charge is involved in the inhibition. If D132 were normally involved in a salt bridge holding the II-III loop in a stable conformation, loss of its negative charge would make this region more conformationally flexible and could alter its sensitivity to changes in the electrical/pH gradient. Alternatively, the loss of D132 as an entry point for protons could bring into play a new, less efficient pathway that might respond differently to an electrochemical potential.

Conclusions None of the residues examined by mutagenesis in helix X, the IX-X extracellular loop or helix VIII preferentially inhibit proton pumping, but since a number retain native spectral properties and have lower or no activity, their involvement in proton transfer remains a real possibility. More definitively, loss of proton pumping with retention of low but significant electron transfer activity in the case of mutants at Asp132 is strongly suggestive of a critical role in proton pumping. It remains to be determined whether Asp132 is a direct participant in a proton relay system or an important determinant of a structural element that is essential for this process.

Chapter 5 - Measurement of membrane potential generation by Asp132Ala and effects of free fatty acids on its activity.

Introduction

In Chapter 4 the inhibitory effect of ionophores was shown on the reconstituted Asp132 mutants. A further understanding of this response may help explain why the Asp132 mutants do not pump protons and provide mechanistic information about the pumping reaction. While some evidence was given in the previous chapter that the ionophores are not inhibiting the enzyme by a direct effect but through changing the membrane potential or pH gradient, further confirmation of this would be useful. Experiments in this chapter help to confirm this result and show another interesting feature of the mutation, the addition of free fatty acids can partially overcome the defect. This 'chemical rescue' by fatty acids was unique to this mutant. The fluorescent dye diSC-3,5 was used to measure the membrane potential: its fluorescence decreases when a potential is developed, due to its movement into the hydrophobic membrane environment. This technique of measuring membrane potentials has been well characterized (Bashford and Smith 1979) and used previously on reconstituted beef heart enzyme (Singh and Nicholls 1985). It is difficult to determine exactly how much membrane potential is produced using this technique. By comparing the result between Asp132Ala and controls reconstituted simultaneously using beef heart oxidase that is known to have a normal membrane potential and wild type oxidase that would be expected to have a normal membrane potential, it is possible to establish whether the responses are qualitatively similar. The stimulation of activity by fatty acids was studied extensively by looking at the effects of changing chain length, the alcohol versus the acid, the effect on other mutants, and whether analogues of the fatty acid could also stimulate.

Experimental procedures

Reconstitution was done as indicated in Chapter 3.

Determination of membrane potential Fluorescence intensity was measured on a Perkin Elmer fluorescence spectrophotometer with excitation set to 622 nm, emission set to 688 nm (10 nm slit), and using 0.45 uM diSC 3,5 as the membrane potential probe at 30 ^{o}C. Reconstituted vesicles (50 uL), containing either wild type or Asp132Ala were added to the buffer: 10 mM K^+ HEPES, 20 mM K_2SO_4, 50 mM sucrose, pH 7.4. To reduce the enzyme cytochrome *c* was added to a final concentration of 1.1 uM. Excess reducing equivalents were added, ascorbate to 5.6 mM and TMPD to 280 uM, to continuously turnover the enzyme allowing generation of a membrane potential. Nigericin was added to 0.5 uM to release the pH gradient; this caused only a small change in the diSC-3,5 fluorescence. Valinomycin was then added to 0.33 uM, releasing the membrane potential and causing an increase in fluorescence to the level seen before the addition of ascorbate and TMPD.

Assay of activity with fatty acids, fatty alcohols, and analogues of arachidonic acid Steady state turnover rates were measured as indicated in Chapter 4 with the specific details as listed in the figure legends. The fatty acids, fatty alcohols and analogues of arachidonic acid were purchased from Sigma. Arachidonic acid purchased from either Sigma or Cayman Chemical gave similar results. These reagents, except for indomethacin, were made up as 250 mM stock solutions in ethanol and stored at -20 ^{o}C. Indomethacin was only soluble in ethanol to 50 mM. Stock solutions of 50 mM and 5 mM were prepared. The same amount from each stock could then be added to the stirred polarograph cell during the steady state turnover assay to give final

concentrations of 5 uM, 50 uM, and 250 uM. Wild-type and mutant enzymes were purified as described in Chapter 4.

Results

Determination of membrane potential of reconstituted Asp132Ala, wild type, and beef heart oxidases As can be seen in Figure 5.1, a similar response is observed in the fluorescence changes of diSC-3,5 for wild type and the Asp132Ala. Beef heart oxidase (not shown) also has the same type of response. For an unknown reason, the addition of oxidized cytochrome *c* causes some decrease in fluorescence. Then ascorbate and TMPD are added to continuously reduce the cytochrome *c* and maintain a steady state turnover of the oxidase causing the build up of a membrane potential. This causes the diSC-3,5 to be pulled into the vesicle membrane resulting in a decreased fluorescence. Nigericin induces an electrically neutral exchange of protons for potassium across the vesicle membrane, resulting in loss of the pH gradient without loss of the membrane potential. Valinomycin allows K^+ to equilibrate across the membrane and thus releases the membrane potential causing the fluorescence to increase to the intensity before the addition of ascorbate/TMPD. Reconstituted Asp132Ala shows the production of a membrane potential like that of the wild type and the beef heart enzymes.

Stimulation of Asp132 mutants by free fatty acids Figure 5.2 shows the polarographic trace of Asp132Ala stimulated by cytochrome *c*, ascorbate and TMPD. Addition of arachidonic acid (5 uM, 50 uM, and 250 uM) caused significant stimulation of the enzyme with the amount of stimulation

Figure 5.1 Reconstituted Asp132Ala shows a normal membrane potential. Addition of 1.1 uM oxidized cytochrome *c* as the substrate caused an initial decrease in fluorescence unrelated to turnover. To generate steady state turnover 5.6 mM ascorbate and 280 uM TMPD were added. This led to a decrease in the fluorescence of the probe, indicating the formation of a membrane potential. Nigericin was added to 0.5 uM to release the pH gradient. Valinomycin was then added to 0.33 uM which releases the membrane potential causing the fluorescence to increase to the same level as before generation of a membrane potential.

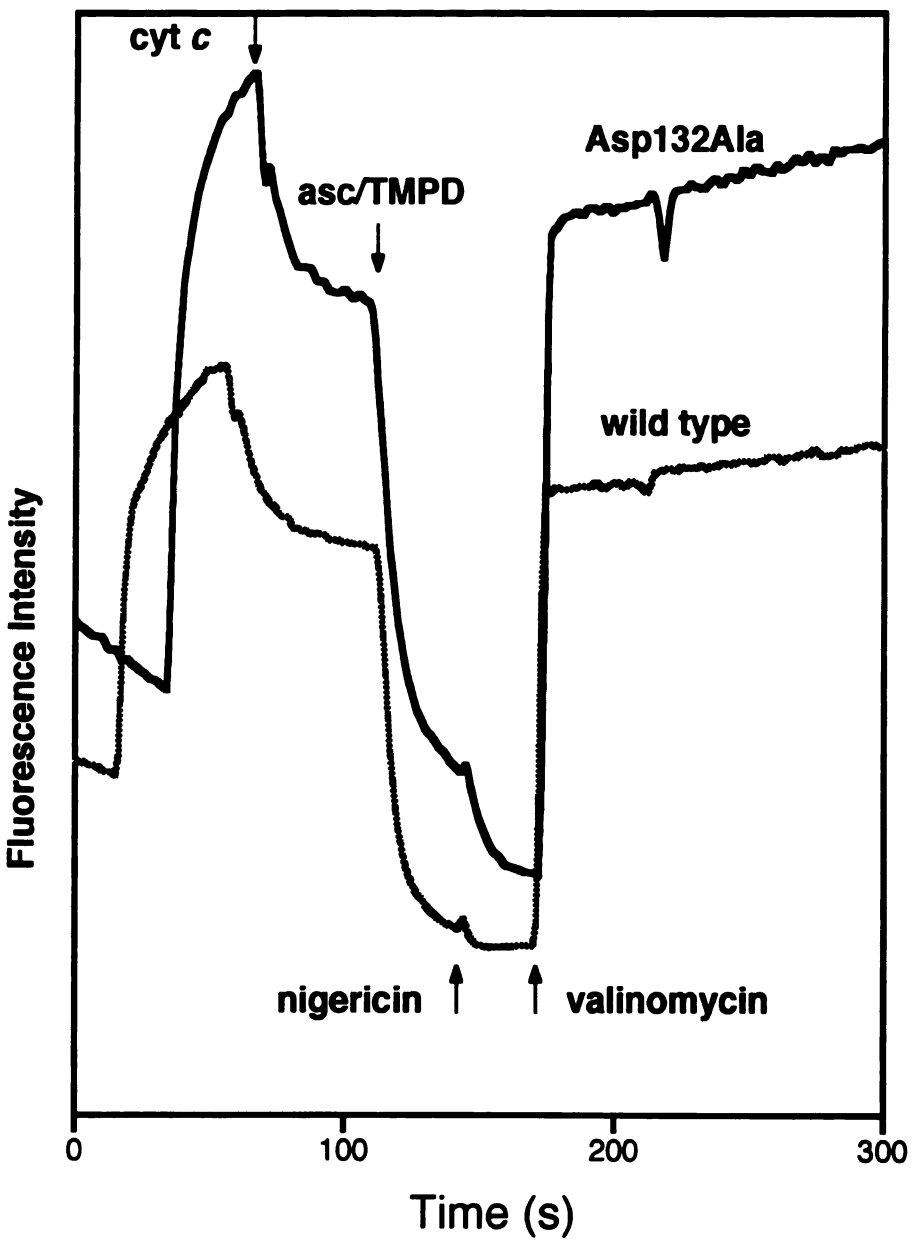

cyt c
asc/TMPD
Asp132Ala
wild type
nigericin
valinomycin
Fluorescence Intensity
0
100
200
300
Time (s)

Figure 5.1

Figure 5.2 Arachidonic acid stimulation of the Asp132Ala mutant. Activity was assayed as indicated in Table 5.1. A. shows stimulation of activity by multiple additions of arachidonic acid and inhibition by 2 mM cyanide. B. shows a single addition of arachidonic acid with inhibition by 9 mM azide. Activity per second is listed along the trace.

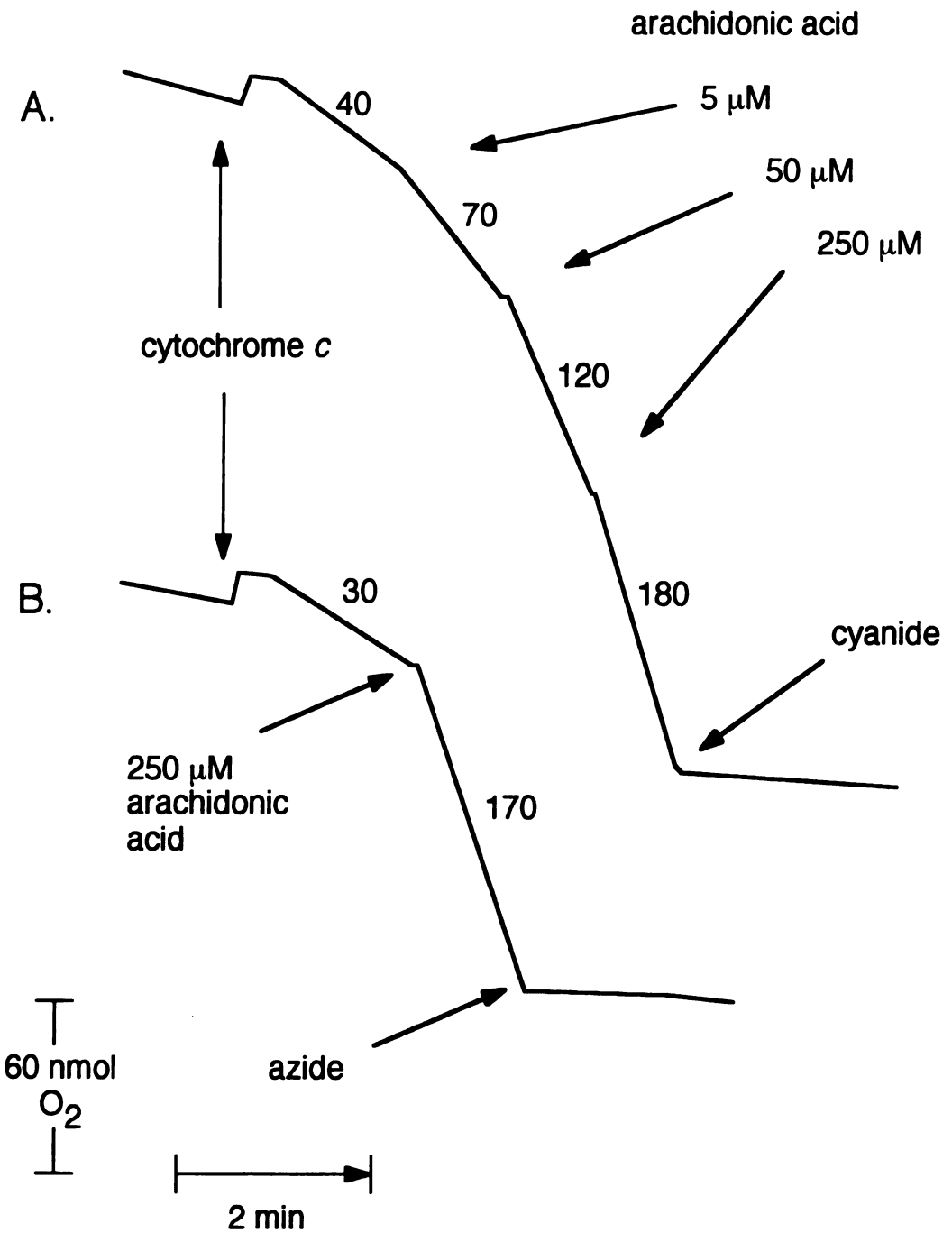

arachidonic acid
A.
40
5 μM
50 μM
70
250 μM
cytochrome c
120
B.
30
180
cyanide
250 μM
arachidonic
acid
170
60 nmol
O2
azide
2 min

Figure 5.2

increasing along with the increasing concentration of the fatty acid, bringing the turnover from 30 sec^{-1} to near 200 sec^{-1}. Inhibition of all cytochrome *c* oxidase activity by cyanide (part A) or azide (part B), inhibitors that bind at the active site of the oxidase, provides evidence that the fatty acid is stimulating the oxidase activity and is not just causing an artifactual secondary reaction with other reagents. Further evidence of this is given by tests of the fatty acid under the same assay conditions excluding oxidase that showed no significant changes in the background rate of oxidation. Both Asp132Ala and Asp132Asn showed stimulation by fatty acids to a similar degree.

Importance of the fatty acid carboxyl and the chain length on stimulation of Asp132Ala This fatty acid effect was more thoroughly characterized to determine whether the carboxyl group on the fatty acid was important for the stimulation and also to determine the effect of the chain length (See Table 5.1). The measurements were made with consecutive additions of low, medium, and high concentrations of each fatty acid or fatty alcohol. It showed that no stimulation occurred with acetate or propionate, indicating that more than just the acidic group was required. Medium chain fatty acids such as octanoic acid and lauric acid showed some stimulation, while the fatty alcohols of the same chain length did not show an effect. The long chain fatty acids from 16-22 carbons showed the most significant effects with the greatest stimulation from arachidonic acid and docosahexanoic acid. Again, alcohols of these two fatty acids did not show any stimulation of the activity. Clearly, the stimulation is caused by more than just a hydrophobic interaction with the protein.

Stimulation of activity by fatty acids is unique to the Asp132 mutants To determine if the fatty acid stimulation was specific for the Asp132 mutants, the same tests with arachidonic acid and its alcohol were performed on wild type, beef heart, and other mutant oxidases (See Table 5.2). Since wild type and many of the mutant oxidases are stimulated by soya bean phospholipids (a mixture of mainly phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol), these enzymes were tested to see if fatty acid stimulation might relate to the stimulation by phospholipid. Asolectin had a stimulatory effect on wild type, beef heart, Thr359Ala, Asp412Asn, and Phe391Gln. No effect was observed on the Asp132 mutants or on Lys362Met, Met262Leu, or His259Asn. In contrast, there was no significant stimulation by fatty acid or fatty alcohol seen with the wild type and beef heart enzymes. Similarly no effect was observed with several of the mutants including Asp412Asn, Lys362Met, and His259Asn. Thr359Ala and Met262Leu showed some stimulation by fatty acid but a similar effect was seen with the alcohol. Phe391Gln showed stimulation by the fatty alcohol, but not the fatty acid. None showed nearly the level of stimulation as seen with the Asp132 mutants. There was little correlation between the stimulatory effect of the phospholipids and that of the fatty acids, indicating the unique role the fatty acid has in stimulating the Asp132 mutants.

Stimulation of Asp132Ala by analogues of arachidonic acid Arachidonic acid is a substrate for prostaglandin synthase, and various inhibitors that bind in the active site of that enzyme, where arachidonic acid normally binds, have been characterized (Laneuville *et al.*, 1994). Several of these were tested

Table 5.1 Fatty acid stimulation of Asp132Ala activity increased with longer alkyl chains, while fatty alcohols had no effect. Activity was assayed using a Gilson oxygraph that measured oxygen consumption of the 13 nM Asp132Ala mutant at 30 uM cytochrome *c*, 1.1 mM TMPD, and 2.8 mM ascorbate in 50 mM potassium phosphate, pH 6.5 at 25 ^{o}C. All fatty acids or alcohols were dissolved in ethanol. For each fatty acid or alcohol consecutive additions were made to a final concentration of 5 uM, 50 uM, and 250 uM to the enzyme during turnover. The numbers in the table are relative to 100 which represents a turnover 50 sec^{-1} for the Asp132Ala mutant.

Table 5.1

	% activity with sequential addition of umol concentration of f. a. or f. alc.		
	5	**50**	**250**
Fatty Acid or Fatty Alcohol			
Na acetate	90	80	70
propionate	80	70	70
propanol	90	90	90
octanoic acid	90	100	120
octanol	100	80	80
lauric acid	120	120	150
lauryl alcohol	110	100	70
palmitic acid	140	210	150
hexadecanol	120	90	80
oleic acid ($18:1^{\Delta 9}$)	120	160	140
linoleic acid ($18:2^{\Delta 9,12}$)	190	260	310
linolenic acid ($18:3^{\Delta 9,12,15}$)	130	170	150
arachidonic acid (20:Δ5,8,11,14)	**180**	**300**	**410**
arachidonoyl alcohol	100	90	100
docosahexanoic acid (22:Δ4,7,10,13,16,19)	**260**	**380**	**330**
docosahexanol	90	90	130

Table 5.2 Stimulation of activity by arachidonic acid is unique to Asp132Ala. Other mutants were tested by the protocol in Table 5.1 for stimulation by arachidonic acid or arachidonyl alcohol. They were also tested with 2 mg of cholate solubilized phospholipid. Two mutations were in subunit II (noted in parenthesis), while the others are all in subunit I.

Table 5.2

	initial activity	maximum % stimulation of activity with addition of arachidonic acid	arachidonyl alcohol	asolectin
oxidase				
wild type	1400	0	0	20
beef heart	450	0	0	10
Asp132Ala	50	300	0	0
Thr359Ala	350	20	30	30
Asp412Asn	370	0	0	40
Phe391Gln	30	0	70	70
Lys362Met	20	0	0	0
Met262(II)Leu	130	40	20	0
His259(II)Asn	10	0	0	0

on the Asp132Ala mutant to see if they might have a stimulatory effect on the activity. Table 5.3 indicates the results of testing these. Benzoic acid although not a characterized inhibitor of the prostaglandin synthase is similar in structure to aspirin. Aspirin may not be the best analogue since it blocks the activity through a covalent modification, whereas the other two tested, flurbiprofen and indomethacin are competitive inhibitors . Benzoic acid showed the least stimulatory effect, indicating that more than just a hydrophobic group and a carboxyl are needed for stimulation. Flurbiprofin showed a small amount of stimulation at the highest concentration. Indomethacin showed more significant levels of stimulation at the lowest concentrations and a moderate level of stimulation at the highest concentration. In each experiment after the addition of the highest concentration of fatty acid, arachidonic acid was added. In all cases this showed further stimulation, and did not show more than the normal amount of stimulation after indomethacin, indicating that the effect was not cumulative. Thus it seems likely that indomethacin is stimulating by the same mechanism as arachidonic acid.

Restoration of reconstituted Asp132Ala respiratory control by addition of fatty acids The unusual inhibitory effect on the reconstituted activity of Asp132Ala by ionophores was discussed in Chapter 4 (see also Figure 5.3 B). It was found that with the addition of fatty acid to the reconstituted enzyme before the addition of the valinomycin and CCCP, the inhibitory effect could be prevented (Figure 5.3 A). Since the fatty acid is capable of acting as an uncoupler (Sharpe *et al.*, 1994), the concentration was kept lower than that required for complete uncoupling. While it appears that this may have restored the respiratory control, the effect is complicated because of the dual role of uncoupling and of stimulation of the activity. As

shown in the previous chapter after CCCP is added to the Asp132Ala vesicles it causes inhibition. If this is followed with the addition of valinomycin a stimulation in activity occurs. It seems possible then that the stimulation with valinomycin as seen in Figure 5.3 A could be the same effect. It is still not clear how the removal of one gradient (pH or membrane potential) can cause inhibition of activity, then if followed by removal of the other gradient, a stimulation occurs.

Experiments were also done to see if proton pumping might be restored. Higher concentrations such as that used for the effect seen in Figure 5.3 A started to cause uncoupling, seen as a faster rate of alkalinization. While it is possible that some proton pumping could have been restored, it was not measurable because of this uncoupling.

Discussion

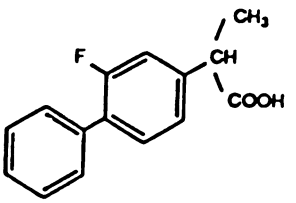
The result that the reconstituted Asp132Ala generates a normal membrane potential under steady state turnover conditions indicates that the enzyme is inserted correctly in the membrane and protons are taken up normally from the interior of the vesicles. This is the first direct evidence that protons are being taken up from inside of the vesicles during steady state turnover.

Fatty acids were shown to significantly increase the electron transfer activity of the Asp132Ala mutant. Longer chain length and the carboxyl of the fatty acid are required for the stimulation with the largest effect from arachidonic acid and docosahexanoic acid. This effect was only seen

Table 5.3 Stimulation of Asp132Ala activity with analogues of arachidonic acid. Both indomethacin and flurbiprofen are prostaglandin synthase inhibitors. Benzoic acid is similar to aspirin that can covalently modify the active site of prostaglandin synthase. Three consecutive additions of fatty acid were made to the assay with a final addition of arachidonic acid. Conditions for the assay are indicated in Table 5.1.

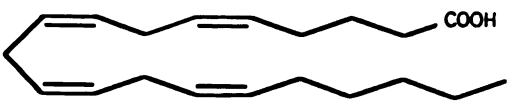
Table 5.3

Flurbiprofen



Indomethacin

Arachidonic Acid



	%activity with sequential addition of umol concentration of analogue			followed by 50 umol arachidonate
	5	50	250	
Analogue				
benzoic acid	110	110	120	260
flurbiprofin	110	110	140	370
indomethacin	120	130	200	290

Figure 5.3 Restoration of respiratory control by the addition of arachidonic acid. Respiratory control ratios were determined on an oxygen polarograph at 25 ^{o}C in 1.8 mL of 10 mM HEPES, 41 mM KCl, 38 mM sucrose pH 7.4. Ascorbate (10 uM final concentration) and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) (0.5 uM final concentration) were used to keep cytochrome *c* fully reduced. The substrate cytochrome *c* at 30 uM final concentration was added to stimulate turnover. To get maximal activity 2 nmol of valinomycin and 10 nmol of carbonyl cyanide m-chlorophenylhydrazone (CCCP) are added. A. the restoration of respiratory control with the addition of arachidonic acid to 20 uL of vesicles giving a final concentration of 7 nM oxidase. B. the inhibition of activity with the addition of valinomycin when fatty acid is not added, using 50 uL of vesicles giving a final concentration of 18 nM oxidase.

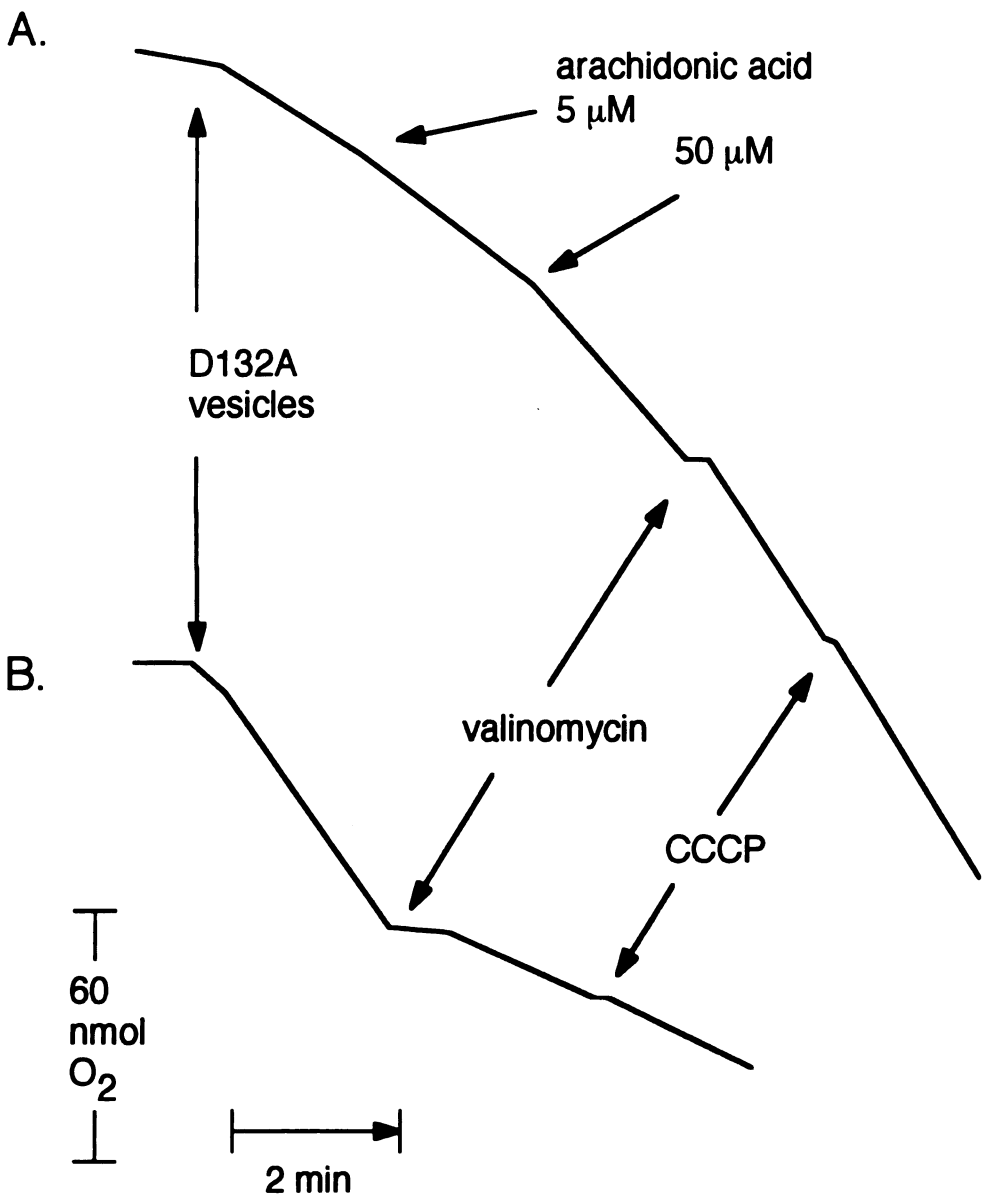

A.
arachidonic acid
5 μM
50 μM
D132A
vesicles
B.
valinomycin
CCCP
60
nmol
O_2
2 min

Figure 5.3

with the Asp132 mutants, not with other mutants, nor with the wild type or beef heart enzymes, indicating a unique interaction with the Asp132 mutants. Since the Asp132 mutants are not stimulated by phospholipids but instead by fatty acids, while wild type and the other mutants show stimulation by phospholipid but not by fatty acid, it can be concluded that the fatty acid and phospholipid stimulatory effects occur by different mechanisms.

Although two analogues of arachidonic acid, benzoic acid and flurbiprofin, showed very little stimulatory effect, indomethacin was able to stimulate up to two fold, indicating that the binding site of the fatty acid may have some similarity to the prostaglandin synthase active site. This provides some evidence that the fatty acid may be in a folded conformation. Since the stimulation was not as great as with the fatty acids, the greater flexibility of the fatty acid may be required for the maximal effect.

A nonphysiological effect of the fatty acids A possible explanation for the restoratory effect of the fatty acid is that it is binding in a position so it can supply the missing carboxyl of the Asp132 mutants. Alternatively, it could be interacting at another site on the enzyme to improve proton access to the active site. This mechanism is similar to that observed in bacteriorhodopsin (Otto *et al.*, 1989; Tittor *et al.*, 1989) and reaction center (Takahashi and Wraight 1991) where the activity of mutants with deleted acidic residues is restored by addition of soluble acids. Such acids have not been tested on the Asp132 mutants because most are inhibitory to the oxidase.

If the fatty acids are improving activity by restoring proton access through a different pathway the fatty acid might be expected to stimulate activity of other mutants which are limited by proton access. Since none of

the other mutants tested showed any significant stimulation with fatty acids, we may conclude that either these mutants are not limited by proton access or if access is limited the fatty acids are not capable of binding at the mutation site but bind only at the Asp132 mutation site. Further experiments will be required to clarify the exact role of the fatty acids in the stimulatory activity on the Asp132 mutants.

Could there be a physiological explanation for the stimulation of Asp132 mutants by fatty acids? In considering the possibility of a physiological effect, evidence exists that addition of fatty acids can stimulate the activity of the beef heart cytochrome oxidase (Labonia *et al.*, 1988; Thiel and Kadenbach 1989). This has not been shown to occur with the bacterial wild type enzyme. However, these data show that noncovalently attached fatty acids are capable of stimulating activity of the Asp132 mutants. If one or more fatty acids are important to the activity of the oxidase then mutation of the Asp132 residue might decrease the affinity for fatty acids, so that higher concentrations are required for stimulation. This mechanism can be compared to the uncoupling protein in brown fat that passively transports protons (Jezek *et al.*, 1994). For proton transport to occur fatty acids must be associated with the uncoupling protein, but are not covalently attached (Rial *et al.*, 1983). Theoretical models have been proposed in which the fatty acid is bound in a stable conformation, with its carboxyl being used to move protons (Klingenberg 1993). Other models propose that the fatty acid itself is moved by the protein and in the process carries a proton across the membrane (Skulachev 1991). More recent experiments by Garlid and coworkers indicate that the fatty acid anion is transported by the uncoupling protein (Garlid, personal communication). Whether this type of fatty acid role can relate to oxidase activity or regulation remains to be determined.

One last possibility to consider is whether the fatty acid could play a physiological role through a covalent attachment to the oxidase. Since fatty acids have been found covalently attached through the N-terminal amino group of glycine (Casey 1994; Milligan *et al.*, 1995), it might be possible that a fatty acid could attach to the oxidase through one of the two conserved asparagines that have been shown to interact with the Asp132 (García-Horsman *et al.*, 1995). Covalent binding of fatty acids has also been shown to cysteines in membrane incorporated G proteins (Milligan *et al.*, 1995), and in the case of rhodopsin the alkyl chain appears to act as an anchor in the membrane (Moench *et al.*, 1994). This type of mechanism could not be easily reconciled with the results in the Asp132 mutants because of the evidence that the carboxyl group of the fatty acid, which would be unavailable if covalently attached, is necessary for the stimulatory effect.

Reverse respiratory control The studies in this chapter have not provided a much clearer explanation of the unusual respiratory control of the Asp132 mutants. However, a possible explanation for the unusual effect of ionophores on the reconstituted Asp132Ala/Asn was proposed through a personal communication with Marten Wikström, and is worth some discussion at this point. During turnover of the reconstituted enzyme the mutant may still allow some proton uptake from the vesicle interior as in wild type, generating a membrane potential and pH gradient. Formation of the membrane potential may make it easier for the mutant to draw protons into the active site from the outside of the membrane allowing oxygen reduction to go faster. When either the pH or the electrical gradient is removed, proton uptake from the outside would be less favorable, causing a decrease in the activity of the enzyme.

This dual access model is harder to reconcile with the observation that the rate shows a decrease with the removal of one gradient ($\Delta\psi$ or ΔpH) and then when this is followed by the removal of the other gradient, an increase in rate occurs. However, the amount of inhibition and stimulation varies with the preparation of enzyme from a very small stimulation (Figure 5.3B) to almost total restoration of the rate to that observed before addition of ionophores (Figure 4.7). It is therefore likely that two opposing effects are involved: first, a normal inhibition of the enzyme activity by the gradients, and second, a stimulation of the activity caused by the gradients promoting easier proton uptake from the outside. Thus release of both gradients removes the normal inhibitory effect and allows the rate to increase to the extent that proton access routes will allow. Further studies are needed to characterize this effect which could provide new information about the mechanism of proton pumping and respiratory control in cytochrome oxidase.

Chapter 6 – Further mutagenesis of residues in the IX-X Loop

Introduction

As discussed in Chapter 4, the mutation of three of the four highly conserved residues in the IX-X extracellular loop caused some inhibition of electron transfer, but did not inhibit proton pumping. The lowered activity could be indicative of a role in proton pumping, if the normal proton pathway could be circumvented by another pathway or the mutated residue replaced by a water. Other studies have shown that the loop overlays the active site in subunit I, with His411 and Asp412 being near the heme a3/Cu_B site and the Thr413 and Tyr414 close to heme *a* (Hosler *et al.*, 1994a; Hosler *et al.*, 1994b). It was also shown that mutation of His411 to Ala, or Asp412 to Asn, causes the loss of a nonredox active manganese and inhibits the electron transfer activity by about 50% (Hosler *et al.*, 1995). Studies of this manganese in *Rb. sphaeroides* show that it can be replaced by a magnesium (Hosler *et al.*, 1995). Low levels of manganese (Fee *et al.*, 1986; Numata *et al.*, 1989; Lauraeus *et al.*, 1991; Haltia 1992) or stoichiometric levels of magnesium (Buse and Steffens 1991) are found in other bacterial oxidases, and all eukaryotic enzymes so far examined, suggesting some general role of this non-redox active metal site. Because the soluble portion of subunit II containing the binuclear Cu_A must closely approach the binuclear center, it was proposed that the manganese/magnesium might be involved in stabilizing the interaction of subunits I and II to facilitate electron transfer between the binuclear Cu_A and heme *a*(Hosler *et al.*, 1995). Using X-band and Q-band EPR, and ESEEM the manganese has been shown to be a six-coordinate metal (Espe *et al.*, 1995). This study also showed that the redox state of the enzyme had only a small effect on bond lengths or angles at this metal center. Since it appears to be

located near the active site, the Mn might be expected to be sensitive to conformational changes that occur during reduction of the enzyme. Thus it was concluded that the protein does not undergo major conformational rearrangements between the two states in the area of the active site. A model of the Mn site based on the crystal structure of a manganese site with similar EPR characteristics in lectins (Espe *et al.*, 1995), predicts that His411 is a direct ligand to the metal, while Asp412 may act as the ligand to a water that is a direct ligand to the metal.

Mutation of Thr413 and Tyr414 did not have an effect on manganese binding. But while mutation of Thr413 to Asn showed insignificant spectral effects, mutation of the Tyr414 to Phe caused a shift of the alpha band from 605.4 to 610 nm. This agrees with data from the oxidase in *Tetrahymena pyriformis* that has a phenylalanine in place of the tyrosine and an alpha band at 617 nm (Ziaie and Suyama 1987).

This IX-X loop is located where residues may be involved in transferring protons away from the binuclear center. Thus the possibility exists that the Mn/Mg could play some role in this process. To further investigate the importance of these residues, four more mutations were made including, His411 to Gln and Asp412, Thr413, and Tyr414 to alanine. A preliminary characterization is reported in this chapter.

Experimental procedures

Any basic molecular biology details not listed below followed those in Sambrook *et al.* (1989). Collection of visible absorbance spectra and determination of steady state electron transfer activity were done as indicated in Chapter 4 with any other details listed in the figure legends.

Materials Restriction enzymes and ligase were obtained from either Boehringer Mannheim Biochemicals or Gibco BRL. The reagents for the mutagenesis procedure were as follows: T4 DNA polymerase and Exonuclease III (USB), 5Me-dCTP (Pharmacia), T4 Kinase and T4 Ligase (NEB or Amersham), MspI (BRL), Hha I (NEB), Nucleotides (Pharmacia), ATP (Sigma). Sequencing was done using the Sequenase version 2.0 kit from USB Large scale purification of oxidase was done as indicated in Hosler *et al.* (1992), except that no NaBr wash was done, and only a single DEAE anion exchange chromatography on an FPLC was used for His411Gln, and Tyr414Ala. For Thr413Ala and Asp412Ala two anion exchange chromatography steps were used.

Mutagenesis and subcloning The Batt Method of mutagenesis was used to make the mutants (Vandeyar *et al.*, 1988), with some modifications to the procedure (Lemieux *et al.*, 1992). Primers 18-21 bp in length were made for each of the mutants. For the Thr413Ala mutant a primer was made that introduced the MluI restriction site, to allow easier detection of this mutant. The procedure required the use of several previously used plasmids (Shapleigh and Gennis 1992). Single stranded DNA was prepared from M13 containing a 220 bp Sal I/Sph I piece from the subunit I cytochrome oxidase gene from *Rb. sphaeroides*. T4 DNA polymerase was used to extend the primer using dATP, dGTP, dTTP, and 5-methyl-dCTP as indicated in (Lemieux *et al.*, 1992). The methylation served to protect this strand from MspI and HhaI which could then be used to nick the non-mutant strand. Exonuclease III was used for complete cleavage and removal of this strand. This was transformed into *E. coli* and allowed to propagate. Single stranded DNA was prepared from multiple plaques. Sequencing of this DNA was done using the Sequenase 2.0 kit from USB to detect those colonies which contained the mutant of interest.

Next the Sal I/Sph I piece was subcloned into a larger piece of pND38 (see Figure 6.1). This was required as an intermediate step because the complete subunit I sequence contains 2 Sal I sites, preventing a direct ligation into that piece. From pND38 a Bgl II/Hind III segment was removed and ligated into pJS3. Next the entire subunit I piece was put into pRK415-1 using HindIII/EcoRI, and screened using XGAL and IPTG (Sambrook *et al.*, 1989). This plasmid is stable in *E. coli* and *Rb. sphaeroides*, allowing it to be a shuttle to the *Rb. sphaeroides*. A Sal I/Hind III piece was moved into M13 and sequenced using the Sequenase 2.0 kit, to confirm that the mutation was still present. Then the pRK415-1 was transformed into S17-1, a strain of *E. coli* capable of conjugating with *Rb. sphaeroides*. After conjugation with JS100, the subunit I deletion strain of *Rb. sphaeroides*, the mutant DNA from *Rb. sphaeroides* was transformed into DH5α'. Again the Sal I/Hind III piece was moved into M13 to allow a final confirmatory sequencing.

Conjugation These strains were conjugated based on the procedure in Donohue *et al.* (1988) as follows. *E. coli* S17-1 containing the mutant in pRK415-1 was grown overnight in 3 mL LB, 25 ug/mL tetracycline at 37 ^{o}C. *Rb. sphaeroides* (JS100) was grown in 50 mL of Sistrom's media at 30 ^{o}C without antibiotics. On the second day 500 uL of the overnight culture of S17-1 was added to 50 mL of LB, 25 ug/mL tetracycline and grown for 1.5 hours. From the *E. coli* and the *Rb. sphaeroides* cultures, 1 mL of each was spun down at 5000 x g for 1 min in a microcentrifuge. To each pellet, 500 uL of LB was added, and then both samples were mixed together. Following centrifugation again at 5000 x g for 1 min, the pellets were resuspended in 100 uL of LB. For the actual conjugation, Millipore HA filters (0.45 u) were placed

Figure 6.1 Site-directed mutagenesis and subcloning to change residues in subunit I of *Rb. sphaeroides*. Site-directed mutagenesis was done in M13 using the Batt Method. Subcloning through pND38 containing a piece of the subunit I gene was necessary because pJS3 contains two Sal I sites. The entire subunit I gene is contained in pJS3. Vector pRK415-1 can propagate in *E. coli* and *Rb. sphaeroides*, allowing it to be used in the conjugation. *E. coli* strain S17-1 can conjugate with the Rb. sphaeroides to transfer the subunit I gene.

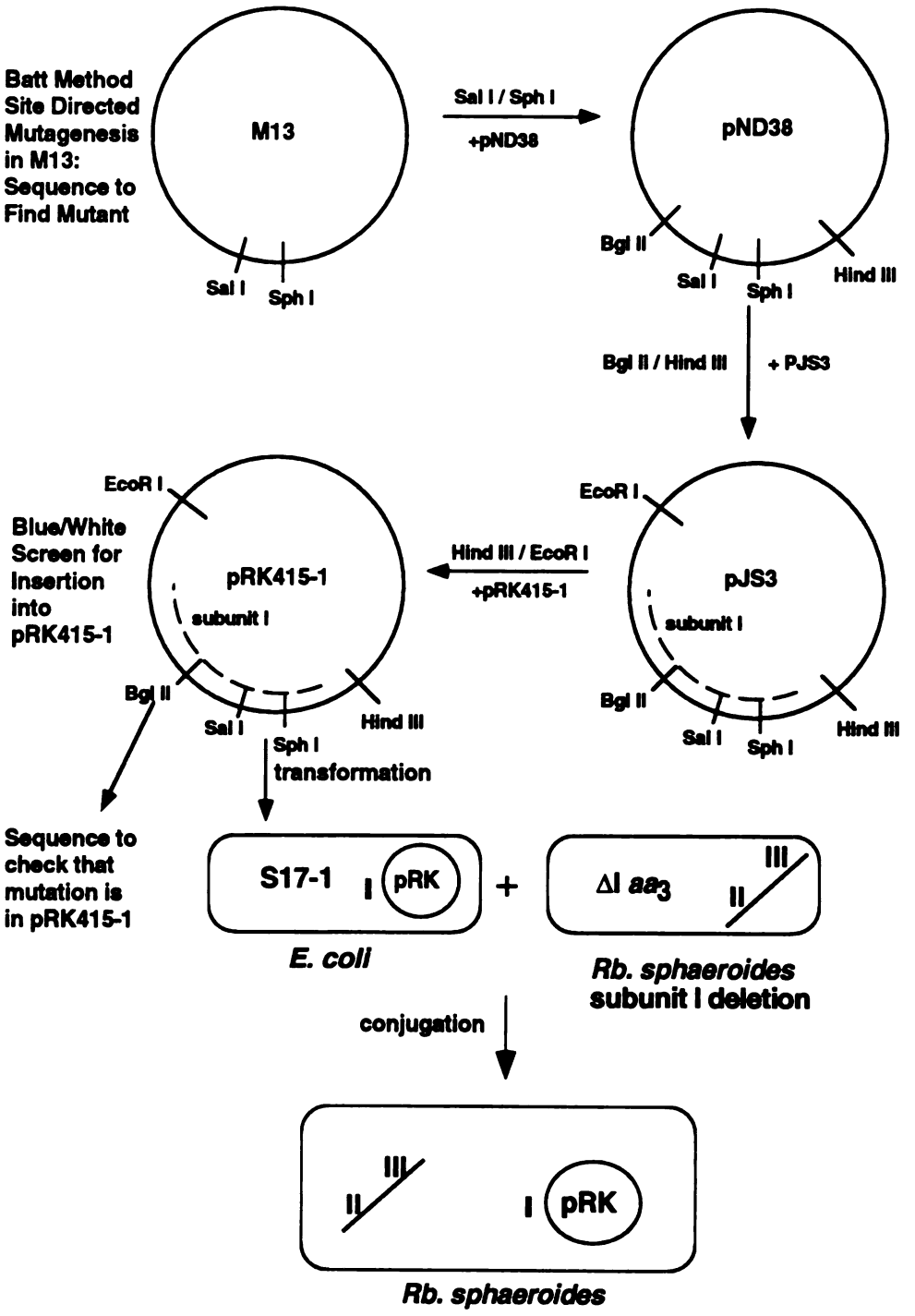

Batt Method
Site Directed
Mutagenesis
in M13:
Sequence to
Find Mutant
M13
Sal I
Sph I
Sal I / Sph I
+pND38
pND38
Bgl II
Sal I
Sph I
Hind III
Bgl II / Hind III
+ PJS3
EcoR I
Blue/White
Screen for
Insertion
into
pRK415-1
pRK415-1
subunit I
Bgl II
Sal I
Hind III
Sph I
transformation
Hind III / EcoR I
+pRK415-1
EcoR I
pJS3
subunit I
Bgl II
Sal I
Sph I
Hind III
Sequence to
check that
mutation is
in pRK415-1
S17-1
I
pRK
+
ΔI aa3
III
II
E. coli
Rb. sphaeroides
subunit I deletion
conjugation
III
II
I
pRK
Rb. sphaeroides

Figure 6.1

on an LB plate, to which the cells were added and allowed to grow at 30 °C for 4 hours. The filter was put in a tube with 1 mL of Sistrom's media, that was shaken for 10 minutes to resuspend the mixture of *E. coli* and *Rb. sphaeroides*. The solution was centrifuged at 1500 x g for 5 min, and resuspended in 1 mL of Sistrom's. From this sample 25 uL were plated on Sistrom's plates containing 1 ug/mL tetracycline, 50 ug/mL streptomycin, and 50 ug/mL spectinomycin, and grown for 3 days at 30 °C.

Small scale membrane preparations Two colonies from each mutant conjugation were grown in 100 mL Sistrom's in 500 mL Erlenmeyer flasks with shaking at 300 rpm at 30 °C for 2 days. Preparation of membranes for spectra followed the protocol in Hosler *et al.* (1992) except that the cells were broken by a single pass through a French press without the addition of protease inhibitors. After the low speed spin to remove cell debris at 20,000 x g for 20 min, the membranes in the supernatant were pelleted at 200,000 x g for 1.5 h. The pellet was solubilized in 50 mM potassium phosphate buffer, pH 7.2 and 2% lauryl maltoside. This was spun in a microcentrifuge at 16,000 x g for 10 minutes and the supernatant was used for the spectra. Dithionite reduced-ferricyanide oxidized spectra were taken to confirm the presence of cytochrome oxidase. An extinction coefficient of 24 $mM^{-1}cm^{-1}$ at 606-650 was used to determine the concentration.

Results

Thr413Ala was the first mutant to be completed, since it had the introduced MluI restriction site, which allowed screening for the mutation after each step of subcloning and ligation. Once in *Rb. sphaeroides*, a large scale preparation of this enzyme was done, and it showed almost identical characteristics to that of the wild type enzyme, like that observed for

Thr413Asn (Hosler *et al.*, 1994b). This mutant was reconstituted and showed normal proton pumping (data not shown).

The other three mutants were somewhat more difficult to work with since in moving the first piece into pND38 and then into pJS3, the wild type insert was already present in the vector, making it impossible to distinguish the wild type from the mutant until the sequencing was done.

Figure 6.2 shows the reduced-oxidized difference spectra of the mutants in the detergent-solubilized membranes. It appears that the spectrum for the His411Gln is shifted to 607 nm, while the other mutants look normal. However, it was discovered in looking at previous membrane preparations, that the slope of the baseline could cause spectra to shift, making it difficult to obtain an accurate peak wavelength.. Thus spectra at this stage of preparation can only be used to indicate the presence of an intact oxidase, not to assess spectral properties.

Large scale purifications were done to isolate each enzyme. Only a single purification over a DEAE anion exchange was used for His411Gln and Asp412Ala. Thr413Ala and Tyr414Ala were purified twice with the DEAE column. The spectra of the purified samples are shown in Figure 6.3. The wild type sample was purified from an overexpression strain, omitting the sucrose gradient and the hydroxyapatite chromatography steps. This gave a more active enzyme than what is normally isolated. This sample looks spectrally pure as can be seen by the lack of contaminating C heme at 416 nm in the Soret. This C heme is seen in the other spectra, and comes from either the bc_1 complex or from the cbb_3 oxidase or membrane bound *c* that is associated

Figure 6.2 Spectra of IX-X loop mutants in detergent-solubilized membranes. Thr413Ala is from a large scale purification (12 L) after the sucrose gradient, while the others are from small scale (100 mL) purifications. Only 1% of the large scale preparation was used for the spectra but the entire sample from the small scale preparations were used. All were solubilized in 50 mM potassium phosphate, pH 7.2 and 2% lauryl maltoside detergent. After centrifugation, the supernatant was divided into two cuvettes. Dithionite was used to reduce one sample and ferricyanide was used to oxidize the other. Spectra are displayed as reduced-oxidized. The shape on the blue side of the Thr413Ala peak is likely due to a scattering artifact. Its peak position is normal, and its purified spectrum in Figure 6.3 looks normal.

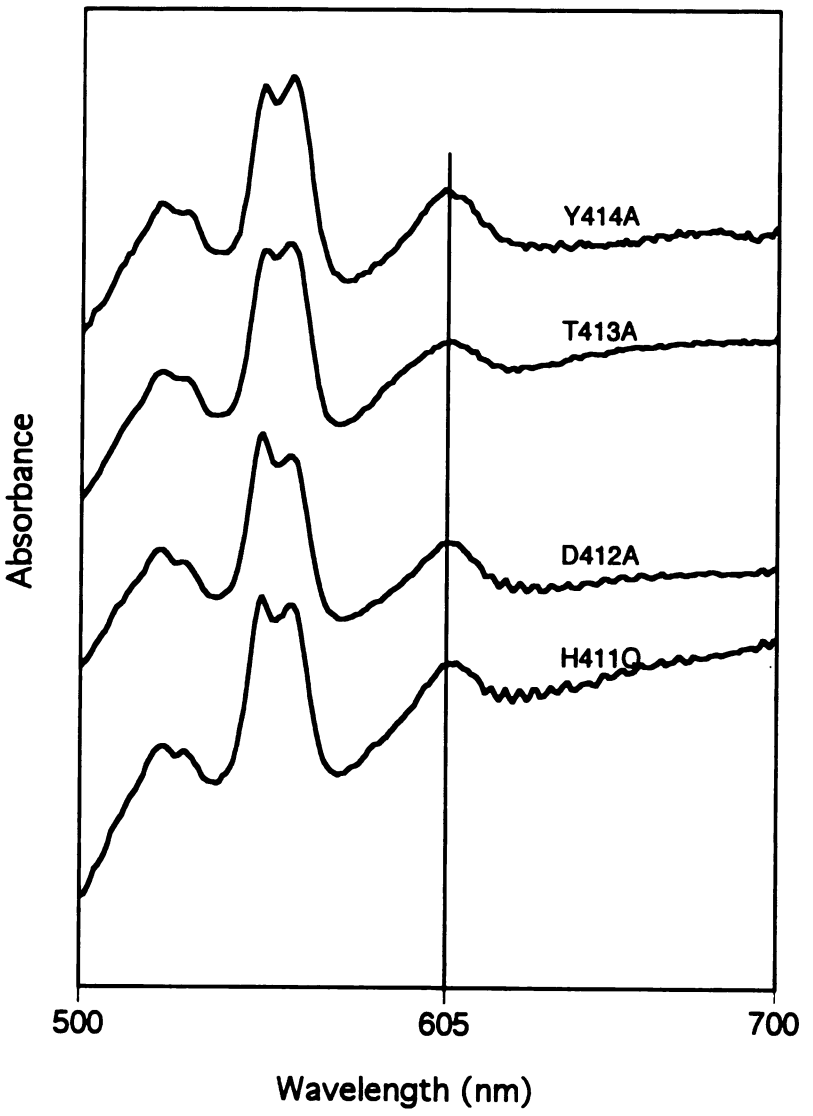

Y414A
T413A
D412A
H411Q
Absorbance
500
605
700
Wavelength (nm)

Figure 6.2

Figure 6.3 Spectra of the purified IX-X loop mutants compared to wild type. Samples were solubilized in 100 mM potassium phosphate buffer, pH 7.2, 0.2% lauryl maltoside, giving a range of final concentrations from 0.3-0.9 uM. Samples were allowed to reduce to completion after the addition of dithionite. Spectra were normalized using DeltaGraph software so that each was on the same scale. Most of the spectral contribution at the 605 nm peak is from heme *a*, while the peak at 445 nm has an equal contribution from heme *a* and heme *a*3. Small blue shifts at 605 nm can be seen for the Asp and Thr mutants; the His mutant shows no shift at 605 nm; and the Tyr mutant shows the largest shift to 604 nm.

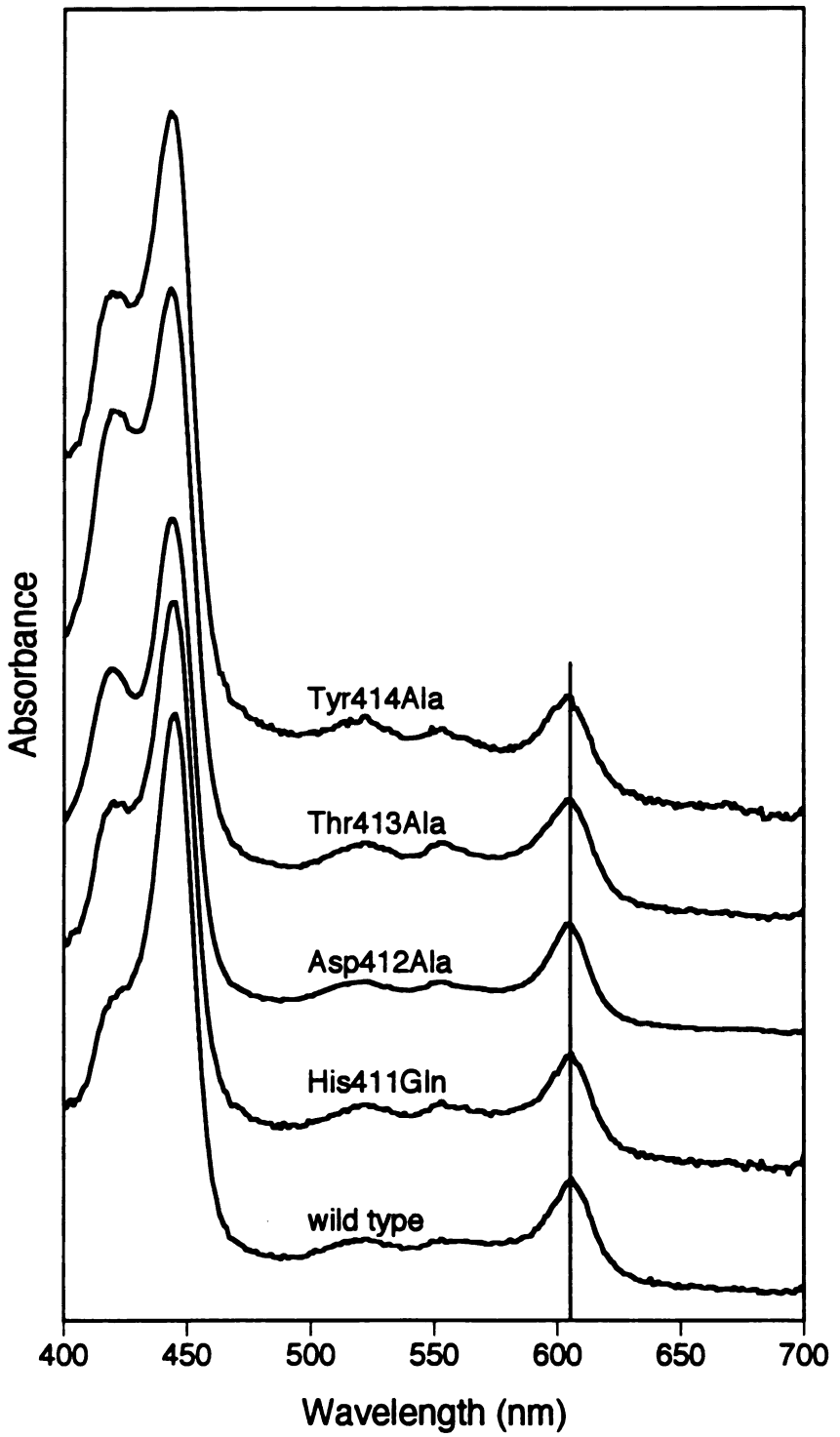

Absorbance
Tyr414Ala
Thr413Ala
Asp412Ala
His411Gln
wild type
400
450
500
550
600
650
700
Wavelength (nm)

Figure 6.3

with the *aa*3 oxidase. This contaminant makes it difficult to reconstitute the enzyme and assay for proton pumping. Steady state electron transfer activity can still be determined.

Electron transfer activity for the mutants and visible spectral characteristics are shown in Table 6.1. Results are also given for the previously characterized mutants in this loop, along with the results for wild type enzyme purified by the complete procedure that gives somewhat lower activity. All of the newly made mutants showed significant electron transfer activity. Asp412Ala showed the greatest inhibition. Although Tyr414Ala showed high electron transfer activity, it had the largest spectral shift, to 604 nm. This, however, is a shift in the opposite direction to the previously characterized Tyr414Phe mutant which shifts to 610 nm (Hosler *et al.*, 1994b). Doing the same assay with the addition of a cholate/phospholipid mixture before addition of the enzyme gave higher activity in each case. Previously characterized activities were only done only with the mixture of cholate/phospholipids added.

Discussion

The mutants were cloned out of *Rb. sphaeroides* into *E. coli.* and then into M13 one last time to do sequencing. None of the mutants previously studied by this lab had been resequenced after being put into *Rb. sphaeroides,* but it was done this time as an extra precaution. Thr413Ala was not checked since it showed normal characteristics. It was also decided to check the previously studied His411Asn because of its complete lack of activity even though Asn is considered to be a good replacement for His. The mutants His411Gln, Asp412Ala, and Tyr414Ala were shown to have the

Table 6.1 Visible spectral shifts and activity of the IX-X loop mutants. The upper table contains data from the newly made mutants. In the bottom is data from previous mutants with the activity from Fetter *et al.* (1995) and the spectral characteristics from Hosler *et al.* (1994b). Peaks for the alpha and Soret region were determined from the spectra in Figure 6.3. Steady state electron transfer activity was assayed as indicated in Table 4.1 except that an assay was also done without the addition of phospholipids.

Table 6.1

	α_{max} (nm)	Soret$_{max}$ (nm)	activity (sec^{-1})	activity plus lipids (sec^{-1})
newly characterized enzymes				
wild type	605.4	444.4	1500	2200
His 411Gln	605.4	443.8	1200	1500
Asp412Ala	604.8	443.5	400	600
Thr413Ala	604.8	443.1	1200	1400
Tyr414Ala	604	443.3	1200	1500

	α_{max} (nm)	Soret$_{max}$ (nm)	activity plus lipids (sec^{-1})
previously characterized enzymes			
wild type	605.4	444.4	1700
His411Ala	604.8	443.8	800
His 411Tyr	604.2	441.1	0
Asp412Asn	605.4	443.8	300
Thr413Asn	605.4	443.8	1300
Tyr414Phe	610	445.6	700

expected sequence. His411Asn was found to actually have the sequence for Tyr at the position of 411. This better explains the results of its loss of activity, its visible spectral shifts, and its loss of the Fe-His stretch in the resonance Raman spectra (Hosler *et al.*, 1994b). It seems likely that the larger Tyr (Chothia 1975) with aromatic character would interact with the protein environment in a different way than a His, or Asn and be less likely to be a substitute ligand for Mn or Mg.

His411Gln shows only a slight shift in the Soret spectra and high activity. Since the previously characterized mutant at this site, His411Asn, had major spectral alterations and had lost all activity, Gln might have been expected to be similar, but with the information from resequencing that the mutant was actually a Tyr, the result is more interpretable. It is interesting that the His411Gln retains such high electron transfer activity (1500 sec^{-1}), higher than the previously characterized His411Ala (800 sec^{-1}). Since the His411Ala had lost the nonredox active manganese, it would seem that the Gln may still be providing a ligand for that metal site, keeping the enzyme almost as stable and active as the wild type enzyme.

Asp412Ala shows some small visible absorbance spectral shifts and significantly lower electron transfer activity (600 sec^{-1}), similar to that of the previously characterized Asp412Asn (300 sec^{-1}). Since Asp412Asn was shown to lose the non-redox active manganese (Hosler *et al.*, 1995), it seems likely that this is also occurring with the Asp412Ala, and that this introduces some structural instability that is causing the lower electron transfer activity. These results might be compared to those found with reaction center, in which mutation of the ligands to the Fe that lies between Q_A and Q_B in the electron transfer path slows down the steady state activity, but does not completely inhibit it (Williams *et al.*, 1991).

It is very interesting that Tyr414Ala shows only a small shift in the alpha band (to 604 nm) and in the opposite direction to the large shift of Tyr414Phe (to 610 nm). We previously hypothesized that either loss of the hydroxyl and a possible hydrogen bond, or repositioning of the aromatic moiety close to heme *a*, caused the shift in the heme *a* spectrum. Since Tyr414Ala shows a much smaller effect on the alpha band and would also have lost a potential hydrogen bond, the shift to 610 nm caused by Tyr414Phe seems likely to be the result of a new interaction of the phenylalanine with heme *a* as was previously proposed (Hosler *et al.*, 1994b).

These results support the previous data for the residues in the IX-X loop that showed that this loop lies over heme *a*, heme *a*3/Cu_B, and are consistent with the model for the active site that has been proposed (Hosler *et al.*, 1994b). Further analysis of the mutants will provide new information on their functional or structural importance.

Summary and Perspectives

A reconstitution and proton pumping assay were developed as indicated in Chapter 3. Many variables in the reconstitution make it difficult to get reproducible and accurate results in the proton pumping assay. Of the factors found to be potential problems, the most important are the purity of the enzyme, cholate, and the phospholipids. One potential for improvement would be to switch to purified lipids instead of the variable mixtures of soya bean phospholipids that are now used. Other factors were found for which it is more difficult to determine an optimum such as the details of the pretreatment of the enzyme with cholate, the ratio of enzyme to lipid used, and the concentration of enzyme used during the reconstitution. Since the main goal of this project was to be able to measure proton pumping and not to understand reconstitutions, time constraints did not allow a complete characterization of these variables.

Much of the project focused on purifying and characterizing various mutant oxidases that might show some inhibition of proton pumping. Until interesting mutants are found this is a repetitive search through many mutants that may not show a significant effect on proton pumping. This was the approach used on bacteriorhodopsin, where the focus of the mutagenesis was not limited by looking at conserved residues as has been done in oxidase. Hundreds of residues were mutated to find several that showed the strongest effects on proton pumping (Krebs and Khorana 1993). This may say something about the resiliency of proton pumping enzymes to mutation, and may indicate the possibility for either multiple proton paths through the protein, or that water molecules may be able to replace lost residues in the path. Many of the mutants in *Rb. sphaeroides* oxidase show some inhibition in electron transfer, so that they could not be completely ruled out of an

involvement in proton pumping. Of course with the present data, it also can not be ruled out that the mutations have altered the structure in a way to slow electron transfer without inhibiting proton pumping. Better techniques for determining the effects of the mutations need to be developed to determine the cause of the lower electron transfer activities.

One possibility is to develop a stopped-flow assay to measure proton pumping on a faster time scale, allowing a more quantitative measurement in changes in the rate of proton pumping. Unfortunately to increase the time resolution simultaneously requires a greater optical absorption signal. Thus more enzyme is needed to make larger batches of vesicles to generate larger changes in proton pumping. This will require better overexpression of the oxidase as is currently being developed by others in the lab. A possible way around the requirement may be to use a steady state proton pumping assay. While this can cause uncoupling of proton pumping from electron transfer (Capitanio *et al.* 1991; Papa *et al.* 1991), it may still be possible to determine differences in the proton pumping activity of the mutants.

It may also be of interest to apply to the aa_3 oxidase any of those techniques used to measure fast electron transfer, either by optical absorption or by resonance Raman. These should be ideal to determine how specific mutations are affecting the enzyme. Presently, studies will need to focus on those techniques such as rapid electron transfer from ruthenated-cytochrome *c* which require smaller amounts of sample (Pan *et al.* 1993). Techniques such as the information-rich flow flash resonance Raman spectroscopy will require much larger amounts of enzyme than are now available. Hopefully, improvements in overexpression of the *Rb. sphaeroides* oxidase will also make this feasible.

It is exciting when an obvious effect on proton pumping is found as is the case with the loss of proton pumping with mutation of Asp132. Studies of a single mutant can provide many insights into the proton pumping mechanism in cytochrome oxidase. Study of this mutant is only now in the beginning stages, but has already shown something interesting with the discovery that it has a unique stimulatory response to fatty acids not seen with the other proton pumping-inhibited mutants. Experiments showed that a longer alkyl chain length gave the greatest stimulation of the Asp132 mutant, and that the carboxyl of the fatty acid was required for this effect. Further experiments can be done to provide more detail about this interaction. Where exactly on the enzyme is the carboxyl of the fatty acid interacting? Where is the hydrophobic tail interacting? What is the conformation of the tail? The studies in Chapter 5 showing that indomethacin can have a two-fold stimulatory effect indicate that the fatty acid may be folded. Yet, the fatty acid has a larger effect implying that some flexibility may be important. Determination of the crystal structure by Michel opens up new possibilities for study. Perhaps the crystals could be soaked with fatty acids, or with analogs of the fatty acids to see if they bind in a specific region of the oxidase.

Finally, four more mutants were made in the loop between helices IX and X. This was important training in allowing me to gain experience in doing site-directed mutagenesis, DNA sequencing, and other molecular biology techniques. It has also provided new information about the loop. The low activity of the Asp412Ala indicates that it may also have lost a manganese (magnesium), and that probably because of a structural effect is slowing electron transfer between Cu_A and heme *a*. A mutant in which His411 is converted to Gln retains high activity, and likely still retains the manganese

(magnesium). It will be of interest to see how changing the ligand from a histidine to a glutamine has affected the EPR properties of this metal center.

All together the mutations have provided some information about the regions that are required for proton pumping. The II-III loop appears to be important as the proton entry point into the oxidase. On the other side of the membrane the IX-X loop appears to have an interaction with all of the metal centers of the enzyme, to have ligands to a manganese (magnesium) ion, and to lie between the Cu_A and the active site. Clearly protons that are exiting on this side of the membrane must be nearby, and further studies of this loop, and of conserved residues in other loops on this side of the membrane with comparison to the crystal structure may clarify where the protons are exiting. One interesting loop that has not received much attention yet is the XI-XII loop that contains two conserved arginines, and a conserved tyrosine, proline and aspartic acid. In the middle of the membrane, mutation of residues such as Thr352 and Lys362 cause complete inhibition of electron transfer. They may very well play a role in proton transfer, but it will require more subtle methods to determine their role. Sitting between the two hemes Tyr422 has been shown not to be critical for proton pumping as proposed by Rousseau *et al.* 1993, narrowing down the search for those residues that are. With the crystal structure of oxidase, the ability to make mutations, and the advances in the various spectroscopic techniques used to study the enzyme, many interesting discoveries about the mechanism of this enzyme should be made in the ensuing years.

APPENDIX

Publications and Abstracts

APPENDIX

PUBLICATIONS

D.S. Wagner, A. Salari, D.A. Gage, J. Leykam, J. Fetter, R. Hollingsworth, and J.T. Watson (1991) "Derivatization of Peptides to Enhance Ionization Efficiency and Control Fragmentation During Analysis by Fast Atom Bombardment Tandem Mass Spectrometry" *Biological Mass Spectrometry* **20**, 419-425.

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ABSTRACTS

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