The Role of Cu_A in the Cytochrome *c* Oxidase Proton Pump

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All is permanence and beauty,
And all is melody and reason,
And all, like diamonds rather
Than light, is coal.

-Jose Marti
Versos Sencillios
"Simple Poetry"
Translated from the Spanish by Elinor Randall.

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Abstract

Cytochrome *c* oxidase plays a central role in energy transduction in most aerobic organisms. It catalyzes the transfer of electrons from ferrocytochrome *c* on the cytosolic side of the inner mitochondrial membrane, to dioxygen. The protons consumed during this reaction are derived exclusively from the matrix space, resulting in a charge separation that contributes to the transmembrane electrochemical gradient. In addition to the dioxygen reduction activity, cytochrome oxidase is also a proton pump that can pump up to four protons from the matrix side of the inner membrane to the cytosolic side for every molecule of dioxygen reduced. This thesis investigated the role of the structure and function of Cu_A in the proton pumping function of the enzyme.

The structure of the Cu_A site was studied using Extended X-ray Absorption Fine Structure (EXAFS). Using the *p*-(hydroxymercuri)benzoate (*p*HMB)-modified enzyme and a Cu_A-depleted form of the enzyme, we assigned the ligand structures for Cu_A and Cu_B in the resting form of the enzyme. The best fit model for the coordination environment at Cu_A was found to be 2 (N,O) ligands at 1.99 Å (presumably from histidine) and 2 (S,Cl) ligands at 2.3Å (presumably from cysteine). The EXAFS curve fitting techniques were further refined to investigate the copper sites in both the resting and fully reduced forms of the enzymes. These results indicated that the resting form of the enzyme contains a "long" 2.6Å Cu-(S,Cl) in addition to the 2.3Å Cu-(S,Cl) interactions previously reported. Using the curve-fitting results from the Cu_A-modified and Cu_A-depleted enzymes, we were able to assign this interaction to one of the two Cu-(S,Cl) interactions at the Cu_A site. The curve-fitting results for the reduced enzyme showed no "long" interaction and indicated an average of one less sulfur per two coppers, suggesting that a a ligand rearrangement occurs upon reduction of Cu_A.

The role of Cu_A in proton pumping was assessed by reconstituting the *p*HMB-modified enzyme into artificial phospholipid vesicles, and measuring its proton pumping activity. We found that this form of the enzyme, which contains a perturbed Cu_A site, exhibits a rapid proton leak. This leak is not associated with *p*HMB modification of the protein surface sulfhydryl groups, but appears to be specifically correlated with the modification of the Cu_A site. We also developed a method for specifically perturbing the Cu_A site using gentle heating at 43°C. It was found that heat treatment causes a specific modification of the Cu_A site. We showed that the products of this reaction are native Cu_A, a type 2 Cu, and novel "blue copper" species. Furthermore, reduction of the enzyme as well as ligand binding to the binuclear center were found to protect the Cu_A site from heat-induced modifications. When the heat-treated enzyme was reconstituted into vesicles, it displayed proton pumping behavior similar to the *p*HMB-modified enzyme, again pointing to a role for Cu_A in the proton pumping machinery of the enzyme. These results also suggest that the Cu_A site is different in the oxidized and reduced forms of the enzyme, and that a strong allosteric interaction exists between the Cu_A site and the binuclear center.

Based on indications that there is a strong interaction between the binuclear center and Cu_A, we probed the protein matrix for a conformational change that could be caused by ligand binding to the binuclear center. Using the flow-flash technique, we identified a transient conformational change that appears to be associated specifically with dioxygen binding and reduction. This conformational change occurs rapidly enough to be involved in the turnover of the enzyme, and can be influenced by the redox state of cytochrome *a* and Cu_A. We conclude this thesis with a model for the turnover cycle of the enzyme, which utilizes Cu_A as the site of redox linkage, and a conformational switch which engages the pumping cycle.

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Abbreviations

CCCP, carbonylcyanide m-chlorophenylhydrazone;

CO, carbon monoxide;

COFR enzyme, CO-inhibited, fully reduced form of cytochrome *c* oxidase;

COMV enzyme, CO-inhibited, mixed valence form of cytochrome *c* oxidase;

Cys, Cysteine;

DCCD, dicyclohexylcarbodiimide;

EDTA, Ethylenediamine tetraacetic acid;

EGTA, (ethylenebis(oxyethylenenitrilo))-tetraacetic acid;

ENDOR, Electron nuclear double resonance;

EPR, Electron paramagnetic resonance;

EXAFS, Extended X-ray absorption fine structure;

FT, Fourier transform;

Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid;

His, Histidine;

*p*HMB, *p*-(hydroxymercuri)benzoate;

HTR enzyme, cytochrome *c* oxidase that was heat treated while fully reduced;

HTO enzyme, cytochrome *c* oxidase that was heat-treated while fully oxidized (resting);

IR, Infrared;

MOPS, 3-[N-Morpholino]propanesulfonic acid;

PMT, photomultiplier tube;

RCR, respiratory control ratio;

SDS-PAGE, Sodium dodecyl sulfate - polyacrylamide gel electrophoresis;

Tris, Tris(hydroxyethyl)aminomethane;

Tween 20, poly(oxyethylene)-sorbitan monolaurate;

XAS, X-Ray Absorption Spectroscopy.

I Introduction

It has been estimated that nearly 90% of the O_2 consumed by aerobic organisms participates in the dioxygen chemistry of cytochrome *c* oxidase and becomes reduced to water in the terminal step of respiration. Cytochrome oxidases of the *aa3* type (having two *a*-type cytochromes) are found in a wide variety of aerobic organisms including bacteria, fungi, single-celled eukaryotes, plants, and animals. It is an integral membrane protein complex comprised of two or three subunits in the simplest bacterial systems and as many as twelve dissimilar subunits in mammals. In eukaryotes, subunits I-III are coded for and synthesized in the mitochondrion, while the smaller subunits (IV-XII) are coded in the nuclear genome, synthesized in the cytoplasm, and assembled with the mitochondrially coded subunits at the inner membrane of the mitochondrion. The three major subunits (subunits I, II, and III) exhibit a high degree of homology, despite the diversity of species (for a review, see Wikström et al., 1981).

All *aa3*-type oxidases contain four redox-active metal centers (two iron hemes and two copper ions) and catalyze the four-electron reduction of molecular oxygen to water using reducing equivalents derived from cytochrome *c:*

$$4\ \text{Cyt}\ c^{2+} + O_2 + 4\ H^+ \text{----------}> 4\ \text{Cyt.}\ c^{3+} + 2H_2O$$

The electrons enter the protein from the cytosol side of the mitochondrial inner membrane and the protons consumed in the dioxygen reduction reaction are taken up from the matrix. In this manner, the sidedness of the membrane is exploited to convert redox free energy into a proton electrochemical gradient across the inner mitochondrial membrane. In addition, cytochrome oxidase is also an electrogenic proton pump capable of transporting up to four protons from the matrix side of the mitochondrial membrane to the cytosol side for every dioxygen molecule reduced. This "vectorial"

proton pumping activity augments the "scalar" proton consumption associated with dioxygen reduction, increasing the efficiency of the free energy conversion from redox energy to the synthesis of ATP (for a review, see Krab and Wikström, 1987).

Cytochrome oxidase has been extensively studied for nearly five decades with quite reasonable success. However, our understanding of the enzyme has now reached a level where further real progress will require an improved molecular definition of the problem. Cytochrome oxidase is an extremely complex enzyme, both structurally and functionally. In fact, it is quite impressive that we have come this far in our understanding of the biochemistry of this enzyme without a detailed picture of the assembly of the subunit composition and three-dimensional structure of the protein complex.

On an elementary level, there is still uncertainty regarding the polypeptide composition and the minimal molecular weight, which has been reported to range from 70 to 120 kDa (Brunori et al., 1987a). Moreover, we do not yet have an unequivocal definition of the functional unit. As suggested by Brunori et al. (1987a), the simplest view of the enzyme is that the basic unit is comprised of all the polypeptides and prosthetic groups that co-purify with the electron transfer and dioxygen reduction activity. On the other hand, because the enzyme is also a proton pump, this definition of the functional unit must be revised to include the minimum number of additional polypeptides that are necessary to carry out redox-linked proton translocation, if any. As these authors point out, a clear-cut distinction between these two viewpoints is difficult because our knowledge of the electron transfer processes and the mechanism of the redox-linked proton pumping activities is still quite rudimentary. The discovery of cytochrome oxidases of the *aa3* type from several bacterial strains with only three subunits that are homologous to the subunits I, II, and II of the eukaryotic oxidases has prompted many workers to consider these three polypeptides as the minimal functional

unit. However, because the enzyme exists as a dimer in the lipid membrane, there may still be problems with this definition. In fact, one of the active areas of investigation with respect to this problem is whether the monomor unit by itself can support redox-linked proton pumping.

Finally, cytochrome oxidase represents a distinct class of proton translocation device whose principle of operation is not well understood. In simplest of terms, it is a molecular machine, capable of existing in a large number of conformational states, but which must operate according to an ordered sequence of conformational transitions to achieve kinetic competence as electrons flow from one metal center to another and dioxygen is reduced to give a series of intermediates at the dioxygen reduction site. Whether the problem is sufficiently tractable and amenable to description, in terms of a small subset of these conformational states, remains to be seen.

The purpose of this chapter is to summarize our current understanding of the structure and function of cytochrome oxidase, particularly those aspects that bear on the proton pumping function. We begin with a general introduction to the structural biochemistry of cytochrome oxidase, and review our current knowledge of the structures of the redox-active metal centers. Because the proton pumping function of the enzyme is linked to the electron transfer from ferrocytochrome *c* to dioxygen, we also discuss the chemistry of dioxygen reduction at the binuclear center as well as the role of the other redox active metals in regulating the electron flow. Finally, we attempt to illustrate how information about the structure and function of the enzyme limits the choice of mechanisms to describe the redox-linked proton translocation.

Structural Biochemistry.

Metal centers. Cytochrome oxidase contains four redox-active metal centers, which are important to its catalytic activity. These four centers may be distinguished on the basis

of function. One pair, cytochrome *a3* and Cu_B, forms a binuclear cluster where dioxygen is bound and reduced during the catalytic cycle. The other pair, cytochrome *a* and Cu_A, mediates the flow of electrons from ferrocytochrome *c* to the binuclear center. Spectroscopically, cytochrome *a* and Cu_A interact only weakly and are often treated as independent electron acceptors. Based on spectroscopic evidence as well as sequence homology data, it is now generally agreed that these redox-active metal centers are located in subunits I and II (Mueller et al., 1988). Specifically, it has been suggested that cytochrome *a*, cytochrome *a3*, and Cu_B reside in subunit I while Cu_A is associated with subunit II (For a model, see Holm et al., 1987).

Recently, there has also been a report that cytochrome oxidase contains three copper ions (Steffens et al., 1987; Yewey et al., 1988). This additional copper (Cu_X) does not appear to be redox active, and has been suggested to be co-purified adventitious copper (Li et al., 1989). In addition, there is evidence that cytochrome oxidase binds zinc and magnesium (Yewey et al., 1988). The zinc has been identified as a Zn^{2+} ion ligated almost exclusively by the cysteine sulfurs of subunit V (Naqui et al., 1988). Naqui et al. have suggested that the zinc ion plays a structural role in the enzyme. The role of magnesium is not yet well understood.

Cytochrome *a* is generally assumed to be the primary acceptor of electrons from ferrocytochrome *c*. However, this position is not unambiguous and must be re-evaluated in light of the rapid electron equilibration between cytochrome *a* and Cu_A recently reported by Morgan et al. (1989). The reduction potential of cytochrome *a* can be near that of cytochrome *c*. It has been argued that if this center is the primary point of entry for electrons, then the degradation of the redox free energy should be minimized in this electron transfer step. However, there is now compelling evidence that this redox potential does vary as the enzyme is turning over (Thörnström et al., 1988; Wikström et al., 1981). In the resting form of the enzyme, cytochrome *a* has a

fairly high midpoint potential (ca. 350 mV) but it decreases to ca. 280 mV when the dioxygen binding site becomes reduced. The redox potential of cytochrome *a* also displays a moderate pH dependence of ca. 30 mV /pH unit. The source of this pH dependence has been localized to the titration of a protonatable group on the inner side of the mitochondrial membrane (Artazabanov et al., 1978). This pH-dependent midpoint potential has led to the suggestion that cytochrome *a* is the site of redox linkage, i.e., electron transfer from cytochrome *a* to the dioxygen binding site is linked to proton translocation. However, it must be noted that the pH dependence of the cytochrome *a* midpoint potential appears to change in response to the state of the enzyme. For example, in the CO mixed-valence form of the enzyme, the pH dependence decreases to ca. 9 mV / pH unit (Blair et al., 1986).

Cytochrome *a* is a six-coordinate low spin heme A, axially ligated by the nitrogens from two neutral imidazoles. Direct evidence for the structure of cytochrome *a* has come from Electron Nuclear Double Resonance (ENDOR) studies of the ferric cytochrome *a* in the oxidized state (Martin et al., 1985) and from resonance Raman (RR) studies of the oxidized and reduced states of cytochrome *a* (Babcock et al., 1981). This type of bis-imidazole coordination is quite typical of cytochromes that serve as electron carriers.

Copper A (Cu_A) is the low potential copper of cytochrome oxidase, having a midpoint potential of ca. 285 mV. It has been suggested that the role of this metal center is to transfer electrons from cytochrome *a* to the dioxygen reduction site. However, the possibility that Cu_A is the primary electron acceptor from ferrocytochrome *c* has been implicated recently. Capaldi and Wikström have noted that Cu_A is the metal cofactor most exposed to the cytosolic side of the membrane, and the putative metal binding site is near a patch of negatively charged amino acid residues that could serve as the docking site for ferrocytochrome *c* (Millet et al., 1982; Holm et al., 1987).

Irrespective of the resolution of these issues, Cu_A is an unusual metal center as evidenced by its enigmatic spectroscopic signatures. In the oxidized state, Cu_A exhibits a weak optical transition in the near infra-red (830 nm), which has been assigned to a charge transfer transition between the copper ion and a sulfur ligand (Beinert et al., 1962). In addition, Cu_A displays an EPR spectrum atypical of Cu complexes and copper sites in proteins. There is no resolvable hyperfine splitting when the spectrum is recorded at X-band (g=2.18, 2.03, and 1.99). Of particular interest is that one g-value is below the free electron g-value, a situation atypical of simple Cu^{2+} centers (Aasa et al., 1976). X-ray absorption spectroscopy indicates that the oxidized Cu_A site is in a highly covalent ligand environment and that there is considerable charge transfer from the ligands to the copper ion (Hu et al., 1977; Powers et al., 1981).

ENDOR studies have identified hyperfine and superhyperfine interactions between the unparied electron of Cu_A and various nuclei. The copper hyperfine interaction is unusually small and isotropic (Stevens et al., 1982 and the references therein). EPR and ENDOR studies using ((^{2}H)Cys) and (^{15}N)His) substituted yeast cytochrome oxidase have implicated at least one histidine and at least one cysteine ligand to Cu_A. The proton hyperfine couplings from the cysteine β-CH_2s are unusually strong (12 and 19 MHz) whereas the corresponding His ^{14}N superhyperfine interaction is significantly smaller than in blue copper proteins. (Stevens et al., 1982, Martin et al., 1988). More recently, EXAFS measurements comparing native, chemically modified, and Cu_A-depleted cytochrome oxidases have shown that two cysteine sulfurs are probably involved in the Cu_A ligation structure (Li et al., 1987). Accordingly, we have proposed that Cu_A is ligated by two cysteine sulfur atoms and two histidine nitrogen atoms. Comparison of amino acid sequences for cytochrome oxidases across a wide variety of organisms does show the presence of two highly conserved cysteine residues in subunit II (Steffens et al., 1987; Hall et al., 1988), and

it has been surmised that these are the two cysteine ligands to Cu_A. No other cysteine residues are conserved.

The structure of the binuclear center has been studied for many years using a variety of spectroscopic techniques, particularly in conjunction with the binding of externally added ligands. The binuclear center coordinates a variety of ligands, including F^-, CN^-, formate, and peroxide in the oxidized state, and O_2, CO, and NO in the reduced state. Cytochrome *a3* is a high spin ferric heme in both the oxidized and reduced forms of the enzyme, with one histidine nitrogen ligand in the axial position distal to Cu_B (Stevens & Chan, 1981). The other axial ligand is variable depending on the state of the enzyme. In the resting enzyme, the ferric heme of cytochrome *a3* is strongly antiferromagnetically coupled to Cu_B yielding a net S=2 paramagnetic species (Brudvig et al., 1986). The bridging ligand is not known, but it is possibly a μ-oxo, μ-hydroxyl, or μ-chloro species. In the reduced state, cytochrome *a3* is a high spin ferrous heme (the Cu_B center is d^{10} and hence diamagnetic) and also S=2. The Cu_B ion has been less well characterized because there is no visible absorption from this metal center. However, ENDOR and EPR studies on intermediates trapped during the turnover cycle indicate that Cu_B is ligated by at least three histidine nitrogens in a fashion similar to the type 3 copper centers (Cline et al., 1983; Reinhammar et al., 1980).

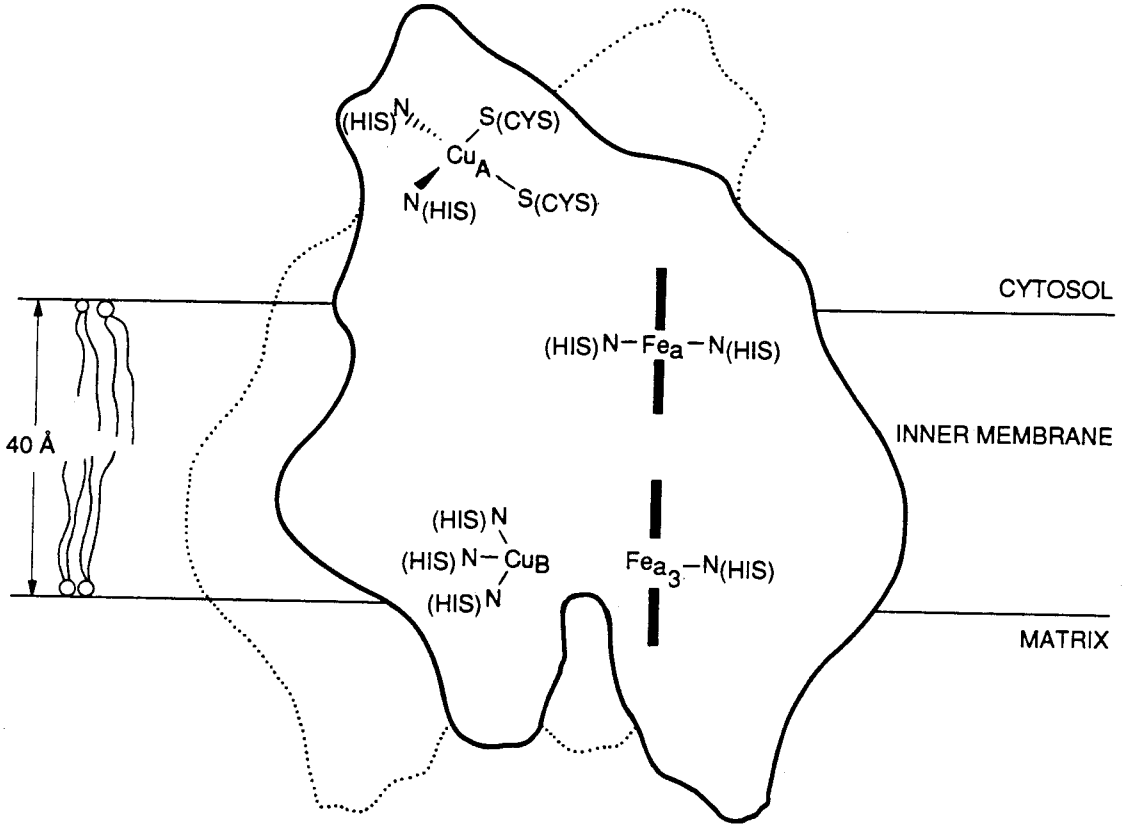
In the resting form of the enzyme, the binuclear center has a reduction potential of ca. 350-400 mV. When dioxygen and partially reduced dioxygen intermediates are bound, the reduction potential increases dramatically, approaching that of the bound dioxygen (ca. 800 mV).

Interactions amongst the metal centers. There is considerable evidence that all four metal centers interact with one another to varying degrees. These interactions are either magnetic or electrostatic by virtue of their spatial proximity or conformational by

virtue of their spatial and conformational linkage. Both types of interactions occur in the binuclear center. The electrostatic interactions are strong here, and manifest themselves in terms of an exchange interaction between the cytochrome *a3* and Cu_B spins, which behave as a magnetic unit. The other two centers can be treated as isolated centers, although they do interact magnetically with each other and with the binuclear center (as a unit), albeit weakly. These magnetic interactions have been used to infer the spatial distribution of the metal centers (Leigh et al., 1974; Brudvig et al., 1984).

The conformational interactions are reflected in the redox behavior of the metal centers. It is well known that cytochrome *a* exhibits an anticooperative interaction with cytochrome *a3* and Cu_B. By contrast, this type of redox interaction has not been observed between Cu_A and the binuclear center. However, Cu_A does interact *allosterically* with the binuclear center, specifically cytochrome *a3*. When Cu_A is chemically modified, the iron-histidine stretching and formyl stretching modes of cytochrome *a3* change in frequency as observed by resonance Raman spectroscopy (Larsen et al., 1989). Also, the rate of cyanide binding to cytochrome *a3* is accelerated when Cu_A is modified by heat treatment (Li et al., 1988). Recently, spectroelectrochemical experiments have also uncovered an anticooperative interaction ~40 mV between cytochrome *a* and Cu_A which may be either electrostatic or conformational in nature (Blair et al,. 1986). In support of this, Brudvig et al. (1984) and Scholes et al. (1985) have reported that the reduction of cytochrome *a* affects the EPR and ENDOR spectra of Cu_A. Interactions of this type between cytochrome *a* and Cu_A and between the dioxygen reduction site and the other metal centers in the enzyme are of considerable interest because allosteric coupling is undoubtedly involved in the regulation of intramolecular electron transfer in the proton pumping reaction. Figure I.1 shows our current view of the redox-active metal centers in cytochrome oxidase.

Figure I.1: A pictorial representation of the cytochrome oxidase dimer in the inner mitochondrial membrane, including the approximate relative positions of the redox-active metal centers. The non-redox-active metal centers (Zn, Mg) are not shown. It should be noted that the intersite distances are not drawn exactly to scale.


(HIS) N
S(CYS)
Cu A
N (HIS)
S(CYS)
CYTOSOL
(HIS) N – Fe a – N (HIS)
40 Å
INNER MEMBRANE
(HIS) N
(HIS) N – Cu B
(HIS) N
Fe a 3 – N (HIS)
MATRIX

Subunits. Mammalian cytochrome oxidase has been shown to contain at least twelve (12) inequivalent subunits, three of which are coded by the mitochondrial DNA and synthesized on the mitochondrial ribosomes (subunits I, II, III), and the remaining subunits (IV-XIII) coded by the nuclear DNA and synthesized in the cytosol. Most eukaryotic organisms have multi-subunit cytochrome oxidases containing more than the three mitochondrially coded polypeptides, while the prokaryotes tend to have simpler oxidases with two or three subunits, which are homologous to the mitochondrially coded subunits I, II, and III.

As mentioned earlier, it is tempting to define the mitochondrially coded subunits as the minimum functional unit of the enzyme. The four redox-active metal centers are located in these subunits, which appear to support both the electron transfer and proton pumping activities. These three subunits also form the membrane-spanning core of the enzyme. The remainder of the subunits in the more complicated oxidases presumably perform a regulatory function. However, because all the redox-active metal centers are contained in subunits I and II, there has been some ambiguity as to the function of subunit III. At one time subunit III was proposed to be involved in the proton translocation process because it is a membrane-spanning polypeptide that is sensitive to the carboxyl reagent dicyclohexyl carbodiimide (DCCD). DCCD has been shown to inhibit proton translocation in the ATP synthase system, and it also inhibits proton translocation in cytochrome oxidase, albeit to a lesser extent. For this reason, it has been argued that subunit III plays a role in proton translocation. In any case, there is good evidence that subunit III plays a role in the oligomerization of the protein (Finel & Wikström, 1988).

Several methods of subunit III removal have now been developed. These subunit III-less enzymes often display no proton pumping activity when assayed using a pH-sensitive glass electrode. However, it was found that these enzyme species

yielded poorly coupled phospholipid vesicles, and the loss of proton pumping activity could be correlated with lowered respiratory control ratio (RCR). Fast kinetics methods have determined that the subunit III-less enzyme retains 47% of the proton pumping activity, more than observed in the glass electrode experiments (for extensive reviews, see Prochaska and Fink, 1986; Brunori et al., 1987b). In our laboratory, we also find that subunit III depletion does not abolish proton pumping activity, so long as the Cu_A site is not modified during the subunit III depletion procedures. In these experiments, the H^+/e^- stoichiometry is reduced to ca. 0.5 (Li et al., 1988). Recently, a bacterial cytochrome oxidase with only two subunits has been purified from *Paracoccus denitrificans*. Reconstitution of this enzyme into phospholipid vesicles resulted in coupled proteoliposomes that displayed proton pumping behavior, albeit with a lowered stoichiometry of ca. 0.5 (Solioz et al., 1982). This result has led to the suggestion that subunit III is not part of either the essential proton pumping or electron transfer machinery, but may be involved in a regulatory role of some kind. However, this interpretation may be oversimplified (see below).

Bacterial oxidases. With the advent of new DNA technologies, the DNA sequences for a number of bacterial oxidases have been obtained. A few of these bacterial oxidases have also been isolated in quantities large enough for spectroscopic studies. These include *aa3*-type cytochrome oxidases from *Paracoccus denitrificans* (Ludwig & Schatz, 1980), *Thiobacillus novellus* (Yamanaka & Fujii, 1980),*Thermus thermophilus* (Fee et al., 1980) and PS3 (a thermophile) (Sone et al., 1979). As isolated, all of these oxidases contain either two or three subunits that seem to be homologous to the three mitochondrially coded mammalian oxidases. Of these bacterial enzymes, the one from *Paracoccus denitrificans* is the most thoroughly studied. It also most closely resembles the mitochondrially coded bovine heart subunits, as does the *Thiobacillus novellus* enzyme. The structure of the cytochrome oxidases from the

thermophilic bacyeria, however, seem to be different. The *Thermus thermophilus* and PS3 enzymes have only one major polypeptide, and some oxidases of this type have an intrinsic cytochrome *c* associated with the oxidase as well. However, all of these bacterial *aa3*-type oxidases exhibit similar functional characteristics and have spectroscopic properties almost indistinguishable from the bovine enzyme. It is clear from these bacterial studies that the catalytic core of cytochrome oxidase is comprised of at most three major subunits.

The isolation of two and three subunit bacterial cytochrome oxdiases provides a simpler and more versatile system for studying redox-linked proton pumping in cytochrome oxidase. Because these oxidases are bacterial in nature, one can take advantage of techniques not accessible to the mammalian oxidase system. Recently, Yamanaka and co-workers reported the purification of copper-deficient *aa3*-type oxidases from *Psuedomonas* AM1 (Fukomori et al., 1985), *Nitrosomonas europaea* (Numata et al., 1989) and *Halobacterium halobium* (Fujiwara et al., 1989). Using bacterial growth under copper-deficient conditions they were able to isolate a Cu_A-deficient enzyme from the *Pseudomonas* AM1 and *Nitrosomonas europa* strains capable of oxidizing ferrocytochrome *c* and reducing dioxygen. The cytochrome oxidase from *Halobacterium halobium* grown under copper-deficient conditions contains no copper as isolated. This enzyme was found to be devoid of electron transfer and dioxygen reduction activity when ferrocytochrome *c* was used as the substrate. These experiments show that the electron transfer between cytochrome *a* and the binuclear center is viable, and that electrons can enter the enzyme via cytochrome *a*. Similar conclusions have been derived from the chemical modification experiments on the bovine enzyme (Gelles and Chan, 1985). Unfortunately, it is not known whether these Cu_A-less enzymes are capable of pumping protons. Finally, a cytochrome oxidase from *Thermus thermophilus* has been recently purified that contains a *b*-type

cytochrome instead of the cytochrome *a* (Zimmermann et al., 1988). This oxidase has a copper site spectroscopically similar to Cu_A, but it contains only one cysteine residue. Again, it is not known whether this enzyme is capable of pumping protons at this time.

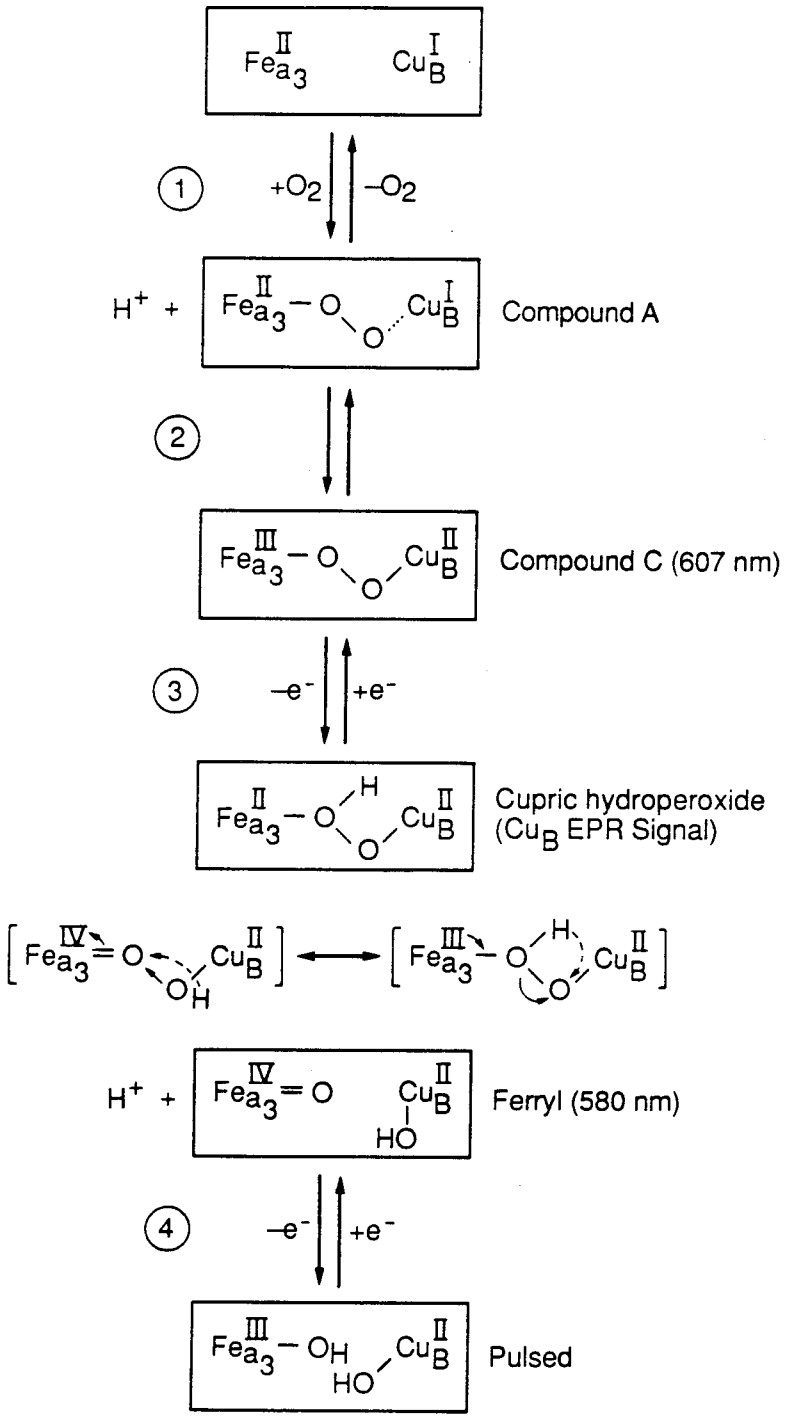
Another exciting development in the bacterial oxidase field is the cloning of the cytochrome oxidase genes for *Paracoccus denitrificans* (Raito et al., 1987) and PS3 (Sone et al., 1988). These studies have already proven useful in identifying a subunit III in *Paracoccus denitrificans* that was not observed in the initial purification. The advent of an expression system and site-directed mutagenesis techniques will allow for specific perturbations of any of the active sites in the protein complex, and the opportunity to delineate more clearly structure-function questions that are not amenable to traditional biochemical and biophysical techniques.

Dioxygen Chemistry.

In cytochrome oxidase, the proton pumping reaction of the enzyme is coupled to the highly exergonic enzyme-mediated reduction of dioxygen at the binuclear center. Specifically, we expect the details of the electron transfers that drive the proton pump to be controlled by the chemistry of dioxygen reduction. Accordingly, the chemistry of the dioxygen reduction reaction is of great importance and this subject has been a major focus of research for the past decade. In this section, we highlight some of the more recent work in this area. The chemistry of dioxygen reduction has been reviewed in detail recently (Hill et al., 1987; Chan et al., 1988), so we will be brief here and refer the reader to these more extensive treatises.

Our current view of the dioxygen reduction mechanism is summarized in Figure I.2. In step 1, dioxygen binds to ferrous cytochrome *a3*, forming a dioxygen adduct called compound A (Chance et al., 1975a,b). This species is short-lived, lasting no

Figure I.2: A scheme depicting the four electron reducing steps of the dioxygen reduction reaction based on the available chemical and spectroscopic evidence. (See text for details.)


Fe$^{II}_{a_3}$ Cu$^{I}_{B}$
1
+O2
–O2
H+ +
Fe$^{II}_{a_3}$ – O O Cu$^{I}_{B}$
Compound A
2
Fe$^{III}_{a_3}$ – O O Cu$^{II}_{B}$
Compound C (607 nm)
3
–e-
+e-
Fe$^{II}_{a_3}$ – O H O Cu$^{II}_{B}$
Cupric hydroperoxide
(Cu$_B$ EPR Signal)
Fe$^{IV}_{a_3}$ = O O H Cu$^{II}_{B}$
Fe$^{III}_{a_3}$ – O H O Cu$^{II}_{B}$
H+ +
Fe$^{IV}_{a_3}$ = O Cu$^{II}_{B}$ HO
Ferryl (580 nm)
4
–e-
+e-
Fe$^{III}_{a_3}$ – OH HO Cu$^{II}_{B}$
Pulsed

more than 10 μs according to a recent Resonance Raman investigation (G. Babcock personal communication). The frequency of the O-O stretch observed in the Raman experiment suggests some electron redistribution from the iron toward the bound dioxygen. Further electron redistribution at the binuclear cluster, particularly from Cu_B, converts compound A to the peroxidic adduct, compound C (step 2). This species is characterized by an optical difference spectrum (compound C minus resting) with a distinctive maximum feature at 607 nm. Compound C is stable in the absence of the transfer of additional reducing equivalents from the low potential centers. Further reduction by one electron (step 3) generates two intermediates at the three electron-level of dioxygen reduction. The first is a cupric hydroperoxide species in which the O-O bond is still intact. The second intermediate is an O-O bond cleavage product of the first (Blair et al., 1985). There is now compelling experimental evidence that the second intermediate is an oxy-ferryl cytochrome *a3* / cupric Cu_B species. It exhibits an optical spectrum with difference features (ferryl minus resting) at 580 nm and 428 nm. In addition, the EPR (Witt & Chan, 1987; Witt & Chan, 1989), resonance Raman (Witt, 1988), Mössbauer (Fee et al., 1988), and EXAFS (Chance & Powers, 1984; Kumar et al., 1988) experiments are consistent with an oxy-ferryl structure. Finally, in step 4, further reduction by the fourth electron yields the pulsed enzyme and water.

Confirming evidence for some of these intermediates has come from the activation of the enzyme by H_2O_2. Here, the chemistry is less heterogeneous, and can be controlled to obtain compound C and the oxyferryl intermediates in high yields (Chan et al., 1988). In particular, the reaction of the pulsed enzyme with stoichiometric amounts of H_2O_2 gives compound C and the reaction with excess H_2O_2 produces nearly quantitative yields of the oxyferryl intermediate. This technique has allowed for the spectroscopic characterization of the oxyferryl species mentioned above.

Finally, Wikström (1981) has shown that the catalytic cycle of cytochrome *c* oxidase can also be partially reversed in mitochondria. When mitochondria are poised in a highly oxidizing environment (in the presence of high ferricyanide / ferrocyanide) the addition of high concentrations of ATP can generate a sufficient membrane potential to induce the sequential formation of two optically distinct water oxidation intermediates. These are identical to the two intermediates generated at the two- and three-electron reduced levels of dioxygen reduction in the experiments that proceed in the forward direction. The first intermediate observed upon charging the membrane is a species with the 580 nm absorption band, spectroscopically identical to the oxy-ferryl species generated by Chan et al. (1988) at the three-electron level of dioxygen reduction. In the reverse reaction, this species corresponds to a one-electron oxidation product of the pulsed enzyme. The second intermediate exhibits an intense absorption difference band at 607 nm and is spectroscopically indistinguishable from the peroxidic intermediate compound C. These experiments are particularly important because they are performed at equilibrium, and allow the midpoint potentials of these intermediates to be measured: 939 mV for the oxyferryl intermediate and 801 mV for compund C (Wikström, 1988).

From these studies, it is clear that the chemistry of dioxygen reduction is quite complicated, and that each electron-level of dioxygen reduction yields chemically distinct oxygen intermediates at the binuclear center, each with a different chemical reactivity and affinity for reducing equivalents. It is therefore improbable that one of the binuclear center metals is involved in redox linkage. Nevertheless, it is important to emphasize the important role that the dioxygen chemistry plays in the proton pumping reaction. At the very least, formation of the peroxide and oxyferryl intermediates increases the redox potential of the binuclear cluster by ca. 500 mV. This can have profound effects on the intramolecular electron transfer reaction rates. In addition, the

formation of these intermediates can trigger conformational changes in the protein that can result in electron gating and proton gating. Consistent with these ideas, Wikström has recently reported that only the electron transfers from the low potential centers to the highly oxidizing compound C and oxyferryl intermediates are involved in translocating protons (Wikström, 1989). This result is supported by the observation that dioxygen binds only to ferrous cytochrome *a3*, implying that at least one step in the cycle involves an electron transfer to the "unloaded" oxidized binuclear center. Such an electron transfer does not contain enough free energy to pump protons against an electrochemical gradient, and cannot be expected to be involved in proton pumping. However, although some electron transfer steps may not be involved in proton pumping, it is clear that the details of all the electron transfer reactions leading to dioxygen binding and reduction must play a major role in the catalytic cycle.

The Kinetics and Pathways of Electron Transfer.

The available body of literature on the kinetics of cytochrome oxidase is copious, and reviews on this subject are available (see Brunori et al., 1981; Wikström et al., 1981; and Hill et al., 1984a,b). In this section, we merely point out the major questions that we feel remain unanswered about the kinetics of the enzyme, focusing on the kinetics of the intramolecular electron transfer events. Understanding the details of these electron transfers has become particularly important in relating electron transfer to proton pumping, because it is most probably the intramolecular electron transfer from either cytochrome *a* or Cu_A to the dioxygen intermediates that is linked to proton translocation.

Under typical experimental conditions, the cytochrome *c* oxidase molecule can catalyze the oxidation of 30 to 600 molecules of cytochrome *c* per second. The oxidation of ferro-cytochrome *c* can be monitored using optical spectroscopy (Smith,

1955), and the consumption of dioxygen can be monitored polarographically (Ferguson-Miller et al., 1976). In addition, the redox states of the hemes and Cu_A can be monitored during turnover using optical spectroscopy (Gibson and Greenwood, 1963).

Two classes of experiment have been used to investigate the turnover cycle of the enzyme. One is the steady state experiment, which is designed to study the rate-limiting step of the enzyme, and the events involved in the approach toward the steady-state. The second class involves transient kinetics, designed to follow the enzyme through one turnover cycle in real time. These latter experiments have been particularly valuable in the study of the intramolecular electron transfers between the low potential centers and dioxygen bound at the binuclear center.

Steady state kinetics. The steady state behavior of cytochrome oxidase is complicated. It is well known that the oxidation of ferrocytochrome *c* by oxidase exhibits non-hyperbolic kinetics (Malmstöm & Andréasson,1985 and the references therein). When the cytochrome *c* concentration is varied in these experiments, two distinct kinetic phases of cytochrome *c* oxidation are often observed, each with a characteristic turnover number and K_m. These values also depend on ionic strength, pH, and the detergent used to solubilize the enzyme (Singorjo et al., 1986). The biphasic kinetics were initially used to argue for the presence of two catalytically competent cytochrome *c* binding sites (Ferguson-Miller et al., 1976). Recently, however, it has been suggested that there may be only one catalytically competent cytochrome *c* binding site but that there are two conformations of the enzyme (E_1 and E_2), which can accept electrons (Malmström & Andréasson, 1985; Thörnström et al., 1985). Malmström and co-workers have proposed that the enzyme can pump protons only in one of these two conformations (E_2) (Brzezinski et al., 1987). They further

note that the existence of two conformations is an intrinsic property of ion pumps displaying alternating access.

One of the newer developments in the the field of steady state kinetics has been a set of experiments in which the reduction levels of cytochrome *a* and Cu_A were monitored during turnover. It has been shown that under turnover, cytochrome *a* is significantly more reduced than Cu_A (Brzezinski et al., 1986; Thörnström et al., 1987). Furthermore, after complete oxidation of the cytochrome *c* at the end of the reaction, Cu_A appeared to be oxidized completely while cytochrome *a* remained partially reduced. To account for these observations, these authors have proposed a model advocating that the enzyme switches from the E_1 (non-pumping) to the E_2 (proton pumping) conformation only when cytochrome *a* and Cu_A are reduced and that intramolecular electron transfer from cytochrome *a* and Cu_A to the "unloaded" binuclear center is a concerted two-electron process. However, because it now appears that protons are pumped only when dioxygen is bound to the enzyme, some of these ideas may need to be revised.

Transient kinetics. When stopped-flow techniques are used to follow the concomitant reduction of cytochrome *a* and Cu_A by ferrocytochrome *c*, biphasic kinetics are also observed. Most of the available literature suggests that these two metal centers are reduced synchronously, in two distinct phases (Antalis and Palmer, 1982; Andréasson et al., 1972), with some reports indicating that Cu_A may lag slightly (Wilson et al., 1975). To date, all of the experiments that measure the electron input have been limited by the binding rate of cytochrome *c*. The best estimates for this rate are between 10^6 and 3×10^7 M^{-1} sec^{-1} (Antalis and Palmer, 1982; Andréasson et al., 1972; Wilson et al., 1975).

Following entry into the enzyme at cytochrome *a* and/or Cu_A, electrons are transferred to the binuclear center (cytochrome a_3 and Cu_B) intramolecularly. Most of

the measurements on the kinetics of this electron transfer reaction have come from flow-flash experiments that monitor the reoxidation of cytochrome *a* and Cu_A in the presence of dioxygen. Accordingly, these electron transfer events are relevant to the proton pumping reaction. These studies have shown that the reoxidation of both cytochrome *a* and Cu_A is multiphasic. Hill and Greenwood (1984a,b) reported that 40% of cytochrome *a* is reoxidized simultaneously with cytochrome a_3 at nearly 30,000 s^{-1}. Following this phase, 60% of Cu_A is reoxidized at 7,000 s^{-1}. Finally the remainder of reduced cytochrome *a* and Cu_A is reoxidized at 700 s^{-1}. These results clearly indicate that the downhill electron transfer events are heterogeneous, depending on the conformation state of the enzyme and the nature of the intermediate at the binuclear site.

Another method used to infer the rate of electron transfer between the low potential centers to the dioxygen reduction site is based on measuring the rate of the reverse electron transfer from the reduced dioxygen binding site to the oxidized low potential centers following CO photodissociation from the CO-mixed valence enzyme. In these experiments cytochrome a_3 and Cu_B are reduced initially. Boelens et al., (1982) have reported that, following CO-photodissociation, a rapid backflow of electrons from cytochrome a_3 to Cu_A occurs in ~5% of the enzyme molecules. Based on these results, they suggested that the electron transfer in the forward direction proceeded from Cu_A to the binuclear center at ~10,000 s^{-1}. Recently, Brzezinski and Malmström (1987) confirmed these observations and obtained a rate of 14,000 s^{-1} for the electron transfer rate from Cu_A to cytochrome a_3. On this basis, these authors argued that Cu_A is the primary electron donor to the oxygen binding site. In addition, they also observed a slower electron transfer from Cu_A to cytochrome *a* (~700 s^{-1}). It should be noted, however, that these electron transfer rates pertain only to the enzyme with an unloaded oxygen binding site, where the redox potential of the binuclear center

is at most marginally (~100 mV) more positive than the low potential metal centers, and thus may not be relevant to the proton pumping forms of the enzyme.

The rate of electron equilibration between cytochrome *a* and Cu_A has also received attention. This is an important issue because if the rate is fast compared to the turnover rate of the enzyme, then the issue of one vs. two cytochrome *c* binding sites, or the issue of one vs. two electron input sites, becomes moot. In addition, a knowledge of this rate under a wide variety of circumstances would facilitate the interpretation of data from the flow-flash experiments alluded to earlier. Toward addressing this question, Morgan et al. (1989) recently studied the electron equilibrium between cytochrome *a* and Cu_A in a partially reduced, CO-inhibited form of the enzyme (where the low potential centers are reduced on the average by one electron) using the perturbed equilibrium method. These workers obtained a value of 17,000 s^{-1} for the sum of the forward and reverse rate constants (cytochrome a^{2+} Cu_A^{2+} <-------> cytochrome a^{3+} Cu_A^{1+}). Thus, the electron equilibration is extremely rapid compared to the turnover rate of the enzyme (30 s^{-1} to 600 s^{-1}), at least in this form of the enzyme. In this experiment, the binuclear site is reduced. It would be of interest to verify that the electron equilibration between the low potential centers is indeed significantly slower when the binuclear center is oxidized, as suggested by a number of stopped flow experiments on the resting enzyme (Wilson, 1975).

It is evident that many questions regarding the electron flow remain unanswered. In particular, it would be important to know whether the protein shuttles electrons from the low potential centers to the dioxygen reduction site through a different pathway depending upon whether the binuclear site is activated by dioxygen or not. The answer to this question has taken on an increased significance and urgency as we attempt to formulate molecular mechanisms to describe the proton pumping process.

Proton Pumping.

Redox loops and proton pumps. Cytochrome oxidase links the electron transfer reaction between ferrocytochrome *c* and dioxygen to a net translocation of proton from the mitochondrial matrix to the cytosol. The concept of linkage between electron transfer and proton translocation in mitochondria was first proposed by Mitchell (1966) as part of the chemiosmotic hypothesis. However, for some time, there was disagreement as to whether the vectorial electron transfer mediated by cytochrome oxidase was coupled to proton translocation (Moyle & Mitchell, 1978). The question now is not whether cytochrome oxidase is involved with linking an electron transfer reaction to proton translocation but rather how this linkage occurs.

For many years, it was thought that cytochrome oxidase was the electron transfer arm of a redox loop. In this model, the electron transfers were catalyzed by the cytochrome oxidase enzyme, with no vectorial translocation of protons (Mitchell, 1961). However, based on proton ejection experiments in coupled mitochondria, Wikström (1977) proposed that cytochrome oxidase is a "proton pump." Wikström argued that cytochrome oxidase uses the free energy of electron transfer to translocate protons vectorially in the opposite direction to the electron transfers. Since that time, Wikström and many others have come to view cytochrome oxidase as a "proton pump" that translocates proton *via* a mechanism other than a "redox loop." However, Mitchell (1988) has maintained that the proton-carrying function of the substrates has been overlooked. As an alternative to Wikström, Mitchell has offered two ligand-based redox loop mechanisms to account for the proton ejections based on the dioxygen substrate and its intermediates as carriers of oxidizing equivalents and the protons. In the first model, H_2O_2 formed during dioxygen reduction is the ligand that accepts the

electrons and delivers the protons to and from the cytosol, respectively (Mitchell et al., 1985). The second model is a Cu_A-based mechanism in which oxidoreduction of the Cu_A is linked to the translocation of an hydroxide from the cytosol to the matrix. These schemes are ingenious. Unfortunately, ligand-based redox loops necessarily have unity H+/e- stiochiometry, and it has been shown that the H+/e- stiochiometry may be variable (Papa et al., 1989).

The distinction between a "redox loop" and a "proton pump" has caused some confusion in the past, and much discussion has ensued concerning the definition of these two terms (Mitchell, 1988; Malmström, 1988). We take a "proton pump" to describe an enzyme that actively translocates a proton *via* a mechanism other than a substrate-based "redox loop", i.e. the coupling between electron transfer and proton transfer does not involve the association and dissociation of protons to and from a redox-active substrate molecule. As a result, the proton pumping function of cytochrome oxidase has gained wide attention because it represents another class of active proton translocation enzymes (probably belonging to the class of active ion pumps) and has general mechanistic implications on how electron transfer can be linked to proton translocation in the respiratory and photosynthetic electron transport chains.

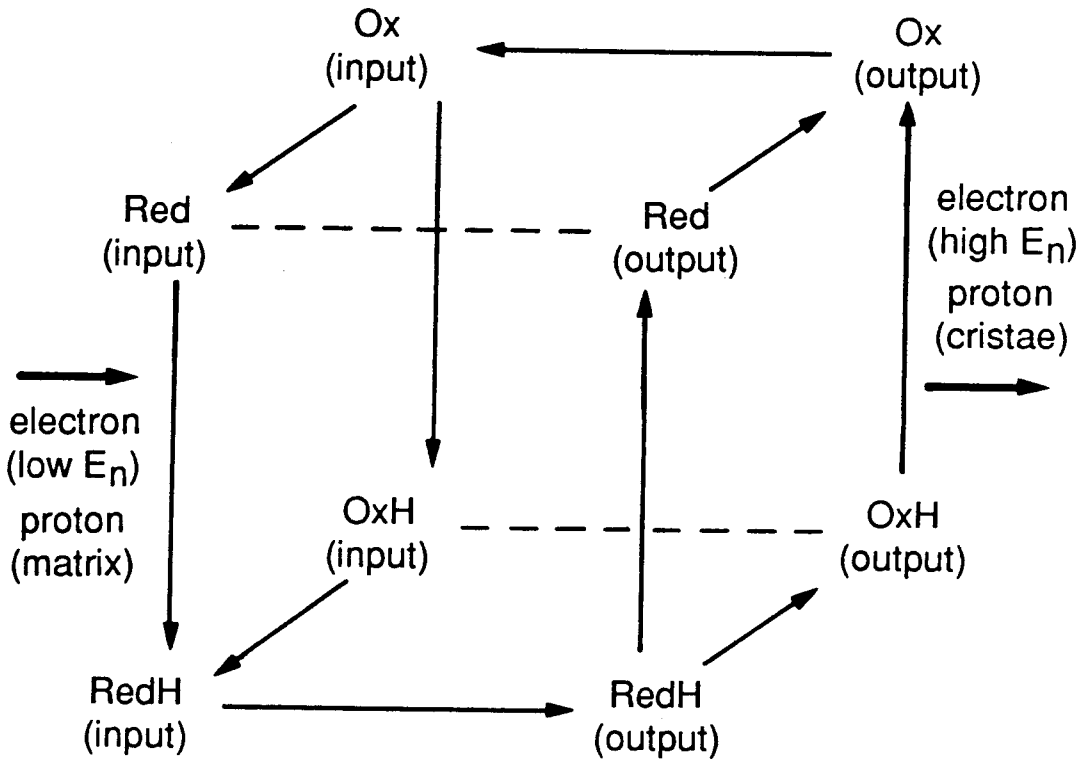
The basic requirements of a proton pump. Any enzyme that couples two reactions must somehow catalyze both reactions in such a way that the "uphill" reaction does not occur in the absence of the "downhill" reaction. In cytochrome oxidase, protons are pumped using energy derived from the exergonic transfer of electrons from cytochrome c to molecular oxygen. Therefore, the driving reaction is electron transfer, and the driven reaction is the energetically unfavorable translocation of a proton against an electrochemical gradient. To ensure that the proton pump does not act as a passive proton transporter, three general requirements must be met for redox-linked proton translocation (Wikström et al., 1981; Malmström, 1985; Blair et al., 1986; for a review

of theoretical proton pumping models, see Krab and Wikström, 1987). We refer to these as: 1) linkage; 2) electron gating; and 3) proton gating. In this section we review these concepts and attempt to clarify the nomenclature that exists in the literature.

Linkage. In order for electron transfer to be linked to proton transfer, one requirement is that these two activites be linked by some common intermediate. Many schemes for linkage have been proposed. The coupling can be direct, with the redox center also being the proton translocator. Or the coupling may be indirect, with the redox element being in conformational contact with the proton translocating element. In both cases, there are two distinct states of the redox center (reduced and oxidized), as well as two distinct states of the proton translocating element (protonated and deprotonated). When the redox and proton translocating elements are linked in a model that includes the sidedness of the membrane, one can envision an eight state "cubic" formalism as proposed by Wikström (1981) shown in Figure I.3.

It is important to note that the "cubic" formalism is not itself a mechanistic model for proton pumping. It is, rather, a formal way of describing the eight possible states in which the transducer of redox free energy to protonmotive energy can exist. As described by Krab and Wikström (1987) the eight states arise from separating the possible redox, protonation, and sidedness states of the transducer along Cartesian coordinates: the "x-axis" describes the redox state of the pump element; the "y-axis" describes the sidedness of the pump element with the input (I) state denoting protonic contact with the matrix space and electronic contact with the electron donor and the output (O) state denoting protonic contact with the cytosol and electronic contact with the electron acceptor; the "z-axis" describes the protonation state of the pump element. There has recently been some discussion on the generality of this treatment. As pointed out by Blair et al., (1986) and Krab and Wikström (1987), the eight state model arises

Figure I.3: The eight state "cubic" formalism that links the two states of the electron translocating element, the two states of the proton translocating element, and the two sides of the membrane, as proposed by Wikström (1981). (See text for a more detailed description).


Ox
(input)
Ox
(output)
Red
(input)
Red
(output)
electron
(high E_n)
proton
(cristae)
electron
(low E_n)
proton
(matrix)
OxH
(input)
OxH
(output)
RedH
(input)
RedH
(output)

from requiring that the electronic and protonic specificities be symmetric. It is not necessary for the electronic sidedness to be the same as the protonic sidedness in any given state (for example, separate proton input and electron input states), in which case there would be sixteen, and not eight states. However, it is argued that the eight-state scheme is the simplest one in which electron transfer, proton transfer, and I/O reactions may be discussed independently, although this scheme seems unnecessarily restrictive (Blair et al., 1986).

To rectify this, Wikström and Krab (1987) recently introduced "specificity of the first kind" to denote the existence of distinct electronic and protonic states on the input and output sides of the "cubic" pump description. These authors attribute the existence of these states to be a consequence of "electron gating." The importance of electron gating in a redox-linked proton pump has recently been emphasized by Chan and co-workers. We assume that specificity of the first kind is implicitly built into the eight-state cubic scheme.

Electron gating, proton gating, and specificity of the second kind. In order for a redox-linked proton pump to attain a maximum H^+/e^- stoichiometry, the electron transfer reaction, which is highly exergonic, must not take place in the absence of proton transfer. Most models for proton pumping argue that this requirement is manifested in a need for two distinct redox states of the pump site. In one, the redox element is in an "electron input state," which is ready to accept electrons, with all of the associated proton movements completed. The other state is an "electron output state," which is ready to pass electrons onto another acceptor, presumably the dioxygen reduction site. And the process by which the protein ensures that electron transfer occurs only into and out of these two states is electron gating (Blair et al., 1986), or specificity of the first kind (Krab and Wikström, 1987). If electron gating is absent, electron transfer may occur in states of the enzyme that are not competent in proton

translocation (i.e. states in which the proton translocating element has not responded to the redox element). These reactions are futile, and lead to electron "leaks," which are not described by the conventional cubic scheme. It is important to distinguish electron leaks from electron slippage (backward movement of electrons), which is represented by the cycle inscribing the left face of the cube. The latter is a non-productive event and does not bear on the stoichiometry.

Implicit in the discussion above is that, in addition to electron gating, the proton flow must be similarly gated. It goes without saying that there must not be proton leaks through the protein that dissipate the transmembrane protonmotive force, even as the enzyme is turning over. The enzyme must also ensure that proton slippage does not occur, such as the cycle that is inscribed on the bottom face of the cube.

Krab and Wikström (1987) have argued that electron and proton gating alone do not ensure a viable catalytic cycle, and that specificity of a second kind is needed. This specificty of the second kind kinetically controls the four input to output conversions so that only two are viable pathways. In our view, the purpose of electron gating and proton gating is to ensure that the coupled processes are kinetically enhanced at the expense of the uncoupled events. In this connection it bears pointing out that a viable pumping model requires that electron and proton movements are synchronized, meaning that both electron and proton gating must be present. Having both types of gating specifies that only two of the four I/O conversions are allowed because both electron and proton transfers must be completed before any I/O conversion may proceed. In other words, only one state on the input side may be I/O competent, and one state on the output state may be I/O competent. Therefore, we feel that electron and proton gating together correspond to "specificity of the second kind."

The site of redox linkage. The largest free energy change of the cytochrome oxidase redox reaction is associated with the electron transfer from the primary

acceptors (Cu_A and cytochrome a) to the dioxygen anchored at the binuclear center. Because this free energy is expended once the electron has reached the oxygen binding site, these "low potential" centers have been considered the most natural candidates for the site of redox linkage. Until recently, the argument that either cytochrome a or Cu_A should be the site of redox linkage was based solely on this idea without experimental support. However, the recent experiments of Wikström and Casey (1985) on whole mitochondria appear to confirm that these two sites are the most likely sites for redox linkage to proton translocation.

Of the two "low potential" centers, cytochrome a has received the most attention as the site of redox-linkage. Three main arguments have been advanced to support cytochrome *a* as the site of linkage. First, the cytochrome *a* midpoint potential exhibits a dependence on the pH of the mitochondrial matrix of ca. 30 mV per pH unit (Arzatbanov, et al., 1978). Second, the reoxidation kinetics of cytochrome a following flash photolysis of the CO-inhibited enzyme has been shown to be heterogeneous (Hill et al., 1984a,b). Third, Moroney et al., (1984) have observed that the steady-state reduction level of cytochrome a is dependent on both the pH and the transmembrane potential. The latter two observations have been rationalized in terms of two different states of cytochrome a, both electron transfer competent, that are presumably the input and output states of the pump site. In support of cytochrome a, Babcock and co-workers have obtained evidence from resonance Raman experiments that the heme A formyl group of cytochrome *a* is hydrogen bonded to a hydrogen-bond donor in the protein, and that the strength of this hydrogen bond increases as the heme iron is reduced (Babcock and Callahan, 1983). This group has offered a proposal for the mechanism of redox linkage based on this result.

More recently, however, some circumstantial evidence from this laboratory seems to implicate Cu_A as the site of redox-linked proton translocation. In these

experiments, Cu_A was chemically modified by *p*-hydroxymercuribenzoate (pHMB) to produce a structurally altered type 2 Cu_A site (Gelles and Chan, 1984). The resultant enzyme exhibited a rapid extravesicular alkalinization when it was reconstituted into membrane vesicles and assayed for proton pumping activity (Nilsson et al., 1988). These authors attribute this behavior to the formation of a facile transmembrane proton conduction pathway through the protein upon Cu_A modification. In subsequent work, Li, et al. (1988) showed that heating cytochrome oxidase at 43°C in the non-ionic detergent lauryl maltoside also results in the structural modification of the Cu_A site. This heat-modified enzyme was shown to contain a mixture of type 1 and type 2 Cu_A sites in addition to native Cu_A. When assayed for proton pumping activity, this modified enzyme preparation displayed either no proton pumping activity (Sone & Nicholls, 1984) or revealed a proton conduction pathway through the protein similar to that of the pHMB-modified enzyme (Li et al., 1988). On the other hand, when the Cu_A site was protected from heat-induced modification by reduction of the enzyme or ligand binding to the binuclear center, proton pumping activity was retained (Li et al., 1988). These results strongly implicate Cu_A as an important part in the proton pumping machinery of the enzyme.

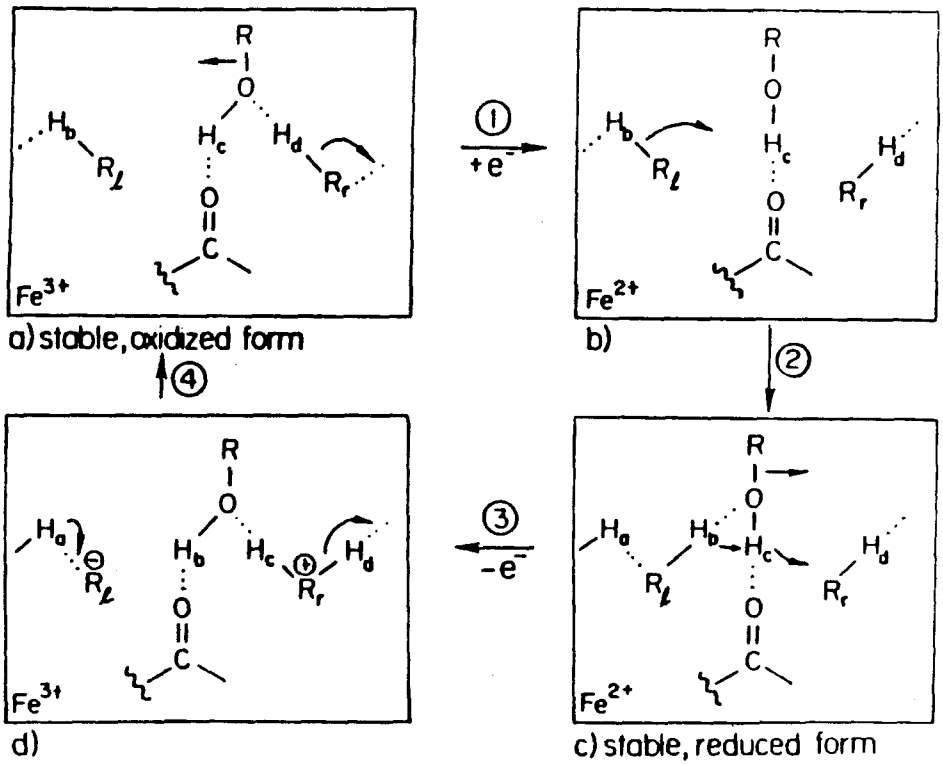
At this time, the biochemical evidence supporting either of the two low potential metal centers as the site of redox linkage is circumstantial at best. Nevertheless, these studies have provided impetus for the development of molecularly based models for redox linkage. In such exercises it seems important to be as explicit about the details of the proposal as possible, so that the various ingredients of the models can be subjected to critical experimental testing. Ultimately, the fate of a particular model must rest on how well predictions match the experimental facts. Depending on the outcome, either a model will have to be abandoned or refined for further assessment.

Models for redox linkage. Although the basic requirements for a redox-linked proton pump have been discussed, there are few mechanistic models of redox linkage that attempt to build in these requirements at the molecular level. Two such models exist in the literature. Here, we present each model and discuss the extent to which each incorporates the requirements of a redox-linked proton pump.

The Babcock model. Babcock and Callahan (1983) observed that the strength of hydrogen bonding between the formyl oxygen of cytochrome *a* and some proton donor(s) in the protein varies between the oxidized and reduced states of the heme center. From the formyl C=O stretching frequency measured by resonance Raman spectroscopy, it was calculated that the hydrogen bond strength differs by 110 mV between the ferric and ferrous forms of cytochrome *a*. Babcock and Callahan proposed that this energy contributes to the total free energy required to drive a proton against the electrochemical gradient (~200 mV) across the inner mitochondrial membrane. The details of the mechanism proposed by Babcock and Callahan (1983) are shown in Figure I.4. When cytochrome a is oxidized, the formyl oxygen is hydrogen-bonded to a protein proton donor lying between two hydrogen-bonded channels. One of these is connected to the matrix side of the membrane, and the other to the cytosolic side. Upon reduction of the site, the hydrogen bond strengh increases between the now electron-rich formyl oxygen and the proton of the donor group. The change in hydrogen bond strength is proposed to cause a change in the geometry of the conjugate base, allowing it to also interact with the proton of an adjacent acidic residue in contact with the matrix-facing hydrogen-bonding chain. As the cycle continues, the hydrogen-bond strength increases between the conjugate base and the matrix-derived proton at the expense of the proton hydrogen-bonded to the formyl group. Eventually, the latter proton leaves to occupy a place on the cytosol-facing hydrogen-bonded channel as it is replaced by the proton from the matrix that is hydrogen-bonded to the conjugate base. The proton hole

Figure I.4: The Babcock model for redox linkage based on cytochrome *a*. (See text for details.)

A Proton Pump Mechanism for Cytochrome a



left in the matrix-facing hydrogen-bonded channel is eventually replenished by a tandem proton migration along the channel toward the pump element, followed by the uptake of another proton into the channel from the matrix space. In this scheme, the redox center is linked directly to the proton binding steps *via* a direct mechanism in which there is alternating access of the "pump site" to the two sides of the membrane. The cytochrome *a* formyl group serves to gate the proton flow in response to a change in the redox state of the center. Although the elements of redox linkage and proton gating are clearly evident here, unfortunately this model makes no provision for the gating of the electron flow to obviate futile cycles.

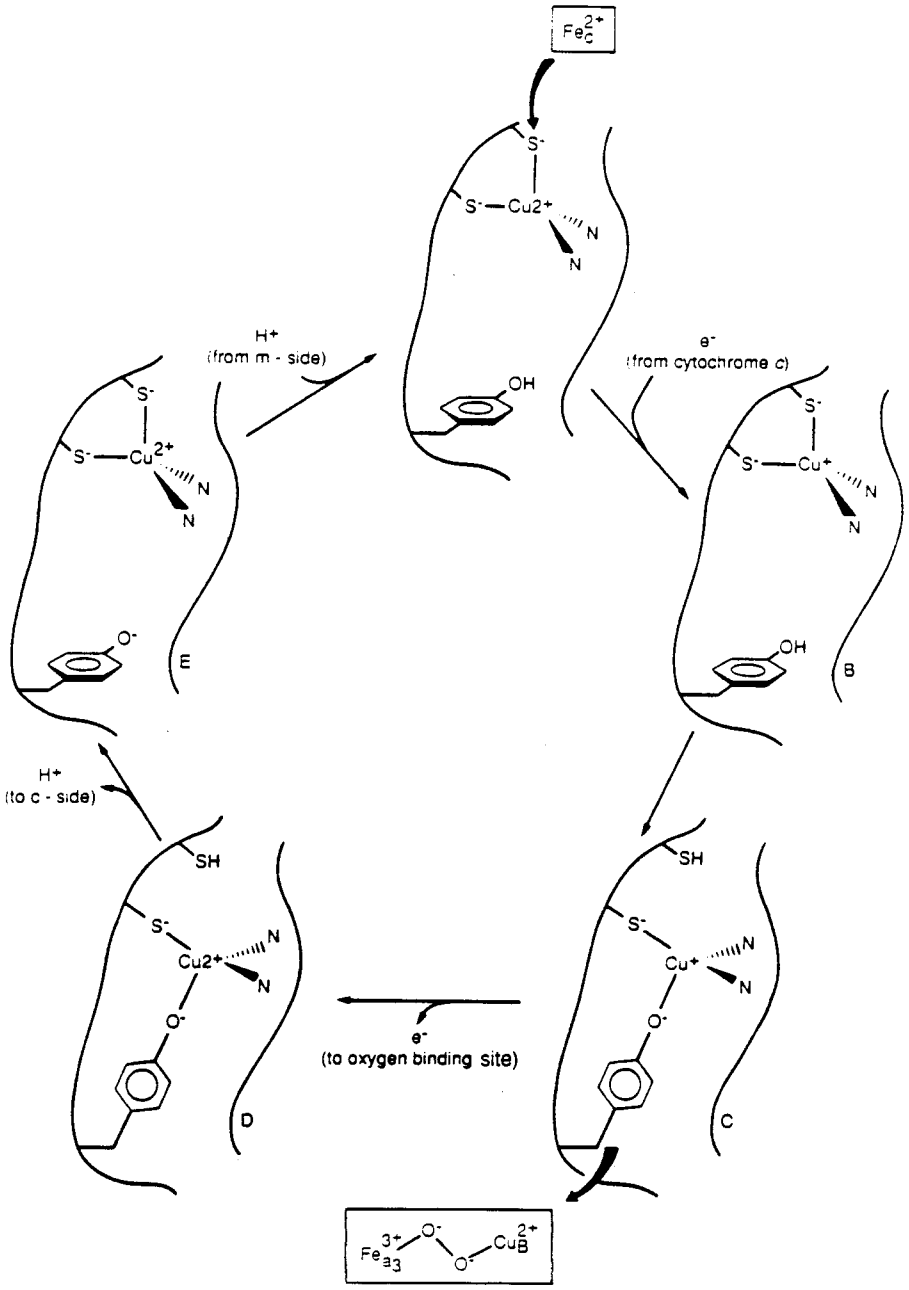
Because a number of treatments of the enzyme have been reported to disrupt proton pumping while leaving the environment around cytochrome *a* intact, Babcock and Callahan recently have offered a revised version of this model in which they allow for the possibility of a redox-linkage of the cytochrome *a* formyl group to a more global conformational change linking the redox element to a distant proton binding and transport element.

The Chan model. Gelles et al. (1986) have proposed an alternate model based on Cu_A as the site of redox coupling to proton translocation. In this model, the redox element is also linked directly to the proton transfer element. However, these authors have explicitly included the gating of electron flow. Gelles et al. (1986) argue that the enzyme must be able to control the rate constants of the possible electron transfers in order to enhance the coupled processes and suppress all of the leak pathways. Conformational switching is proposed as a means of achieving this electron gating. The electron enters the Cu_A site in one conformation (the "input state"), and facile electron transfer out of the site occurs only after the enzyme has switched conformations to the "output state." The redox linkage actually occurs during this switching process.

The Chan model is outlined in Figure I.5. Two hydrogen-bonded channels connect the pump element with the matrix and cytosol. In the oxidized state, the copper ion is ligated by two histidine and two cysteine ligands in a distorted tetrahedral geometry. This is the electron input state. When the site becomes reduced, the bis-dithiolate coordination becomes asymmetric and one cysteine bond lengthens relative to the other (Chan et al., 1988). At this point, a tyrosine (or another residue with a similar pK_a) interacts with the copper ion, displacing one cysteine ligand. The change in pK_as of the incoming tyrosine ligand and outgoing cysteine ligand leads to a proton transfer from the tyrosine to the cysteine. In this way, part of the redox energy from the reduction of the Cu_A site is expended in moving the proton from the matrix side of the pump site to the cytosol side. Following this ligand exchange (or rearrangement) and proton transfer, the reduced Cu_A site is in the electron output state, and transfers the electron to the dioxygen reduction site. Because this is a reduced type 1 site, Gelles et al. (1986) proposed that electron transfer to dioxygen intermediates at the binuclear center is extremely facile. Thus, the process is kinetically driven. When the Cu_A site becomes oxidized, the reverse ligand exchange (or rearrangement) occurs, and eventually, the tyrosinate is returned to the matrix side of the pump to be reprotonated following the tandem migration of the protons in the matrix-facing hydrogen bonding channel toward the pump site. It is this last step that is proposed to be the rate-limiting in the proton pumping cycle.

While there is no direct experimental evidence to support the above model, the Cu_A modification experiments discussed earlier do suggest a central role for Cu_A in proton pumping. In formulating this model, Gelles et al., (1986) have attempted to incorporate all three requirements of a redox-linked proton pump as well as the available information on the ligand structure and electronic structure of the Cu_A site.

Figure I.5: The Chan model for redox linkage based on Cu_A. (See text for details.)


Fe_C^{2+}
H+
(from n - side)
e-
(from cytochrome c)
OH
E
B
H+
(to c - side)
SH
e-
(to oxygen binding site)
D
C
Fe_{a3}^{3+}
Cu_B^{2+}

Identification of the redox linkage site: The search for a crossover point. With the general acceptance of proton pumping in cytochrome oxidase, recent research efforts have been directed toward identifying the site of redox linkage and unraveling the molecular mechanism of the redox-linked proton translocation reaction. The identification of this site is of particular importance because the nature of the redox center(s) involved will dictate the kinds of mechanisms at work. Because there are essentially no other examples of a redox-linked proton pump, and because the theoretical considerations of such a pump are relatively undeveloped, it is unclear what special properties such a center(s) should possess.

One of the most popular approaches toward identifying the site of redox linkage is the search for a classical "crossover point" in cytochrome oxidase (Rich, 1988). The concept of a crossover point was originally established during the effort to identify the energy coupling sites in the respiratory chain. This approach hinges on the assumption that any element in a linear electron transport chain that is involved with proton translocation will necessarily have a steady state turnover rate that is sensitive to a membrane potential. Therefore, when the system is at steady state, and a membrane potential is applied, the proton translocating element will slow down in response, causing the electron carrier upstream in the chain to become more reduced while the electron carrier downstream will become more oxidized. Extended to cytochrome oxidase, this approach makes several assumptions. First, this approach relies on a linear sequence of intramolecular electron transfer events, or that all electron transfers pass through the site of redox linkage. If there is a branched pathway in which one arm is not coupled to proton translocation, one would not necessarily expect the classical "crossover" behavior. Second, it assumes that the electron transfer events are rate-limiting. For a proton pump, it seems more likely that the electron transfer rates are

facile and that the turnover numbers are limited by conformational events subsequent to the electron transfer events. When the electron transfer rates become sufficiently retarded by the membrane potential, the electron transfers can become rate-limiting. Third, the existence of a "crossover point" assumes that the redox elements are non-interactive. If the redox element that is coupled to proton translocation interacts with the upstream and downstream redox elements in such a way as to modulate the rates of electron transfer, depending on the magnitude of the membrane potential, then one will not observe a standard "crossover point" behavior. Fourth, many of these experiments utilize cyanide to slow down the steady-state turnover rate. Under this circumstance, the majority of the cytochrome oxidase molecules are inhibited with cyanide and it is unclear whether the steady state situation is representative of normal turnover. Finally, the interpretation of the steady state kinetics of cytochrome oxidase is complicated, as the analysis involves no fewer than four electron acceptors at the dioxygen reduction site, each with a different affinity for reducing equivalents and different chemical reactivities. Thus, while a "crossover point" may exist in cytochrome oxidase, the complex behavior of the redox element linked to proton translocation in response to a transmembrane potential may obscure its identification, and the absence of such behavior does not necessarily disprove the involvement of a redox center in proton translocation.

The work described in this thesis attempts to address some open questions regarding the role of Cu_A in proton pumping. As described above, this problem is comprised of several facets, structural, and dynamic. In Chapter II, a detailed study of the structure of the Cu_A center using Extended X-Ray Absorption Fine Structure is described. Chapter III describes the results of chemical modifications on the proton pumping activity of the enzyme. This approach is continued in Chapter IV, which describes a method for the modification of the Cu_A site by gentle heating and the effect

of this heat treatment on the proton pumping activity of the enzyme. This chapter also describes allosteric effects between the Cu_A and the binuclear center. The final experimental chapter, Chapter V, deals with probing the protein matrix for conformational changes and interactions that may be important in the proton pumping reaction using time resolved fluorescence measurements. This thesis is concluded in Chapter VI with a model for the complete turnover cycle of the enzyme based on the experimental results obtained in Chapters II-V.

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II Extended X-ray Absorption Fine Structure Measurements of Copper in Cu_A-depleted, *p*HMB-modified and Native Cytochrome c Oxidase

Abstract.

Cytochrome *c* oxidase contains four redox-active metal centers, two heme irons, cytochromes *a* and a_3, and two copper ions, Cu_A and Cu_B. Due to the paucity of spectroscopic signatures for both copper sites in cytochrome *c* oxidase, the ligand structures for these sites have remained ambiguous. We herein describe the specific depletion of Cu_A from the *p*-(hydroxymercuri)benzoate (*p*HMB)-modified cytochrome *c* oxidase recently reported by Gelles & Chan [(1985) Biochemistry 24, 3963-3972]. Characterization of this enzyme shows that the structures of the remaining metal centers are essentially unperturbed by the Cu_A modification and depletion (Li et al., manuscript in preparation). Copper EXAFS measurements on the Cu_A-depleted cytochrome *c* oxidase reveal coordination of 3 (N,O) ligands and 1 (S,Cl) ligand at the Cu_B site. Comparison of EXAFSresults obtained for the Cu_A-depleted, *p*HMB-modified, and "unmodified control" enzymes has allowed the deconvolution of the EXAFS in terms of the inner coordination spheres for Cu_A as well as Cu_B. Based on these data, we find that the structure for the Cu_A site is consistent with 2 (N,O) ligands and 2 S ligands.

Introduction.

In its role as the terminal electron acceptor in the mitochondrial respiratory chain, cytochrome *c* oxidase catalyzes the transfer of electrons from ferrous cytochrome *c* to molecular oxygen. Functional cytochrome *c* oxidase contains four inequivalent redox active metal centers, two heme irons and two copper ions. One copper and one iron, namely Cu_B and cytochrome a_3, together form the site of oxygen binding and reduction. The other two metal centers, Cu_A and cytochrome *a*, serve to accept electrons from cytochrome *c* and pass them on to the oxygen binding site.

Both copper sites in cytochrome *c* oxidase are unique among biological copper compounds. Cu_A exhibits unique spectroscopic properties including an electron paramagnetic resonance (EPR) signal unlike that of any other known copper protein. Cu_B, which is involved in oxygen binding and reduction, is the only known biological example of a copper ion antiferromagnetically coupled to a heme iron.

The unique physical properties of the two copper sites in cytochrome *c* oxidase have prompted keen interest in their local ligand environments. Unfortunately, the inner coordination spheres of both Cu_A and Cu_B remain elusive. Studies on these sites by optical and magnetic resonance techniques have been hampered by the ambiguous spectroscopic signatures of Cu_A and the lack of data for the structure of Cu_B. Despite these drawbacks, electron nuclear double resonance (ENDOR) studies on Cu_A using both the beef heart enzyme (Van Camp et al., 1978) and isotopically substituted (^{2}H)Cys and (^{15}N)His enzymes from the yeast *Sacharomyces cerevisiae* (Stevens et al., 1982) have revealed hyperfine interactions between the copper unpaired electron and a nitrogen from at least one histidine ligand and protons from at least one cysteine ligand. Similar ENDOR studies on a Cu_B EPR signal observed during turnover conditions have revealed hyperfine interactions from three nitrogenous ligands (Cline et

al., 1983). While X-ray absorption measurements in principle can provide some insight into the ligands for both coppers, definite assignment of the ligand atom scattering contributions to each copper site has been difficult due to the overlap of the Cu_A and Cu_B absorption edges.

In this work, we report the specific depletion of Cu_A from *p*HMB-modified beef heart cytochrome *c* oxidase (Gelles & Chan, 1985). Copper EXAFS measurements on the Cu_A-depleted enzyme combined with similar measurements on both the *p*HMB-modified enzyme described by Gelles & Chan (1985) and the "unmodified control" enzyme have allowed the deconvolution of the scattering contributions from each copper. A clear picture of the identity of the ligand structures for both Cu_B and Cu_A has emerged from this study.

Materials and Methods.

Materials. *p*-(Hydroxymercuri)benzoate (*p*HMB), Tween 20, and EDTA were all purchased from Sigma. All reagents and buffers were enzyme grade unless otherwise specified. Anaerobic work was performed under 1 atm of argon gas, which had been scrubbed of residual oxygen by bubbling through 0.1 M vanadium(II) in 2 N HCl.

Cytochrome *c* oxidase was isolated by the method of Hartzell and Beinert (1974). Heme *a* concentration was determined using $\Delta\varepsilon$ (reduced minus oxidized = 24 M^{-1} cm^{-1}) at 605 nm and was taken to reflect the concentration of the total enzyme. The enzyme preparation was stored at -80°C until used.

*p*HMB-modified cytochrome *c* oxidase was prepared using the procedure and reaction conditions outlined in Gelles & Chan (1985) with the exception that mixing was accomplished using an end-over-end stirrer. "Unmodified control" enzyme was prepared under conditions identical to that of the *p*HMB modification except that no *p*HMB was used. As shown by Gelles & Chan (1985), unmodified control enzyme

exhibits no significant differences from the native enzyme in activity, optical, or EPR properties.

Preparation of Cu_A-Depleted Cytochrome c Oxidase. Immediately following the *p*HMB modification procedure, EDTA was added to the preparation to yield a final concentration of 50 mM EDTA. The reaction mixture was then dialyzed against a buffer containing 50 mM Tris-HCl, 50 mM NaCl, 0.5% Tween 20, 50 mM EDTA, pH 7.7 for six hours. After dialysis, the solution was concentrated to approximately 200 μM in enzyme by pressure ultrafiltration in an Amicon 8010 cell with an XM-300 membrane, and subsequently stored at -80°C for further characterization.

Preparation of EXAFS Samples. Samples of "unmodified control," *p*HMB modified, and Cu_A-depleted cytochrome *c* oxidase were concentrated from a 75 mL reaction mixture containing 30 μM enzyme by pressure ultrafiltration to a final volume of 7.0 mL using an Amicon 5105 cell with an XM-300 membrane. The resultant concentrate was pelleted by ultracentrifugation for 2-3 hours at 100,000 g in a Beckman Airfuge equipped with a 7.0 mL batch rotor. The pellet was resuspended into a minimal volume of supernatant, yielding a final enzyme concentration of between 1.5 and 2.0 mM. This suspension was then loaded into a 0.20 mL Lucite EXAFS/EPR cell (Scott et al., 1986). Samples were stored at 77K in liquid nitrogen until used.

EPR Spectroscopy. EPR spectra were recorded on a Varian E-line Century Series X-Band Spectrometer, equipped with a 12 bit analog-to-digital converter used for the computer digitization of the signal. Sample temperature was maintained at 77K by immersion in liquid nitrogen or at 10K by a liquid helium cryostat (Oxford ESR 900). To eliminate baseline artifacts due to dissolved oxygen, the EPR samples were equilibrated with 1 atm of argon gas immediately prior to freezing.

Atomic Absorption Spectroscopy. Cytochrome *c* oxidase samples for atomic absorption spectroscopy were prepared by overnight ashing in concentrated nitric acid

at ~70°C. Each sample was between 50 μM and 100 μM in cytochrome *c* oxidase. Measurements were made on a Varian model AA-6 atomic absorption spectrophotometer and calibrated against standard curves of cupric sulfate and ferric nitrate.

EXAFS Measurements. X-ray absorption spectroscopy (XAS) experiments were performed at the Stanford Synchrotron Radiation Laboratory (SSRL) with the SPEAR ring operating under dedicated conditions (3.0 GeV electron energy, ~70 mA electron current). The data were collected on the wiggler beam line VII-3 using Si[220] monochromator crystals and an ionization chamber fluorescence detector (EXAFS Co., Seattle) containing a six-absorption length Ni filter. Samples contained in the Lucite/Mylar EXAFS/EPR cells were maintained at 4K during data collection using a cryostat custom-designed for XAS fluorescence data collection by Oxford Instruments (Model CF1208). Each XAS spectrum consists of an average of 8-15 sweeps (8660 9700 eV), each sweep requiring 25 minutes. Energy calibration of the XAS spectra was accomplished by the internal calibration technique (Scott, 1985) using a 5 μ thick copper foil.

Data reduction and analysis were accomplished by our standard methods (Scott,1985). Averaged spectra (F/I_0) were formed, and background subtracted by fitting a second-order polynomial to the data in the 9050-9650 eV region, adjusted by a constant to match the data just before the edge (9850eV). The EXAFS data were extracted from the resultant data by fitting a cubic spline to the data over the range 9028-9650 eV (spline points at 9180 and 9400eV), subtracting and normalizing the resultant data to the atomic falloff modeled by theVictoreen formula, which was normalized to match the spline at $k = 0(E_0 = 9000$ eV). Curve-fitting employed backscattering (phase and amplitude) functions empirically derived from model compounds by complex Fourier back-transformation (Scott, 1985). For Cu-N and Cu-

S, these functions were extracted and averaged from a series of Cu(II)compounds as previously described (Scott et al., 1986).

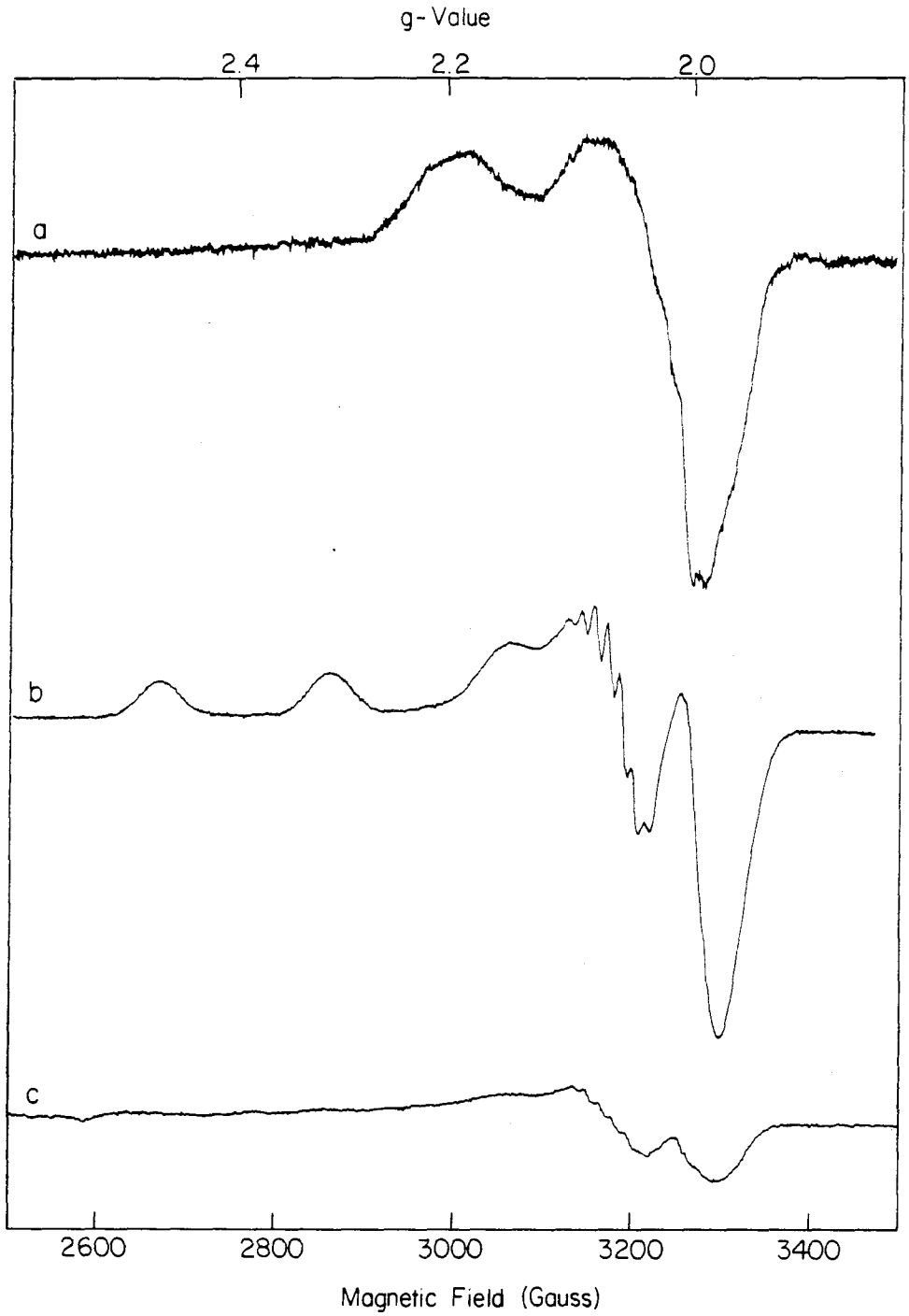
Results.

Cu_A Depletion of Cytochrome c Oxidase. The EPR spectra of native and *p*HMB modified cytochrome *c* oxidase are shown in Figure II.1a,b. As shown by Gelles & Chan (1985), modification of the Cu_A site by *p*HMB results in the quantitative conversion of oxidized Cu_A to a type 2 copper ligated to three spectroscopically equivalent nitrogenous ligands. As shown in Figure II.1c, dialysis of the *p*HMB modified enzyme in the presence of 50 mM EDTA results in the disappearance of the *p*HMB-modified copper EPR signal. Quantitation of the extent of Cu_A depletion by comparing integrated areas of the low field copper hyperfine line indicates that more than 90% of the EPR-visible copper signal in the *p*HMB-modified Cu_A species has been removed.

Further quantitation of the extent of copper depletion was accomplished by atomic absorption spectroscopy. The Cu:Fe ratios determined for the three samples were: "unmodified control," 1.1±0.1; *p*HMB-modified, 1.2±0.1; Cu_A--depleted, 0.5±0.1. Thus, both the "unmodified control" and the *p*HMB-modified enzyme contain two coppers per two irons while the Cu_A-depleted enzyme contains only one copper per two irons within experimental error.

The specific activity of the Cu_A-depleted enzyme as assayed by cytochrome *c* -driven O_2 consumption shows that it has retained 75% activity as compared to the *p*HMB-modified enzyme, which itself has 20% activity compared to native oxidase. In addition, both the Cu_A-depleted and *p*HMB-modified bind CN^- with rates similar to that of resting enzyme. These results suggest that the cytochrome c and oxygen binding sites in the Cu_A-depleted oxidase are functionally intact. The structural integrity of the

Figure II.1: EPR spectra of (a) resting state, (b) *p*HMB-modified, and (c) Cu_A-depleted cytochrome *c* oxidase. Microwave frequency, 9.16 GHz; microwave power, 0.2 mW; modulation frequency, 100 kHz; modulation amplitude; 10.0 Gauss; sample temperature, 77K. The spectra shown were normalized to enzyme concentration.


g-Value
2.4
2.2
2.0
a
b
c
2600
2800
3000
3200
3400
Magnetic Field (Gauss)

Cu_A-depleted enzyme is supported by the observation of no significant changes in the visible absorption spectrum (Li, et al., manuscript in preparation). In addition there are no significant changes in the g=3 EPR signal associated with cytochrome a^{3+} or the g=12 EPR signal assigned to the antiferromagnetically coupled cytochrome $a_3$3+ Cu_B^{2+} site (Li, et al., manuscript in preparation).

Copper EXAFS. The Fourier transforms (FTs) of the copper EXAFS of native, *p*HMB-modified, and Cu_A-depleted cytochrome *c* oxidase are compared in Figure II.2. As we have shown previously for other preparations of the resting state enzyme (Scott et al., 1986), the FT peak at R' ~1.5 Å is assignable to (N,O)-containing ligands, whereas the FT peak at R'~2.0 Å is assignable to (S,Cl) containing ligands. Fourier filtering of these two "first shell" FT peaks yields filtered copper EXAFS data that were analyzed by curve fitting. The results are summarized in Table II.1 and Figure II.3. Criteria based both on the goodness-of-fit parameter f and on $\Delta\sigma^2$ yield the best-fit average copper coordination spheres (per copper) summarized in Table II (third column). [The "best" $\Delta\sigma^2$ (Cu-S) is deemed to be zero because the model compound data were also collected at 4 K (Scott et al., 1986). The $\Delta\sigma^2$ (Cu-N) are observed to be slightly greater than zero, indicating more static disorder in this shell than in the model compound.]

Figure II.2: Comparison of Fourier transforms k=3.0-13.0 Å^{-1}, (k^3 weighting) of Cu EXAFS of cytochrome *c* oxidase samples normalized on a per Cu basis: "unmodified control" (solid line); *p*HMB-modified (long dashes); and Cu_A-depleted (short). The FT peaks at R'~1.5 Å are due to Cu-(N,O) scattering and those at R'~1.9 Å are due to Cu-(S,Cl) scattering.

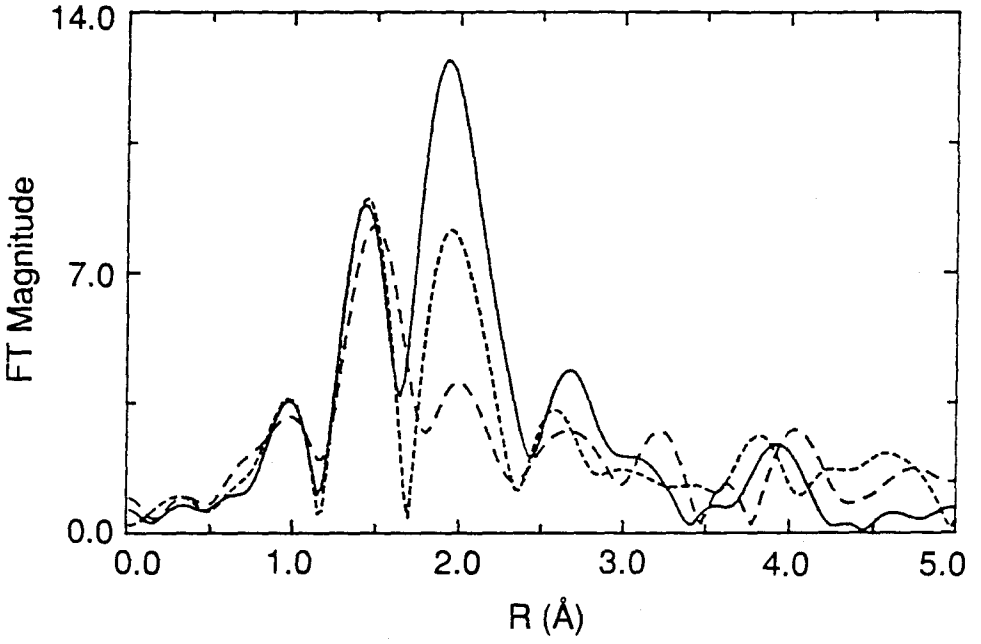

14.0
7.0
0.0
FT Magnitude
0.0
1.0
2.0
3.0
4.0
5.0
R (Å)

Figure II.3: Best-fit simulations of the first coordination sphere Cu EXAFS of cytochrome *c* oxidase samples: (a) unmodifed control; (b) *p*HMB-modified; and (c) Cu_A-depleted. In each, the solid line is the Fourier-filtered EXAFS using a filter window including the two main FT peaks of Figure II.2 (R'=1.15-2.40 Å, 0.1 Å Gaussian half-width) and the dashed line is the best-fit simulation, the details of which are in Table II.1.

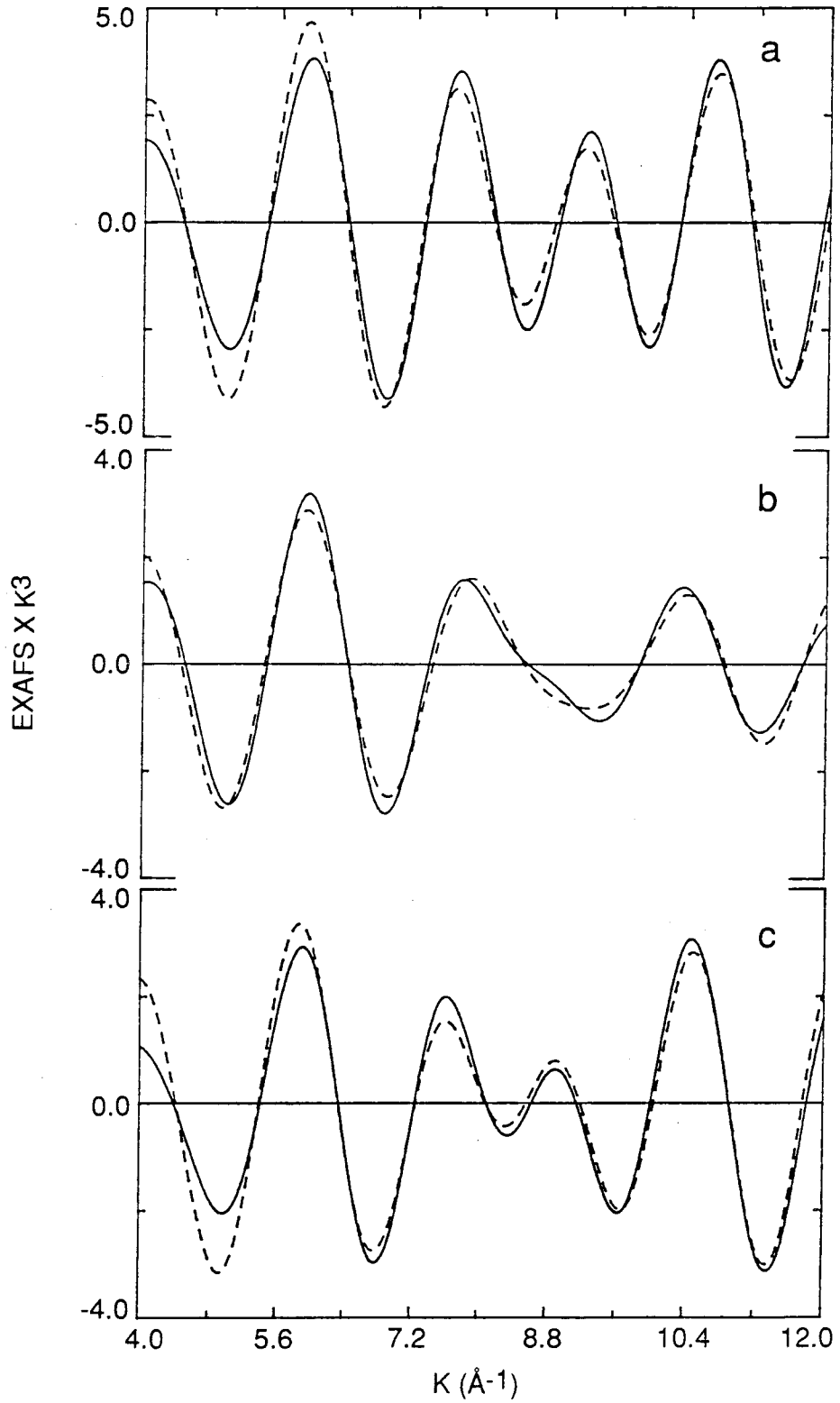

5.0
0.0
-5.0
a
4.0
0.0
-4.0
b
4.0
0.0
-4.0
c
EXAFS X K3
4.0
5.6
7.2
8.8
10.4
12.0
K (Å-1)

Table II.1: Representative Curve-Fitting Results for the First Coordination Sphere of Cytochrome *c* Oxidase Samples.[a]

Sample	fit[b]	Cu(N,O)			Cu(S,Cl)			f[c]
		N_S	R_{as}(Å)	$\Delta\sigma_{as}^2$(Å^2)	N_S	R_{as}(Å)	$\Delta\sigma_{as}^2$(Å^2)	
unmodified	a	(2.5)[d]	1.96	+0.0025	(1.5)	2.28	-0.0019	0.065
control		(3.0)	1.98	+0.0035	(1.0)	2.28	-0.0041	0.048
pHMB-		(3.0)	1.97	+0.0037	(1.0)	2.30	+0.0026	0.041
modified	b	(3.5)	1.98	+0.0046	(0.5)	2.31	-0.0017	0.039
Cu_A-		(2.0)	1.95	+0.0006	(2.0)	2.30	+0.0028	0.100
depleted	c	(3.0)	1.98	+0.0039	(1.0)	2.31	-0.0021	0.069

a N_S is the coordination number per copper; R_{as} is the copper-scatterer distance; $\Delta\sigma_{as}^2$ is a relative mean square deviation in R_{as}, $\Delta\sigma_{as}^2=\sigma_{as}^2$(sample) - σ_{as}^2(reference), where the references are given in Scott et al. (1986).

b The letters in this column refer to the best fits as shown in Figure II.3.

c f' is a goodness of fit statistic normalized to the overall magnitude of the $k^3\chi(k)$ data (Scott et al., 1986):

$$f = \frac{\{\sum_i [k^3(\chi_{(i)obs'd} - \chi_{(i)calc'd})]^2 / N\}^{1/2}}{(k^3\chi)_{max} - (k^3\chi)_{min}}$$

d Numbers in parentheses were not varied during optimizations.

Discussion.

Individual assignment of the Cu_A or Cu_B ligands in cytochrome *c* oxidase using copper EXAFS has always been hampered by the overlapping absorption of the two different copper sites. As shown in Figure II.1, extensive dialysis of *p*HMB-modified enzyme against 50mM EDTA results in 90% depletion of the *p*HMB-modifed Cu_A. Characterization of both *p*HMB-modified and Cu_A-depleted enzymes by optical spectroscopy as well as exogenous ligand binding (Li, et al., manuscript in preparation, data not shown) shows that the specific depletion of Cu_A from cytochrome *c* oxidase has been accomplished with little perturbation of the remaining metal centers. In addition, the Cu_A-depleted enzyme exhibits catalytic activity comparable to that of *p*HMB-modified enzyme, which is further indication of the functional integrity of the remaining metal centers in both electron transfer as well as oxygen reduction. The preparation of a 90% Cu_A-depleted enzyme has allowed the EXAFS from the Cu_B site in cytochrome *c* oxidase to be obtained essentially without interference from the Cu_A site. Combining these results with EXAFS measurements on the *p*HMB-modified and "unmodified control" enzymes allows a definite assignment of the inner coordination spheres of both Cu_A and Cu_B as summarized in Table II.2.

As shown in Table II.2, 3 (N,O) ligands and 1 (S,Cl) ligand may be assigned to the inner coordination sphere of Cu_B. The scattering due to sulfur in the Cu_A-depleted enzyme is much too large to be accounted for by the residual *p*HMB-modifed Cu_A. EPR characterization of the *p*HMB-modified Cu_A site shows a type 2 copper ligated to three equivalent nitrogenous ligands. If we assume that this modified Cu_A site contains at most one sulfur ligand, the upper limit on the sulfur scattering contribution due to residual *p*HMB-modified Cu_A in our 90% Cu_A-depleted enzyme preparation is no greater than 0.1 S per Cu. Sulfur has been implicated as a part of the inner coordination sphere of Cu_B (Powers et al., 1981). Powers et al. have suggested from

Table II.2: Best-fit EXAFS-Derived Coordination Environments for the Copper Sites of Cytochrome *c* Oxidase.·

Sample	Coordination environment		
	Cu_A	Cu_B	Avg. (per Cu)
Unmodified control	2(N,O); 2(S,Cl)	3(N,O); 1(S,Cl)	2.5(N,O); 1.5(S,Cl)
*p*HMB-modified	4(N,O)	3(N,O); 1(S,Cl)	3.5(N,O); 0.5(S,Cl)
Cu_A-depleted	—	3(N,O); 1(S,Cl)	3.0(N,O); 1.0(S,Cl)

similar EXAFS measurements on cytochrome *c* oxidase that the EXAFS FT of Cu_B resembles those for stellacyanin. The EXAFS data we obtain for Cu_B however, bear little resemblance to that of stellacyanin. The Cu-(S,Cl) distance of 2.3 Å (Table I) clearly indicates that Cu_B is not a type 1 or "blue" copper. Therefore, if sulfur is indeed a part of the inner coordination sphere of Cu_B, methionine is a more likely candidate for the ligand than is cysteine. Methionine ligation to Cu_B is attractive in that it is consistent with the relatively long observed Cu-S distance. Another alternative that cannot be dismissed, however, is that a chloride ligand is bound to Cu_B. Binding of Cl^- to the fully oxidized cytochrome a_3-Cu_B site has been previously suggested to explain the spectroelectrochemical data obtained at various Cl^- concentrations (Blair et al., 1986).

The three (N,O) ligands in the inner coordination sphere of Cu_B are in accord with earlier spectroscopic studies on this site. Using ENDOR, Cline et al. (1983) identified three distinctly inequivalent nitrogen hyperfine interactions associated with an EPR-visible Cu_B species trapped during turnover. Experiments on (^{15}N)His substituted yeast oxidase are in progress to identify these nitrogenous ligands.

Our present EXAFS study clearly indicates that the inner coordination sphere of Cu_A consists of 2 (S,Cl) ligands and 2 (N,O) ligands (Table II.2). While S and Cl are indistinguishable in this experiment, Cl ligation to Cu_A would be inconsistent with earlier EPR and ENDOR work on the Cu_A site (see Blair et al., 1983, for a review). The assignment of 2 S ligands to Cu_A is consistent with a more recent ENDOR study that suggested two cysteine ligands to Cu_A (Martin, et al., 1985). Available protein sequence data for all species to date except for that for wheat reveals two highly conserved cysteines in cytochrome *c* oxidase. These conserved cysteines reside in subunit II, three residues apart ,and must provide the Cu_A binding site. The assignment of 2 (N,O) ligands to the Cu_A site is also consistent with previous ENDOR

work. Stevens et al. (1982) identified nitrogen hyperfine coupling between one or two histidine nitrogens and the unpaired electron at Cu_A.

The Cu_A site in *p*HMB-modified cytochrome *c* oxidase shows ligation to 4 (N,O) ligands. This finding is in accord with the previous EPR work of Gelles & Chan (1985) who proposed three spectroscopically equivalent nitrogenous ligands to the chemically modified Cu_A. This result is also consistent with the relatively low reduction potential of the chemically modified Cu_A site (Gelles & Chan, 1985).

A zinc absorption edge was also observed during the course of these copper EXAFS experiments. Several reports have recently identified zinc as a stoichiometric component of cytochrome *c* oxidase with one zinc on per oxidase functional unit (Einarsdottir and Caughey (1984,1985), Naqui et al., 1986). The presence of a zinc edge in both the *p*HMB-modified and Cu_A-depleted enzymes suggests that the zinc component of oxidase is not removed by the chemical modification or depletion of Cu_A. While the structure and function of the zinc site are largely unknown, these data do provide additional evidence for the structural integrity of the chemically-modified and Cu_A-depleted enzyme.

In summary, the preparation of a Cu_A-depleted cytochrome *c* oxidase has allowed the inner coordination sphere of Cu_B to be determined in a straightforward manner. These data, combined with the EXAFS data from the *p*HMB-modified and "unmodified" control enzymes, have enabled us to obtain the ligand structure for the Cu_A site. Our findings are largely consistent with other spectroscopic evidence for the structures of the Cu_A and Cu_B sites in cytochrome *c* oxidase. In particular, the present work clearly establishes the involvement of one heavy ligand (S,Cl) at Cu_B and two sulfur ligands in the inner coordination sphere of Cu_A.

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III The Copper Sites in Resting and Fully Reduced Cytochrome *c* Oxidase: An X-ray Absorption Fine Structure Study

Abstract.

Copper K-edge X-ray absorption spectroscopy (XAS) has been used to investigate the coordination environment of the copper centers in cytochrome *c* oxidase. We have utilized an improved curve-fitting method that assumes two shells of (S,Cl) ligands to fit the copper X-ray absorption fine structure (EXAFS) spectra of seven resting state enzyme samples. Using this method, we find that the first coordination shell of the oxidized copper sites can be best accounted for by the following model: Cu_A with 2 (N,O) ligands at 1.99Å, 1 (S,Cl) ligand at 2.3Å, and 1 (S,Cl) ligand at 2.6Å; Cu_B with 3 (N,O) ligands at 1.99Å and 1 (S,Cl) ligand at 2.3Å. We have also collected EXAFS data for the fully reduced enzyme. These results, combined with earlier EXAFS results [Li, P. M., Gelles, J., Chan, S. I., Sullivan, R. J., & Scott, R. A. (1987) *Biochemistry 26,* 2091-2095] show that the best-fit coordination scheme is: Cu_A with 3 (N,O) ligands at 2.01Å, 1 (S,Cl) ligand at 2.3Å; Cu_B with 3 (N,O) ligands at 2.01Å, 1 (S,Cl) ligand at 2.3Å. Proposed structures for the Cu_A and Cu_B sites are discussed.

Introduction.

Cytochrome *c* oxidase catalyzes the final transfer of electrons from ferrocytochrome *c* to molecular oxygen in the aerobic respiratory chain. In eukaryotes, the enzyme is an integral membrane protein residing in the inner mitochondrial membrane. In the dioxygen reduction reaction, the electrons are derived from cytochrome *c* on the cytosolic side of the membrane, and the protons consumed in the dioxygen reduction are taken up from the matrix. Thus, a charge separation occurs across the membrane, contributing to the transmembrane electrochemical potential. In addition to the dioxygen reduction activity, cytochrome oxidase also functions as a redox-linked proton pump. For every molecule of dioxygen reduced, up to four protons are pumped from the matrix side of the membrane to the cytosol side (Wikstrom, 1977). For a general review, see Wikstrom et al. (1981).

The enzyme contains four redox active metal centers that are involved in catalysis. Two of these, labelled cytochrome a_3 and Cu_B, are involved in dioxygen binding and reduction. These two centers are spatially close and form a binuclear cluster near the matrix side of the membrane. The other two centers, cytochrome *a* and Cu_A, have low midpoint potentials (near that of cytochrome *c*), and are involved in mediating the transfer of electrons from cytochrome *c* to the binuclear site.

Different lines of evidence from previous work on cytochrome oxidase have provided some structural information on the copper sites in cytochrome oxidase. The Cu_A site in cytochrome oxidase has been compared to the blue copper proteins. However, the atypical spectroscopic properties of Cu_A suggest an electronic structure unlike the blue coppers and unusual for Cu^{2+} complexes in general. Electron paramagnetic resonance (EPR) studies have revealed an unusually small and isotropic Cu hyperfine interaction for Cu_A, of which the largest component is not coincident with

the largest *g*-value (Hoffman, et al., 1980). Furthermore, the low field *g*-value is reported to be below the free electron *g*-value at *g*=1.99 (Beinert et al., 1962; Aasa et al., 1976). In light of more recent ENDOR results (Stevens et al., 1982; Martin et al., 1989), this lab has proposed a model for the oxidized Cu_A site in which the copper atom is coordinated by two interacting cysteine sulfur ligands and two histidine nitrogen ligands in a distorted tetrahedral geometry. Protein sequence data reveal that the only two highly conserved cysteines in the enzyme reside in subunit II three amino acids apart. Less is known about the Cu_B site. EPR and ENDOR work have suggested that this site is structurally similar to a type 3 copper site, having at least three nitrogen ligands (presumably from histidines) (Reinhammar et al., 1980; Cline et al., 1983). All indications are that these ligands reside in subunit I.

X-ray absorption spectroscopy (XAS) has also been used to probe the environment of the copper sites in cytochrome oxidase. However, the analysis of these data has been problematic due to the overlapping absorption edges for the two copper sites. Recently, Li et al. (1987) showed that this problem could be alleviated by using Cu_A-modified and Cu_A-depleted forms of the enzyme. From such studies, these authors suggested in the resting oxidized enzyme that the first coordination shell of Cu_A consists of 2 (N,O) ligands and 2 (S) ligands while the first coordination shell of Cu_B consists of 3 (N,O) ligands and 1 (S,Cl) ligand. In this paper, we describe further efforts to verify these conclusions. Specifically, we report a significant refinement of the curve-fitting techniques used to analyze the EXAFS data. These new curve-fitting results have now allowed us to refine our description of the bis-thiolate coordination for Cu_A in the oxidized state. We have also collected EXAFS data for the fully reduced enzyme and present the first spectroscopic evidence for the coordination environment of the reduced Cu_A and Cu_B sites in cytochrome oxidase based on the application of these new curve-fitting methods to EXAFS analysis of this state of the protein..

Materials and Methods.

Materials. All reagents and buffers were enzyme grade unless otherwise specified. Tween 20 was purchased from Sigma. All anaerobic work was performed under 1 atm of argon gas, which had been scrubbed of residual oxygen by bubbling through 0.1 M V^{2+} in 2N HCl.

Enzyme sample preparation. Cytochrome *c* oxidase was isolated in our laboratory by the method of Hartzell & Beinert (1974) with modifications. The cytochrome *a* concentration was determined using $\Delta\varepsilon_{(\text{reduced minus oxidized})} = 24\ M^{-1}cm^{-1}$ at 605 nm, and was taken to reflect the concentration of the total enzyme. Two other Hartzell / Beinert preparations were used (Samples 1 and 6), and were isolated by H. Beinert in Madison, Wisconsin. One enzyme sample (Sample 2) was prepared by the Yonetani method in the laboratory of M. Wilson in Essex (Yonetani, 1961). The monomeric form of the enzyme (Sample 3) was prepared by the method of Nalcez et al. (1983). Sample 4 was Hartzell /Beinert enzyme isolated in our laboratory. A sample isolated by the van Buuren method (Procedure III of Hartzell et al., 1978) was donated by H. Beinert (Sample 5). A sample isolated using the King method (Procedure II of Hartzell et al., 1978) was also prepared in our laboratory (Sample 7). The details of the samples and their curve-fitting results can be found in Scott et al., 1986.

Oxidized EXAFS samples were prepared as described in Li et al., 1987. The EXAFS sample of reduced cytochrome oxidase was prepared in our laboratory as follows. A sample of resting Hartzell-Beinert enzyme (3.0 mL, 250 μM in enzyme) in an anaerobic cell was made de-oxygenated by exchanging the atmosphere over the sample with purified argon several times. The container was then transferred to a glove box where the sample was reduced with a few grains of dithionite, and then loaded into a Beckman Airfuge 7 mL batch rotor. This rotor was equipped with a specially

machined aluminum top, which kept the contents of the rotor anaerobic during the centrifugation procedure. The sample was centrifuged at 100,000 x g for 4 h to pellet the enzyme. After the spin, the rotor was again transferred into a glove box, and the contents of the pellet were suspended in a minimum volume of anaerobic buffer and loaded into a lucite XAS sample holder (see Scott et al., 1986 for details). Typically, the EXAFS samples were ca 2 mM in copper and were stored at 77K in liquid nitrogen until used.

X-ray absorption data collection. All XAS data were collected at the Stanford Synchrotron Radiation Laboratory (SSRL) under dedicated operating conditions (3.0 Gev, 60-70 mA) using Si[220] monochromator crystals. A 2 mm vertical beam aperture was used for the EXAFS scans reported. The data were collected in the 8700–9700 eV range with the high-energy limit being the presence of a zinc edge at 9661 eV. Each scan lasted ~30 min, with 7-21 scans being averaged for each sample depending on the signal-to-noise ration for each individual scan. Data for the resting state samples were collected as described in Scott et al. (1986). The data for the reduced enzyme were collected as fluorescence excitation spectra using an ionization chamber fluorescence detector (EXAFS Co., Seattle) containing a six-absorption length Ni filter.

Data reduction and analysis. The EXAFS data reduction procedure was accomplished by our standard techniques (Scott et al., 1985; Scott et al., 1986). The energy for each individual scan was calibrated by using a copper foil reference (first inflection point at 8980.3 eV) as an internal calibrant (Scott, 1982). After averaging the scans, the background was removed by fitting a second-order polynomial to the data between 9050 and 9650 eV, and subtracting a constant from the polynomial function so that it passed through the data just before the Cu absorption edge. After subtraction of the polynomial, a third-order spline fit was used to fit the data between 9035 and 9650 eV with spline nodes at 9180 and 9400 eV. After subtracting the spline from the data,

the resultant was normalized by dividing by the Cu atomic falloff as modeled by the Victoreen equation (MacGillavry & Rieck, 1968). This falloff function was multiplied by a factor that forced the falloff curve to match the spline polynomial at 9000 eV (k=0). As discussed by Scott et al. (1986), this factor corrects for differences in concentration from one sample to another.

For the curve-fitting procedures used, the following theoretical expression for the EXAFS was used:

$$\chi(k)=\sum_{s}\frac{B_s N_s |f_s(\pi,k)|}{kR_{as}^2}\exp(-2\sigma_{as}^2k^2)\sin[2kR_{as}+\alpha_{as}(k)] \quad (1)$$

Here, the subscripts used are a for the absorber and s for the scatterer. The summation is over all the shells of scatters. For each of these shells, s, N_s, is the number of scatterers at an average distance R_{as} from the absorber (Cu atom). $\Delta\sigma_{as}$ is the root mean square deviation in the absorber-scatterer distance, R_{as}. The curve-fitting procedure uses an average Cu coordination hypothesis. For each shell, the type of scattering atom is chosen by selection of the appropriate backscattering functions. These functions appear in equation (1) as the amplitude term, $[B_s|f_s(\pi,k)|]$ and the phase term, $[\alpha_{as}(k)]$. (B_s is a scaling factor that is used to correct the theoretical amplitude functions). For these studies, we use empirical backscattering functions derived from model compound data. For a more extensive discussion of this technique, see Scott et al. (1986). Equation (1) is used to simulate the expected EXAFS spectrum. The fit of this simulated spectrum to the observed EXAFS spectrum obtained by adjusting N_{as}, R_{as}, and $\Delta\sigma_{as}$ values for each shell to vary in a non-linear least squares optimization.

A modified goodness-of-fit statistic (f') was used to estimate the quality of the curve fitting results:

$$f' = \frac{\{\sum_{i}[k^3(\chi_{(i)obs'd} - \chi_{(i)calc'd})]^2 / N\}^{1/2}}{(k^3\chi)_{max} - (k^3\chi)_{min}} \quad (2)$$

This statistic is normalized for the differences in the magnitude of the $k^3\chi$ data from one spectrum to another.

Results and Discussion.

Copper EXAFS of resting cytochrome c oxidase. Cytochrome *c* oxidase is a multi-site copper protein. Deconvolution of the copper XAS data into contributions from each copper component is non-trivial. Two approaches have been used in the study of the copper EXAFS of cytochrome oxidase. Powers et al. (1979) have used the "site modelling" approach, which involved using the copper XAS spectrum of stellacyanin and other model compounds to model the Cu_A and Cu_B sites of the enzyme. However, Scott et al. (1986) have made an argument against the use of the "site modelling" approach, pointing out that the choice of one site model over another is arbitrary. These authors have pointed out that arbitrarily assigning one subset of scatterers forces the rest of the scatterers to be assigned to the other copper site. As an alternative, these authors have advocated an approach that uses theoretical curve-fitting to determine the average coordination of both copper sites in any enzyme derivative.This approach was recently used by Li et al. (1987) to assign the ligand structure for both Cu_A and Cu_B using a chemically modified and a Cu_A-depleted form of the enzyme. These authors showed that the best coordination scheme for Cu_A is 2 (N,O) ligands and 2 (S,Cl) ligands, whereas Cu_B is most likely to have a ligand structure that involves 3 (N,O)

ligands and 1 (S,Cl) ligand. However, despite the success in identifying the ligands to Cu_A and Cu_B using this approach, one problem has remained in the EXAFS analysis, namely the magnitude of the goodness-of-fit statistics is always considerably inferior to the theoretical fits to the EXAFS data for other metalloproteins (R. A. Scott, personal communication). The values for the goodness-of-fit parameters *f'* for cytochrome oxidase tend to be larger that the similar *f'* values for other metalloproteins by a factor of 2 to 3.

Figure III.1 shows a typical set EXAFS data for resting state preparation of cytochrome oxidase as it is taken through the data reduction and analysis procedure. Eight such preparations were examined. Figure III.1a shows the raw EXAFS data. As discussed by Scott (1986), all the raw spectra are quite similar in their signal-to-noise characteristics as well as their overall shape. Figure III.1b shows a typical Fourier transform of the raw data. In general, the FT consists of two major peaks, one at R'=1.5 Å and one at R'=1.9Å. As discussed by Scott et al. (1986) there is a peak at R'=3.5 that is sometimes discernible, but irreproducible. These authors have assigned this peak to noise in the FT. Because the two major peaks in the FT were sometimes unresolved, they were always selected together using a window shown by the dotted line. Although the FTs differ in their resolution depending on the type of sample and the experimental conditions under which the data were collected, all eight data sets yielded extremely similar curve fitting results (Scott et al., 1981; Scott et al, 1986). Back transformation of the two peaks yielded the filtered data shown in Figure III.1c. The filtered data were then curve-fit by using Cu-N and Cu-S backscattering functions described in the Materials and Methods. According to the method of Scott et al. (1986) an average coordination number was chosen to be integer or half integer for each shell. Then, R_{as} and σ_{as} for each shell were optimized using a least squares optimization routine.

The curve fitting results for seven different resting enzyme samples are summarized in Table III.1. We find that the goodness-of -fit statistics *f'* can be improved dramatically in every case by the use of two coordination shells to describe the Cu - (S,Cl) scattering. When two shells are used, we find that two distinct Cu - (S,Cl) interactions are consistently obtained, one at ~2.27Å and one at ~2.60Å. The Cu- (N,O) scattering appears to be best described by 2.5 (N,O) ligands at a distance of ~2.00Å. Despite the differences in enzyme preparation and data collection conditions, these results are quite reproducible for all the samples examined. It should also be noted that the Cu-(N,O) interaction is unaffected by the use of two Cu - (S,Cl) shells instead of one. The average improvement in the goodness-of-fit statistic for the seven different samples of resting cytochrome oxidase examined is 45%.

Because Cu-(S,Cl) interactions were found for both the Cu_A and Cu_B sites by Li et al. (1987), the location of the "long" Cu-(S,Cl) interaction could also be assigned using these data. These authors found that the *p*-(hydroxymercuri)benzoate-modified enzyme that contained a type 2 Cu_A site and a native Cu_B site could be fit with only one Cu-(S,Cl) interaction at 2.30Å. Furthermore, the Cu_A-depleted enzyme, which contained only Cu_B, could be fit with a Cu-(S,Cl) interaction at 2.31Å. These results indicate that the "long" Cu-(S,Cl) interaction is likely to reside at the Cu_A. The Cu-(S,Cl) distance (R_{as}) was allowed to vary during the curve-fitting procedure for the *p*HMB-modified and Cu_A-depleted EXAFS data.

Copper EXAFS of fully reduced cytochrome c oxidase. A key step toward understanding the role of Cu_A in the turnover cycle of the enzyme would be to obtain structural information on Cu_A in several forms of the enzyme. Unfortunately, the spectroscopic properties of the reduced Cu_A site (Cu^{+1}) are even more limited than for the oxidized Cu_A site. The reduced Cu_A site is diamagnetic, and hence silent to electron paramagnetic resonance techniques. Being d^{10}, reduced Cu_A also has no charge-

Figure III.1: A typical EXAFS data set for resting cytochrome *c* oxidase: (a) raw EXAFS after the background subtraction and spline fit; (b) Fourier transform of the raw EXAFS; (c) Fourier filtered EXAFS (solid line) and the best curve-fitting results (dotted line).

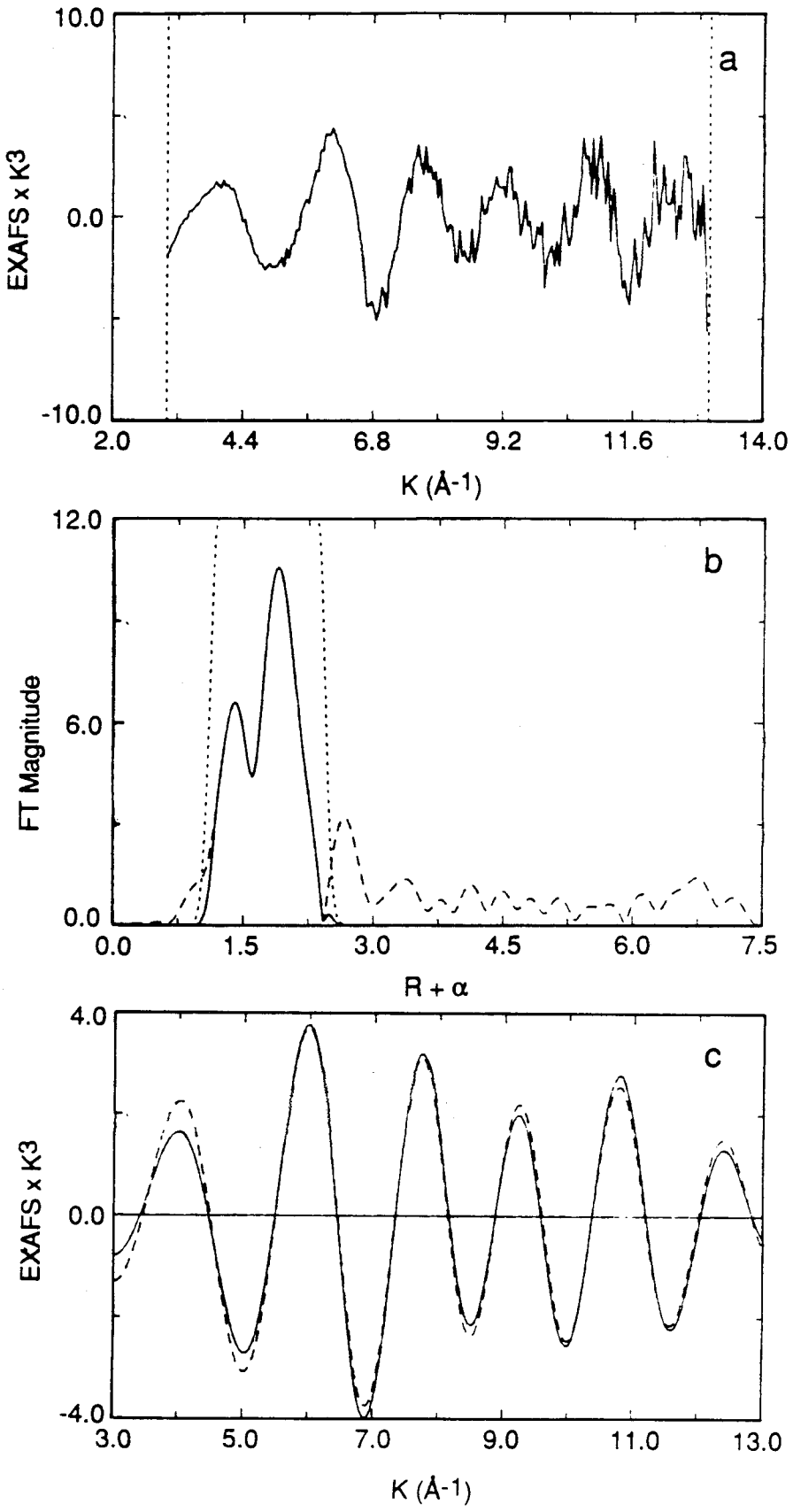

a
10.0
0.0
-10.0
EXAFS x K3
2.0
4.4
6.8
9.2
11.6
14.0
K (Å-1)
b
12.0
6.0
0.0
FT Magnitude
0.0
1.5
3.0
4.5
6.0
7.5
R + α
c
4.0
0.0
-4.0
EXAFS x K3
3.0
5.0
7.0
9.0
11.0
13.0
K (Å-1)

Table III.1: Curve-Fitting Results for the First Coordination Sphere of Resting State Cytochrome c Oxidase Using One and Two Sulfur Shells.

	Cu–(N,O)			Cu–(S,Cl) [short]			Cu–(S,Cl) [long]			
Sample	N_S	R_{as} (Å)	$\Delta\sigma_{as}^2$ (Å2)	N_S	R_{as} (Å)	$\Delta\sigma_{as}^2$ (Å2)	N_S	R_{as} (Å)	$\Delta\sigma_{as}^2$ (Å2)	f
1	(2.5)	1.98	0.1381	(1.5)	2.27	0.1023	–	–	–	0.0553
2	(2.5)	1.99	0.1990	(1.5)	2.28	0.0893	–	–	–	0.0502
3	(2.5)	1.96	0.1290	(1.5)	2.27	0.1012	–	–	–	0.1240
4	(2.5)	1.98	0.1154	(1.5)	2.28	0.0808	–	–	–	0.0629
5	(2.5)	2.00	0.1194	(1.5)	2.29	0.0982	–	–	–	0.0647
6	(2.5)	1.99	0.1270	(1.5)	2.31	0.1033	–	–	–	0.0722
7	(2.5)	1.92	0.1100	(1.5)	2.27	0.0761	–	–	–	0.0482
1	(2.5)	2.00	0.1326	(1)	2.27	0.1327	(0.5)	2.64	0.0960	0.0308
2	(2.5)	1.99	0.1990	(1)	2.28	0.0893	(0.5)	2.67	0.0980	0.0169
3	(2.5)	1.96	0.1290	(1)	2.27	0.1012	(0.5)	2.60	0.1037	0.0302
4	(2.5)	1.98	0.1154	(1)	2.28	0.0808	(0.5)	2.61	0.0988	0.0177
5	(2.5)	2.00	0.1194	(1)	2.29	0.0982	(0.5)	2.60	0.1100	0.0192
6	(2.5)	1.99	0.1270	(1)	2.31	0.1033	(0.5)	2.64	0.1159	0.0361
7	(2.5)	1.92	0.1100	(1)	2.27	0.0761	(0.5)	2.63	0.096	0.0394

[a]N_{as} is the coordination number per copper; R_{as} is the copper-scatterer distance; $\Delta\sigma_{as}^2$ is a mean square deviation in R_{as}; $\Delta\sigma_{as}^2 = \Delta\sigma_{as}^2{}_{(sample)} - \Delta\sigma_{as}^2{}_{(reference)}$ where the reference is $[Cu(imidazole)_4]^{2+}$ at 4K for Cu-(N,O) and $[Cu(mnt)_2]^{2+}$ at 4K for Cu-S. Numbers in parentheses were not varied during optimizations.

transfer bands in the visible region (Beinert et al., 1962). One of the few techniques capable of directly observing the reduced copper sites of cytochrome oxidase is XAS. Using anaerobic sample preparation techniques, we have now been able to generate a sample of the fully reduced enzyme for x-ray absorption studies.

Figure III.2 shows an EXAFS data set for the fully reduced form of cytochrome oxidase. The raw EXAFS data is shown in Figure 2a. The FT of these data is shown in Figure III.2b. At this point in the data reduction, the data appear qualitatively similar to the data for the resting enzyme. The FT spectrum is dominated by two FT peaks at R'=1.5Å and R'=1.9Å. It should be noted, however, that the relative intensities of the two peaks are somewhat different from the same peaks in the EXAFS FT for the oxidized enzyme. The Fourier-filtered EXAFS data for the fully reduced enzyme is shown in Figure III.2c. The dotted line shows the theoretical best-fit to these data. Any attempt to fit these data with amplitude and phase functions derived from Cu^{2+} model compounds were unsuccessful, indicating that the XAS characteristics of the copper sites change substantially upon reduction. Likewise, any attempt to use the amplitude and phase functions derived from Cu^{1+} model compounds to fit the EXAFS for the resting enzyme was equally unsuccessful, Thus, the theoretical fits to the oxidized and reduced forms of the enzyme are unique.

The curve fitting results for the theoretical fits to the data shown in Figure III.2 are summarized in Table III.2. The best fit result indicates an average of 3.0 (N,O) ligands per Cu at 2.03Å and 1.0 (S,Cl) ligands per Cu at 2.31Å. In contrast to the theoretical fits for the resting enzyme, the reduced form of the enzyme appears to be well described by only one type Cu-(S,Cl) scatterer. Further separating the Cu-(S,Cl) scattering contribution into two shells gives some improvements in goodness-of-fit parameter f'. However, the increase in f' was not large enough to justify using a two-

Figure III.2: Typical EXAFS data set for fully reduced cytochrome *c* oxidase: (a) raw EXAFS after the background subtraction and spline fit; (b) Fourier transform of the raw EXAFS; (c) Fourier filtered EXAFS (solid line) and the best curve-fitting results (dotted line).

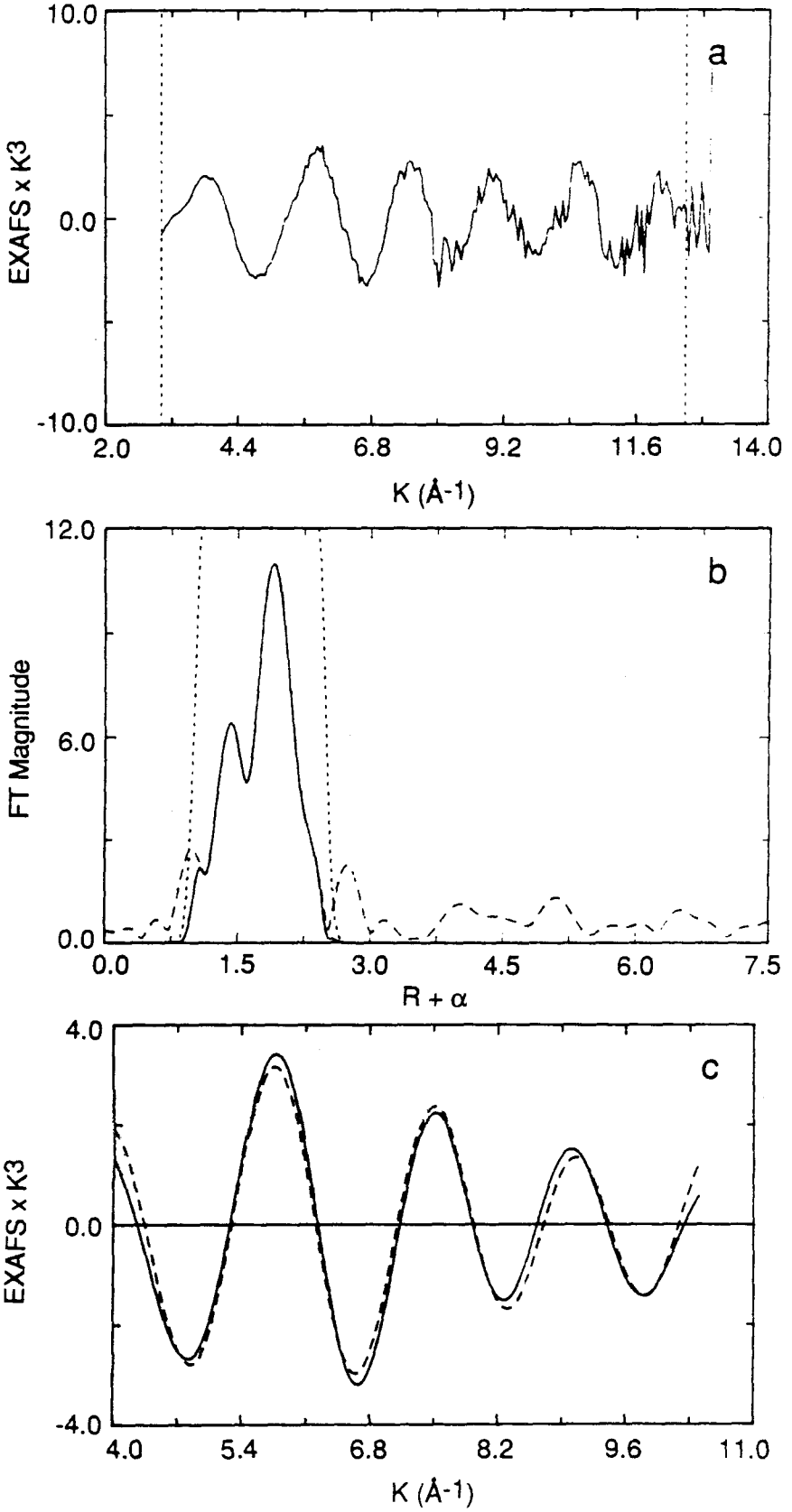

10.0
0.0
-10.0
EXAFS x K3
a
2.0
4.4
6.8
9.2
11.6
14.0
K (Å-1)
12.0
6.0
0.0
FT Magnitude
b
0.0
1.5
3.0
4.5
6.0
7.5
R + α
4.0
0.0
-4.0
EXAFS x K3
c
4.0
5.4
6.8
8.2
9.6
11.0
K (Å-1)

Table III.2: Representative Curve-Fitting Results for the First Coordination Sphere of Reduced Cytochrome *c* Oxidase Samples.[a]

	Cu–(N,O)			Cu–(S,Cl)			
Fit	N_S	R_{as}	$\Delta\sigma_{as}^2$ (Å^2)	N_S	R_{as}	$\Delta\sigma_{as}^2$ (Å^2)	f'
A	(4.0)[d]	2.09	0.1305	–	–	–	0.1628
B	–	–	–	(4.0)	2.29	0.1475	0.0847
C	(4.0)	2.09	0.1949	(2.0)	2.30	0.1243	0.0649
D	(2.0)	2.01	0.1477	(2.0)	2.31	0.1215	0.0601
E	(3.5)	2.05	0.1346	(0.5)	2.32	0.0698	0.0558
F	(2.5)	2.023	0.1434	(1.5)	2.31	0.1110	0.0513
G*	(3.0)	2.03	0.1392	(1.0)	2.31	0.0965	0.0465

[a] N_S is the coordination number per copper; R_{as} is the copper-scatterer distance; $\Delta\sigma_{as}^2$ is a relative mean square deviation in R_{as}, $\Delta\sigma_{as}^2=\sigma_{as}^2$(sample) - σ_{as}^2(reference), where the references are given in Scott et al. (1986).

[b] The letters in this column refer to the best fits as shown in Figure II.3

[c] f' is a goodness of fit statistic normalized to the overall magnitude of the $k^3\chi(k)$ data (Scott et al., 1986):

$$f' = \frac{\{\sum_i [k^3(\chi_{(i)obs'd} - \chi_{(i)calc'd})]^2 / N\}^{1/2}}{(k^3\chi)_{max} - (k^3\chi)_{min}}$$

[d] Numbers in parentheses were not varied during optimizations.

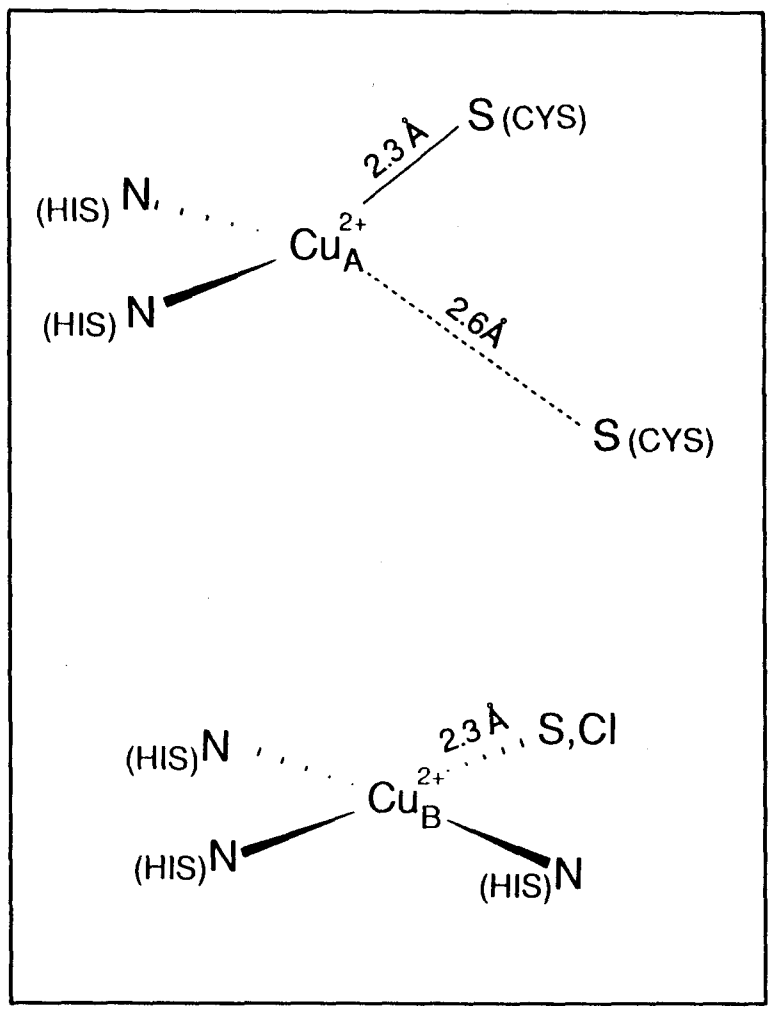
*Choice for "best fit."

shell fitting procedure. As for the resting enzyme, any attempts to include a "blue copper"-like Cu-(S,Cl) interaction at 2.18Å were unsuccessful.

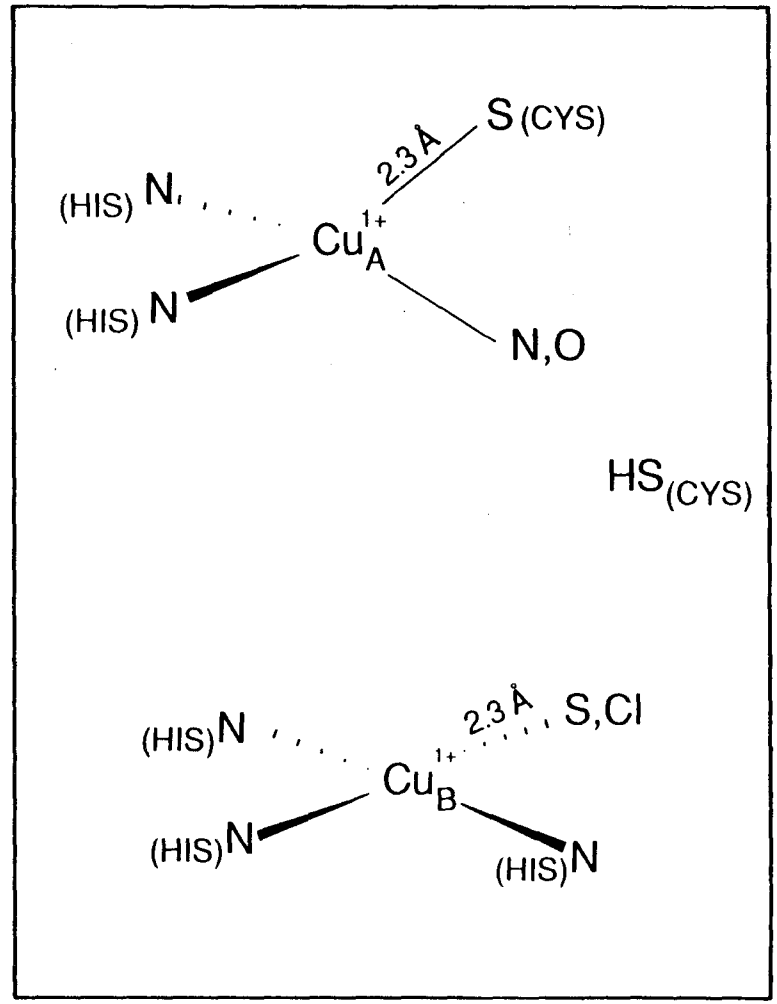
These results indicate that the first shell coordination environment for the copper sites changes when the enzyme becomes fully reduced. The most prominent difference between the resting and the reduced enzymes is the disappearance of the "long" Cu-(S,Cl) interaction that we have assigned to the Cu_A site. In fact, the average coordination for the reduced enzyme suggests that a (S,Cl) ligand is lost upon the reduction of the Cu_A site, and replaced by a (N,O) ligand. Such a ligand rearrangement has been postulated by Chan and co-workers based on the properties of bis-dithiolate Cu^{2+} and Cu^{1+} centers (see Gelles et al., 1986). However, there has been only indirect evidence for the lability of a sulfur ligand at Cu_A. Li et al. (1988) demonstrated that the Cu_A site can be modified by gentle heating at 43°C. One of the products of this heat treatment was a "blue copper"-like Cu_A site. The implication is that the heat treatment disrupts the cysteine coordination to Cu_A. Li et al. (1988) have further demonstrated that reducing the enzyme, or ligand binding to the binuclear center, protects the Cu_A site from this heat-induced modification. They suggested that this effect could result from a different protein environment about the Cu_A site in the reduced and ligand-bound forms of the enzyme.

Figure III.3 shows a pictorial representation of the copper sites in the resting and reduced forms of cytochrome oxidase based on the EXAFS data. We propose that in the oxidized enzyme, Cu_A is coordinated by two cysteine sulfurs and two histidine nitrogens. Unlike the previous proposals for this site, we advocate a somewhat asymmetric cysteine coordination, with a "short" Cu–S bond at ~2.3Å and a "long" Cu–S bond at ~2.6Å. It should be noted that the "short" Cu–S bond proposed here is much longer than the 2.18Å Cu–S interaction in the oxidized "blue copper" centers.

Figure III.3: A model for the oxidized and reduced copper sites in cytochrome *c* oxidase. Left panel: Best EXAFS-derived structures for oxidized Cu_A and Cu_B. Right panel: Best EXAFS-derived structures for reduced Cu_A and Cu_B.



OXIDIZED



REDUCED

In the reduced form of the enzyme, we propose that Cu_A undergoes a ligand rearrangement. Here, the Cu_A site can be described best by ligation to two (or three) histidine nitrogens and one "short" cysteine S. In this model, the "long" cysteine S in the oxidized Cu_A first coordination shell de-ligates upon reduction and moves to an extremely long distance (>2.9Å). This kind of ligand structure is reminiscent of the blue coppers except that the extremely long S is derived from a methionine side-chain and not cysteine.

The most notable feature of this model is the asymmetric bis-dithiolate coordination at Cu_A in the oxidized state. Because this type of coordination is unprecedented, we conclude this section with a short discussion of the proposed structure and some of the unusual spectroscopic features associated with the Cu_A site.

Cu_A is characterized by a small and isotropic copper hyperfine interaction,which cannot be explained totally by an admixture of Cu(4p) orbital character (Hoffman et al., 1980). Based on ENDOR results using [^{2}H]Cys- and [^{13}C]Cys-, Martin et al. (1988) have suggested that the small and isotropic hyperfine observed can be explained by the delocalization of the unpaired spin onto two sulfur ligands. These authors concluded based on these results that there may be two cysteine sulfur ligands to Cu_A, each with a distinct set of β-methylene protons. A somewhat asymmetric cysteine coordination is consistent with these results.

The linear electric field effect (LEFE) data measures the shift in g-value of an EPR resonance in the presence of an applied linear electric field, which is sensitive to the symmetry at the site (Mims, 1976). Mims et al. (1980) reported LEFE data for Cu_A, that suggested that this site is quite unlike the type 1 or type 2 copper sites. They concluded that the ligand field at Cu_A is not tetrahedral, nor is it trigonal planar like the "blue" copper sites. These authors also note that the LEFE data for Cu_A are inconsistent

with a square planar arrangement of ligands. The presence of asymmetric cysteine S coordination to Cu_A is also consistent with these results.

It is important to note that a ligand rearrangement at Cu_A could play a key role in the turnover cycle of the enzyme. In particular, Chan and coworkers have invoked this kind of ligand rearrangement to model Cu_A as the site of redox linkage to proton translocation (Chan et al., 1988). It could also be involved in an allosteric communication between the Cu_A site and the other redox-active metal centers in the protein. This kind of communication may be especially important in controlling the electron transfer pathways and gating the electron flow through the enzyme. We present data in the subsequent chapters of this thesis that support the involvement of these kinds of ligand rearrangement reactions and allosteric interactions in the turnover cycle of the enzyme.

Conclusions.

The Cu EXAFS of the resting and reduced forms of cytochrome oxidase samples allow three major conclusions regarding the ligand structure of the copper sites in cytochrome oxidase:

(1) The ligand structures of the copper sites in resting cytochrome oxidase can be described best by 2 (N,O), 2(S,Cl) ligands at Cu_A, and 3(N,O), 1(S,Cl) ligands at Cu_B, with one of the (S,Cl) ligands at Cu_A having "long" distance of ~2.6 Å.

(2) The ligand structure of the copper sites in the reduced form of the enzyme is quite different from the resting form of the enzyme. In particular, it appears that the "long" (S,Cl) interaction at Cu_A disappears in the fully reduced enzyme.

(3) There is little evidence for a short "blue copper"-like Cu-S interaction.

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IV Chemical Modification of the Cu_A Site Affects Proton Pumping Activity in Cytochrome *c* Oxidase

Abstract.

Cytochrome *c* oxidase in which the Cu_A site has been perturbed by extensive modification of the enzyme with the thiol reagent, *p*-(hydroxymercuri)benzoate has been reconstituted into phospholipid vesicles. The reconstituted vesicles lack respiratory control and the orientation of the enzyme in the vesicles is similar to that of the native cytochrome *c* oxidase. In the proton translocation assay, the vesicles containing the modified enzyme behave as if they are unusually permeable to protons. When the modified and native proteins were co-reconstituted, a substantial portion of the latter became uncoupled as revealed by low respiratory control and low overall proton pumping activity. These results suggest that the modified enzyme catalyzes a passive transport of protons across the membrane. When milder conditions were used for the chemical modification, a majority of the thiols reacted while the Cu_A site remained largely intact. Reconstitution of such a partially modified cytochrome *c* oxidase produced vesicles with respiratory control and proton translocating activity close to those of reconstituted native enzyme. It thus seems that the appearance of a proton leak is related to the perturbation of the Cu_A site. It is suggested that this is a consequence of Cu_A filling a function in the proton translocation activity of cytochrome *c* oxidase.Cytochrome *c* oxidase fills a central function in the energy transduction of aerobic organisms. In eukaryotes, the enzyme, which spans the mitochondrial membrane, catalyzes the reduction of molecular oxygen to water. The reductant, cytochrome *c*, donates the electrons from the cytosolic side of the membrane while the protons consumed in the reaction are taken up from the mitochondrial matrix. In addition, one proton is actively transported from the matrix to the cytosol for each electron transferred (Wikström, 1977; Casey et al., 1979). The reaction is thus

electrogenic and contributes to the electrochemical potential gradient across the inner mitochondrial membrane. It has been suggested (Wikström et al., 1981) that the proton translocating activity is necessary for the full utilization of the redox span between cytochrome *c* and molecular oxygen. While a great deal is known about the oxygen reduction in cytochrome *c* oxidase (see Malmström, 1982; Naqui et al., 1986 and Wikström et al., 1981 for reviews, and Blair et al., 1985), the molecular mechanism of proton translocation remains largely unknown.

Introduction.

The enzyme contains four redox-active metal centers, two irons in cytochromes *a* and a_3, and two copper ions that are usually labelled Cu_A and Cu_B. Cytochrome a_3 and Cu_B form the binuclear site where oxygen is bound and reduced, while cytochrome and Cu_A are the primary acceptors of electrons from cytochrome *c*. A recent investigation of proton translocation by cytochrome oxidase in intact mitochondria (Wikström & Casey, 1985) suggests that the site of energy coupling is one of the two low-potential centers cytochrome *a* and Cu_A. Although cytochrome *a* has been commonly assumed to be the more likely candidate (Wikström et al., 1981; Babcock & Callahan, 1983), Chan and co-workers have presented theoretical arguments that Cu_A being the site of coupling is equally consistent with current knowledge of the enzyme (Gelles et al., 1986). Furthermore, mechanistic considerations by these authors suggest that Cu_A is the center most suited for a role in proton translocation.

Recently, Gelles & Chan (1985) discovered that the Cu_A site may be chemically modified by exhaustive treatment with the thiol reagent, *p*-(hydroxymercuri)benzoic acid (*p*HMB). The protein product is active in electron transfer, albeit with an activity of about 20% of that of the native enzyme. Because the modified Cu_A site is not redox active under the assay conditions used, the residual activity must reflect electron transfers from cytochrome *c* to the oxygen binding site *via* cytochrome *a*.

Reconstitution of this modified enzyme into phospholipid vesicles allows measurement of its proton-translocating activity and therefore offers a possibility to probe the role of Cu_A in energy transduction. We report here the characterization of reconstituted cytochrome *c* oxidase containing a modified Cu_A site with respect to energy coupling and proton translocation.

Materials and Methods.

Materials. 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes); *p*-(hydroxymercuri)benzoic acid (*p*HMB); asolectin (from soybean; type IIs), cholic and deoxycholic acids; carbonylcyanide-m-chlorophenyl hydrazone (CCCP) and cytochrome *c* (from horse heart; type VI) were obtained from Sigma. Dodecyl maltoside and valinomycin were from Calbiochem. Sephadex G-25 was from Pharmacia and Biogel P-100 from BioRad. Amberlite XAD-2 was obtained from Mallinkrodt and washed (Müller et al. 1986) before use. All other chemicals were of analytical grade. The asolectin was purified by acetone-ether fractionation as described by Kagawa & Racker (1971) except that dithiothreitol (1 mM) was used as the antioxidant. Cholate and deoxycholate were recrystallized from 70% ethanol and 80% acetone, respectively.

Cytochrome *c* oxidase was purified from beef heart mitochondria as described by Hartzell & Beinert (1974) and stored at -80 °C. Enzyme concentrations were calculated from $A_{(red\ minus\ ox)}$ at 605 minus 630 nm using an extinction coefficient of 27 $mM^{-1}cm^{-1}$ (Hill & Greenwood, 1984).

Cytochrome *c* was reduced by the addition of solid sodium dithionite and then gel filtered on a short Sephadex G-25 column. The concentration of reduced cytochrome *c* was determined as described by Casey (1986).

Exhaustive modification of cytochrome c oxidase with pHMB. The reaction conditions of Gelles & Chan (1985) were used except that the Tween-20 in the reaction buffer was replaced by 10 mM dodecyl maltoside. After 24 hours at room temperature, the mixture was centrifuged (25,000 g, 15 min) to remove solid *p*HMB. The supernatant was concentrated by ultrafiltration (Amicon XM-300 membrane) to a volume of approximately 0.5 mL. To remove the remaining *p*HMB, the solution was passed down a Bio-Gel P-100 gel filtration column (0.8x15 cm) equilibrated with 0.1

M Hepes/K^+, pH 7.4 containing 0.3% deoxycholate. The protein fraction was collected, kept on ice, and used for reconstitution within a few hours.

Partial modification of cytochrome c oxidase with pHMB. Cytochrome *c* oxidase was diluted to a concentration of 7 μM (calculated from the absorption at 420 nm and $\Delta\varepsilon 141\ mM^{-1}$; Blair et al., 1982) with 50 mM Tris-HCl, 50 mM NaCl pH 7.7 containing 0.5% dodecyl maltoside. Then 156 ul of the same buffer saturated with *p*HMB (41.6 mM *p*HMB as judged from the absorption at 232 nm and $\Delta\varepsilon 16.9\ mM^{-1}\ cm^{-1}$; Boyer, 1954), giving a final concentration of *p*HMB of 0.98 mM. The absorbance change at 250 nm was 0.35 after correcting for the contribution from free *p*HMB. Using $\Delta\varepsilon=7.6\ mM^{-1}cm^{-1}$ (Boyer, 1954), this corresponds to the modification of, on the average, 6-7 thiols per enzyme. To quench the reaction, cysteine (final concentration 1 mM) was added and the reaction mixture was concentrated to approximately 0.5 mL by ultrafiltration. EPR spectroscopy of a sample prepared this way showed that the Cu_A signal is essentially unperturbed. If the reaction was allowed to proceed further, (4 hours at room temperature), a larger absorbance change, corresponding to the modification of approximately 9 thiols per enzyme was obtained. However, in this case the EPR spectrum of Cu_A becomes perturbed, showing the appearance of hyperfine structure in the g region.

Reconstitution of cytochrome c oxidase. The enzyme was reconstituted into phospholipid vesicles by cholate dialysis as described by Casey (1986) with minor modifications. The second and third dialysis buffers were supplemented with 20 g/l Amberlite XAD-2 and a fourth dialysis (15 h) versus 200 volumes of 0.1 mM Hepes/K^+, 22 mM K2SO4 and 55 mM sucrose, pH 7.4 was added. After dialysis, the vesicles were centrifuged (25,000 g, 30 min) to remove large particles. This step was found to improve the respiratory control of the vesicles significantly.

Two different batches of cytochrome *c* oxidase were used in the present work. One, which was used for the majority of the experiments, produced well-coupled vesicles when it was reconstituted. The other, however, gave vesicles with poor respiratory control (RCR = 2.6-2.8). Following Finel and Wikström (1986), we found that this could be much improved by sucrose gradient ultracentrifugation of the enzyme prior to reconstitution. Consequently, an ultracentrifugation step was routinely included in reconstitutions using this batch of enzyme.

Gradients of 5-30% sucrose (w/v) in 0.5 M potassium phosphate buffer pH 7.4 containing 2% cholate in a total volume of 4.5 mL were prepared in 5 mL centrifuge tubes as described by Luthe (1983). Samples (~60 nmol cytochrome *c* oxidase in 0.5 mL) were applied and the tubes were centrifuged for 16 hours at 40,000 rpm in a Beckman SW50.1 rotor. The green band of cytochrome *c* oxidase was collected manually from the top, concentrated by ultrafiltration and reconstituted.

Characterization of reconstituted vesicles. Respiratory control was measured polarographically at 25°C using an oxygen electrode from Yellow Springs Instruments. The reaction medium contained 50 mM K phosphate, pH 6.8, 50 mM KCl, 0.1 mM EGTA, 25 μM cytochrome *c* and 15 mM ascorbate. Reactions were initiated by the addition of reconstituted vesicles. The respiratory control ratio (RCR) was calculated by dividing the rate obtained in the presence of 0.2 μM CCCP and 0.02 μM with that obtained in the absence of the uncouplers.

The orientation of the enzyme in the reconstituted vesicles was determined essentially as described by Thelen et al. (1984), except that anaerobic conditions, instead of cyanide, were used to prevent turnover of the enzyme. The sample, in a Thunberg-type spectrophotometric cell equipped with a septum port and a sidearm, was made anaerobic by repeated evacuation and flushing with purified argon. After a spectrum of the fully oxidized enzyme was taken, the fraction facing outwards in the

active orientation was reduced by the addition of ascorbate. Another spectrum was then taken and the remaining oxidized enzyme (the fraction facing the vesicular lumen) was reduced by solid sodium dithionite added from the sidearm. The fraction of enzyme with the cytochrome *c* binding site facing the external medium was calculated as $A_{(red-ox)}$ at 605-630 nm obtained with ascorbate divided by the corresponding value obtained with dithionite.

Proton translocation assay. Extravesicular pH changes were measured with a Radiometer PHM 82 pH meter equipped with a GK 2401C electrode. The instrument was interfaced to an IBM PC AT for data collection and storage. The data acquisition rate was 2 s^{-1} and the time constant of the system (including mixing time) was approximately 2 s. Reaction mixtures contained 1.1 mL medium (44 mM KCl, 56 mM choline chloride), 0.25 mL vesicles (0.8-1.5 nmol cytochrome *c* oxidase) and valinomycin to a final concentration of 11 μM. The pH of the reaction mixture was carefully adjusted to that of the cytochrome *c* stock solution with 0.5 mM H2SO4 or dilute, carbonate-free NaOH. The stirred reaction vessel was thermostated at 20°C and air, depleted of CO_2 by scrubbing with 10% KOH, was blown over the surface of the solution. Reactions were initiated by the addition of reduced cytochrome *c* in an approximately 15-fold excess over the reconstituted cytochrome *c* oxidase. The external and total buffering capacities were obtained by adding a known amount of dilute standard H_2SO_4 (Casey, 1986). For the determinations of proton translocation stoichiometry, the initial acidifications were obtained from extrapolation of the decays to zero time (Casey, 1986; Müller et al., 1986). Under these assay conditions, we did not find any appreciable dependence of the number of protons transported per electron on the excess cytochrome *c* in the range 10- to 30-fold excess.

Results.

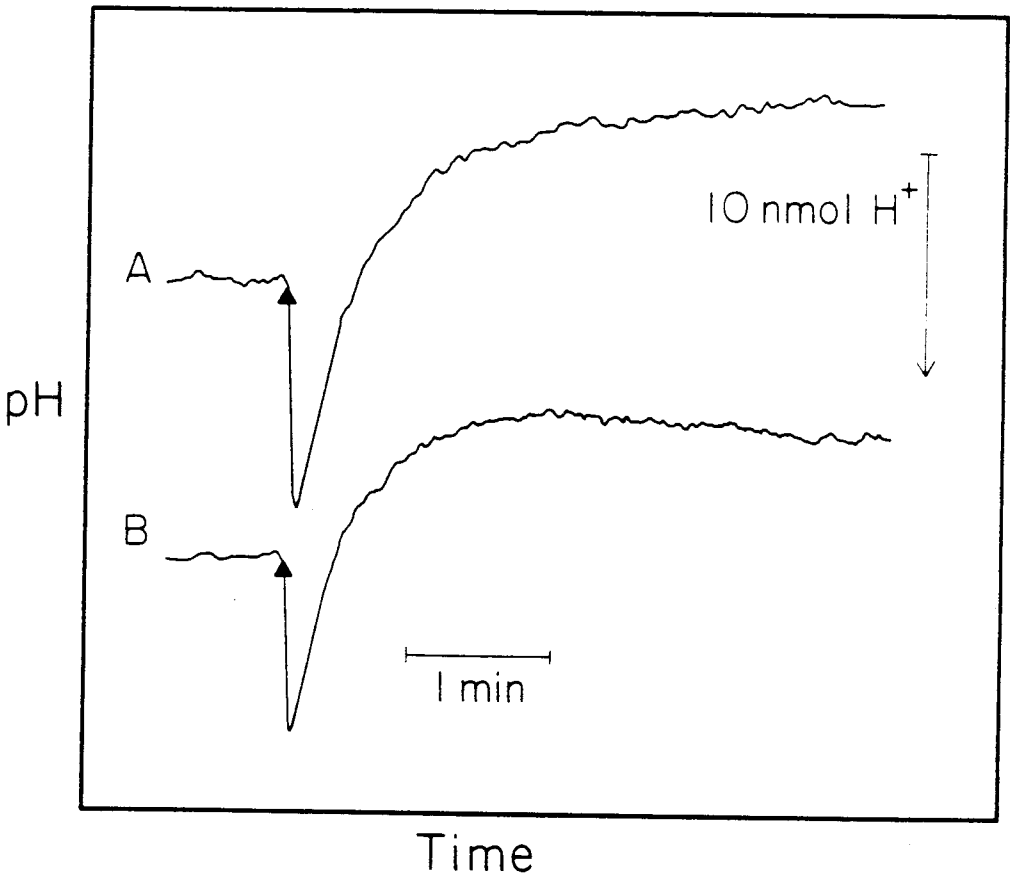
Reconstitution of the modified cytochrome *c* oxidase produced vesicles with a very low degree of coupling. Typically, respiratory control ratios (RCR) of 1-1.2 were obtained. Control samples were treated identically except that *p*HMB was omitted, and gave RCR values of 2.5-3.2 while native oxidase that had been incubated with 0.3% deoxycholate before reconstitution gave values between 4.5 and 6. The orientation assay showed that 75-85% of the enzyme was oriented with the cytochrome *c* binding site facing the external medium in all three cases.

Figure IV.1 shows the behavior of the reconstituted native and control enzymes in the proton translocation assay. With the native enzyme (Trace A), a stoichiometry of 0.85 protons ejected per electron transferred was obtained after extrapolation to zero time. Other vesicle preparations with the native enzyme gave values between 0.7 and 0.9 H^+/e^-. In the presence of 4 μM CCCP, addition of cytochrome *c* resulted in rapid alkalinization corresponding to approximately 1 H^+/e^- (data not shown).

Reconstituted control enzyme (Trace B) gave stoichiometries around 0.5, which is significantly lower than that obtained with the native enzyme. This is similar to what has been observed earlier with cytochrome *c* oxidase depleted of subunit III (Püttner et al., 1984) or treated with dicyclohexylcarbodiimide (Casey et al., 1979). However, at the protein:detergent ratio used in the present work, subunit III is not expected to dissociate from the enzyme (Hill & Robinson, 1986). Another explanation for the low stoichiometry is that prolonged incubation at room temperature may partially monomerize the enzyme. Finel & Wikström (1986) have shown that the apparent stoichiometry of proton translocation is lower with monomeric cytochrome *c* oxidase.

The vesicles containing the modified enzyme behaved dramatically different. Figure IV.2 shows an almost instantaneous alkalinization of the external medium upon the addition of cytochrome *c*. These vesicles clearly do not display any proton pumping

Figure IV.1: Proton pumping by the native and control enzymes. Trace A) the reaction mixture contained 1.3 nmol reconstituted native cytochrome *c* oxidase. Trace B) the reaction mixture contained 1.4 nmol reconstituted control enzyme (treated as described in the text). At times indicated by the triangles, 15.9 nmol ferrocytochrome c was added. The external buffering capacities, indicated by the vertical arrow, were determined in both samples after the cytochrome *c*-induced pH changes had decayed completely. The vertical scales are adjusted so that the initial acidifications brought about by adding 10 nequiv H_2SO_4 correspond to the same vertical displacement for the two traces.

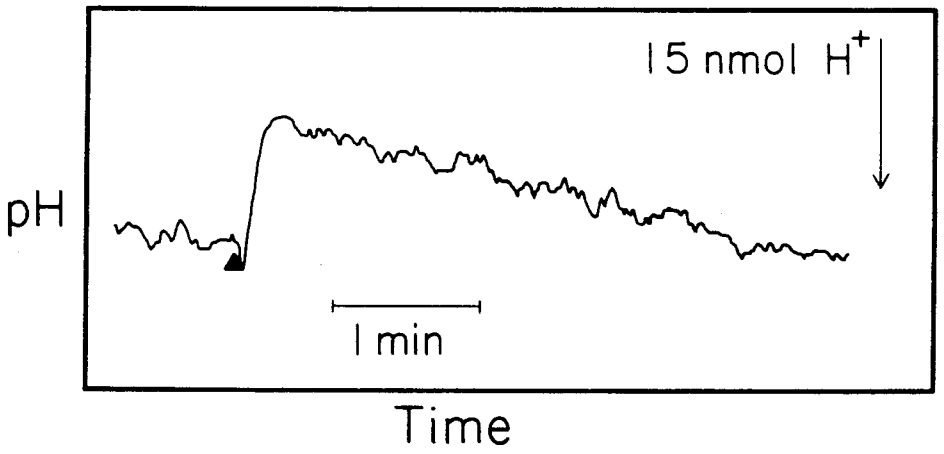

pH
A
B
10 nmol H+
1 min
Time

activity. Moreover, the scalar protons consumed in the vesicular lumen are replenished from the external medium much faster than the permeability of the bilayer would allow. This increased membrane permeability towards protons could be the result of a leak introduced in the enzyme by the chemical modification or due to the presence of a soluble uncoupler (traces of *p*HMB?) in the vesicle preparation. Alternatively, the modified enzyme is not reconstituted at all and only present in solution in the external medium. Any one of these explanations is consistent with the low respiratory control obtained for the reconstituted modified enzyme mentioned earlier. However, we find that the orientation of the reconstituted modified enzyme falls in the same range as those of the native and control samples, indicating that it is incorporated in a similar manner. We also have no evidence that residual *p*HMB is acting as an uncoupling agent (see below) in the vesicle preparation. This leaves the existence of a transmembrane proton-conducting pathway in the modified cytochrome *c* oxidase as the most likely explanation for the observed behavior in the proton pumping assay.

To investigate the proton permeability of the modified enzyme further, we have co-reconstituted it with native cytochrome *c* oxidase. Under the reconstitution conditions used here, a substantial portion of the enzyme is expected to become incorporated into vesicles containing more than one enzyme molecule (Casey et al., 1984). If the modified enzyme were responsible for the enhanced proton permeability, all vesicles that contain at least one molecule of modified enzyme would behave as if completely leaky and give rise to traces similar to that in Figure IV.2. Only vesicles that contain native oxidase exclusively would yield ordinary pumping traces (Figure IV.1, Trace A). The net effect would thus be the uncoupling of that fraction of the native enzyme located in vesicles also containing a modified enzyme.

Native and *p*HMB-modified cytochrome *c* oxidases were mixed in ratios of 0.8:0.2 and 0.6:0.4 (concentrations based on oxidized-reduced difference spectra) and

Figure IV.2: Behavior of the vesicles containing modified enzyme. The reaction mixture contained 0.5 mL vesicles (0.9 nmol enzyme) in a final volume of 1.35 mL and 15.9 nmol ferrocytochrome c was added at the time indicated by the triangle. The vertical arrow reflects the total buffering capacity.


15 nmol H+
pH
1 min
Time

reconstituted. Characterization of the reconstituted vesicles gave RCRs of 1.7 and 1.3, respectively. The outward orientations of the enzyme in the two vesicle preparations were 71% and 77%, respectively. A reconstitution of native enzyme alone carried out in parallel resulted in vesicles with an RCR of 6. In the co-reconstituted samples, the modified enzyme contributes only a small fraction of the total activity. Accordingly, the low RCR values obtained can most probably be attributed to uncoupling of part of the native enzyme rather than merely the presence of uncoupled, modified enzyme.

In Figure IV.3, proton translocation of the reconstituted mixtures (Trace B and Trace C) are compared to that obtained from the vesicles containing native enzyme only (Trace A). The appearances of Traces B and C show that the inclusion of even a small amount of modified enzyme exerts a drastic effect on the net proton translocation. To evaluate the effect of co-reconstitution more precisely, we estimated the proton translocation expected if the native and modified enzyme behaved independently. With the specific activity of the modified enzyme being approximately 20% of that of the native, it may be calculated that the initial acidification obtained from the 0.8:0.2 mixture would be 90% of that obtained from the native enzyme alone.

If f is the fraction of modified enzyme and a its specific activity relative to that of the native enzyme, the fractions of the ferrocytochrome c consumed by the native and modified enzymes are $(1-f)/(1-f+fa)$ and $fa/(1-f+fa)$, respectively. Becausee the fraction consumed by the modified enzyme causes alkalinization, the initial acidification obtained from the mixture relative to that obtained from the native enzyme alone is $(1-f-fa)/(1-f+fa)$. The corresponding estimate for the 0.6:0.4 mixture is 75%.

To verify the validity of this treatment, the experiment was repeated with a mixture of native and modified enzymes that had been reconstituted separately. Figure IV.4 shows that the calculation of expected acidification is correct within 15%. This

Figure IV.3: Comparison of proton pumping by reconstituted mixtures of native and modified enzyme with that of native enzyme alone. Trace A) the reaction mixture contained 1.3 nmol reconstituted native enzyme. Trace B) the reaction mixture contained 1.3 nmol (total) of a reconstituted mixture containing 80% native enzyme and 20% modified enzyme. Trace C) the reaction mixture contained 1.2 nmol (total) of a reconstituted mixture containing 60% native enzyme and 40% modified enzyme. At the times indicated by the triangles, 15.9 nmol ferrocytochrome c was added. External buffering capacities were determined as in Figure IV.1 and are displayed in the same way.

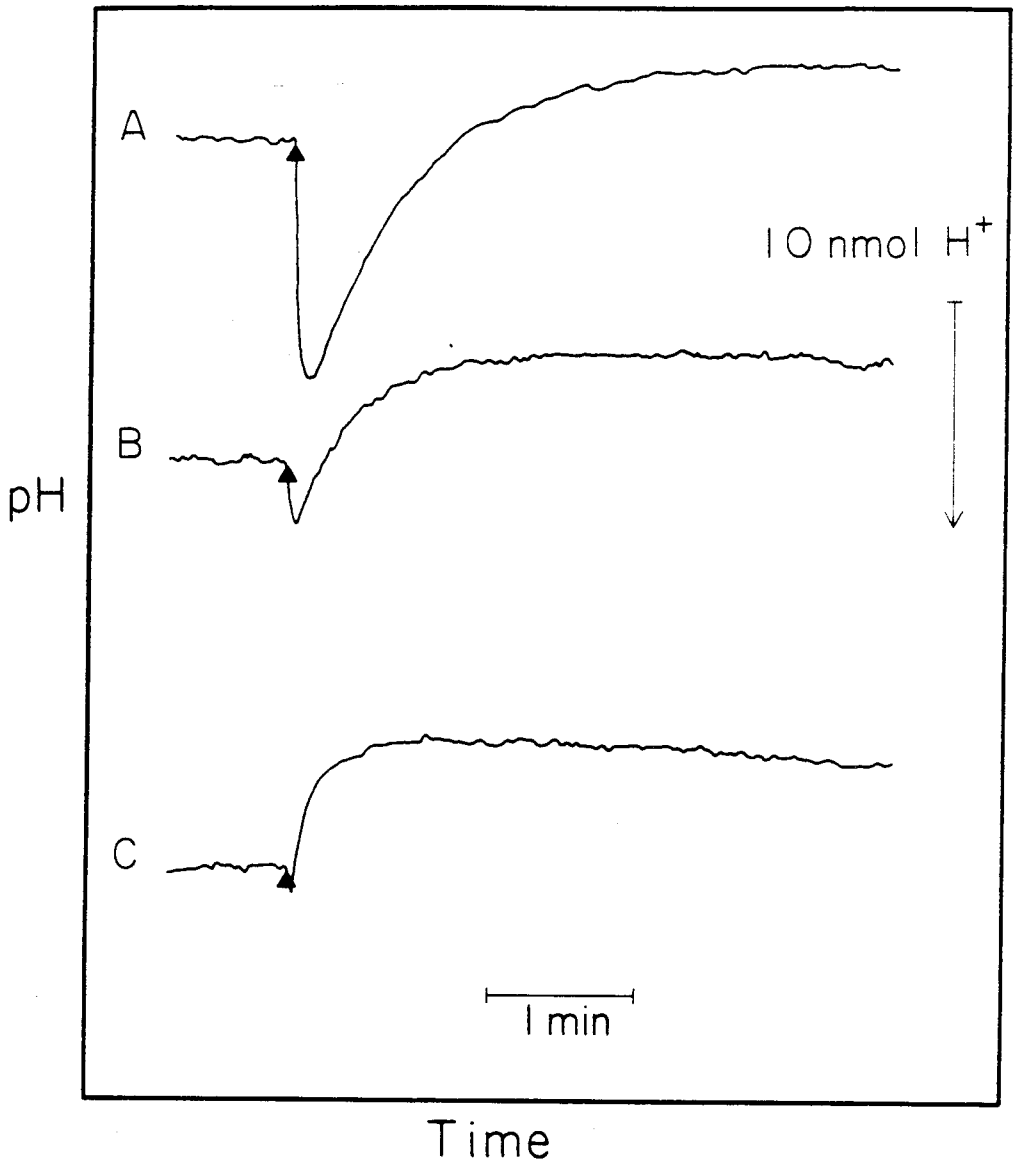
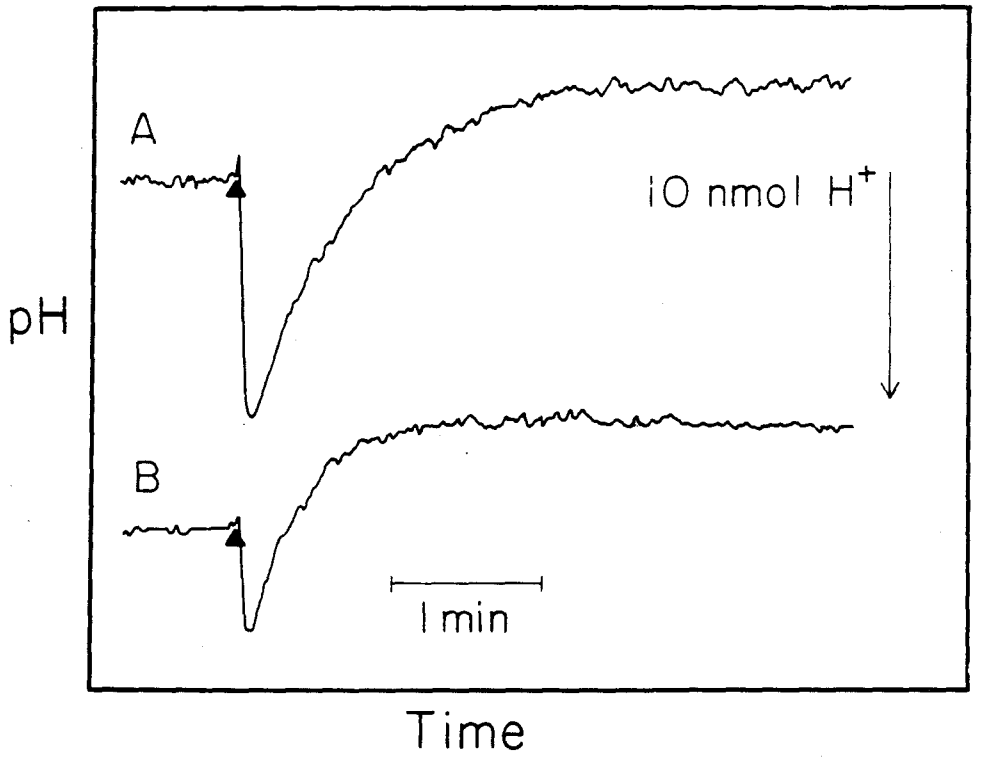

A
B
C
10 nmol H+
pH
1 min
Time

Figure IV.4: Comparison of proton pumping by a mixture of native and modified enzymes that had been reconstituted separately with that of the native enzyme alone. Trace A) the reaction mixture contained 1.3 nmol reconstituted native enzyme. Trace B) the reaction mixture contained 0.63 nmol reconstituted native enzyme and 0.22 nmol reconstituted modified enzyme. Ferrocytochrome *c* additions (15.9 nmol) are indicated by the triangles. External buffering capacities were determined as in Figure IV.1 and are displayed in the same way.


A
B
10 nmol H+
1 min
pH
Time

result also excludes the presence of a soluble uncoupler in the vesicle preparation containing the modified enzyme as the source of its enhanced proton permeability.

A comparison between the experimental and expected initial acidifications obtained from the reconstituted mixtures clearly shows that a substantial part of the native cytochrome *c* oxidase becomes uncoupled when it is reconstituted together with the *p*HMB-modified enzyme. The low RCRs found for the mixtures are in accord with this explanation. These results strongly support the notion that the modified cytochrome *c* oxidase contains a pathway for the transmembrane transport of protons. On the other hand, totally different results would have been obtained had the low RCR and proton pumping activity been due to impaired insertion of the modified enzyme into the vesicles.

Given that a vesicle containing at least one molecule of modified cytochrome *c* oxidase becomes completely uncoupled, the fraction of enzyme reconstituted into vesicles containing more than one enzyme molecule may be estimated from the net acidifications obtained. The trace obtained from the 0.8:0.2 mixture may be deconvoluted into a sum of two contributions: 63% from "pumping" vesicles and 37% from "leaking" vesicles. Assuming a Poisson distribution for the number of enzymes per vesicle (Apell & Läuger, 1986), we estimate that approximately 35% of the enzyme is in vesicles containing at least two enzyme molecules. This is in reasonable agreement with the distribution found by Casey et al. (1984).

Under the reaction conditions used to carry out the *p*HMB modification, all thiols of the protein are expected to become modified by the reagent. However, it has been shown earlier (Tsudzuki et al., 1967) that a majority of the thiols react very rapidly with *p*HMB at a much lower reagent concentration. Gelles & Chan (1985) found that Cu_A is not affected under these conditions. To assess whether the modification of thiols not associated with Cu_A is responsible for the leakiness of the

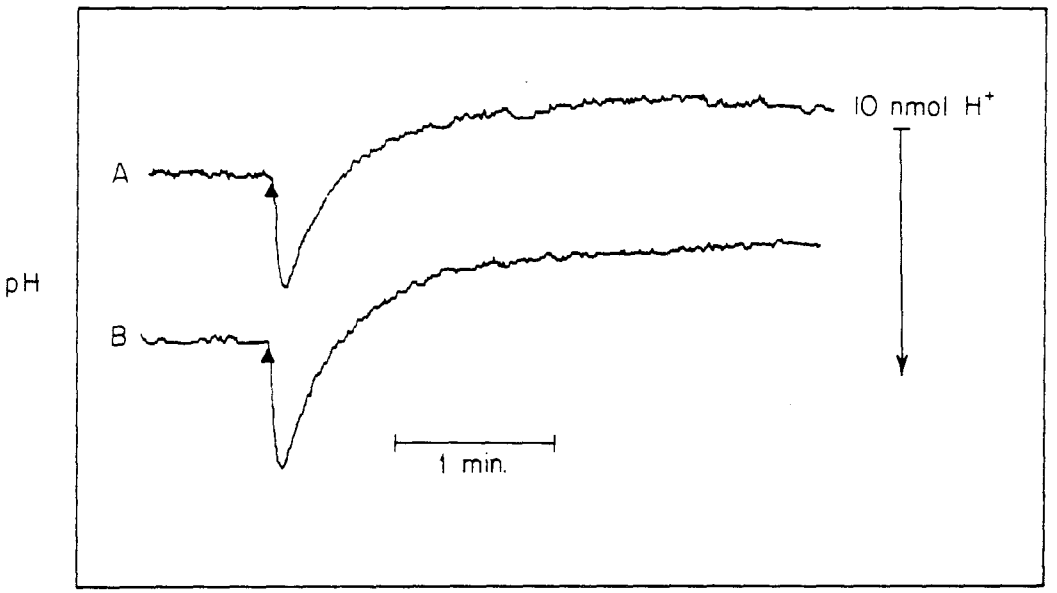
*p*HMB-modified cytochrome *c* oxidase, we prepared and reconstituted a short-time modified sample. The extent of modification was monitored *via* the absorbance change at 250 nm (Boyer, 1954; Benesch & Benesch, 1962).

When a sample that had been exposed to *p*HMB for only a short time was layered on a sucrose gradient, ultracentrifuged, and reconstituted, we obtained vesicles with an RCR of 3.8. A sample of native cytochrome *c* oxidase that had been ultracentrifuged and reconstituted in parallel gave vesicles with RCR = 3.5. The outward orientations were 74% and 71%, respectively. Proton translocation by these vesicles is shown in Figure IV.5. Clearly, modification of the rapidly reacting thiols has not impaired the proton pumping activity. However, the apparent stoichiometry for proton translocation for both vesicle preparations is significantly lower than the vesicles used for the experiment shown in Figure IV.1 (Trace A). The reason for this discrepancy is not clear, but seems to be related to the use of two different batches of cytochrome *c* oxidase.

Discussion.

Taken together, the proton translocation results obtained with partially and exhaustively modified enzymes indicate that the disruption of the Cu_A site is necessary for the appearance of the proton leak. There are, however, a total of 11 free thiols in cytochrome *c* oxidase (Buse et al., 1985), two of which are most probably ligands to Cu_A (Darley-Usmar et al., 1981; Li et al., 1987). Because we were able to modify only 6-7 thiols without affecting the Cu_A site, it cannot be entirely excluded that the modification of the remaining 2-3 non-ligand thiols is causing the appearance of the proton leak. However, we have also found that heating of cytochrome *c* oxidase according to Sone & Nicholls (1984) induces a change in the Cu_A EPR signal as well the loss of proton pumping activity (Li et al., 1988). Although the resulting EPR

Figure IV.5: Comparison of proton pumping by native and partially modified enzymes. Trace A) the reaction mixture contained 1.3 nmol reconstituted native enzyme. Trace B) the reaction mixture contained 1.2 nmol reconstituted, partially modified enzyme. At the times indicated by the triangles, 15.1 nmol ferrocytochrome *c* was added. External buffering capacities were determined as in Figure IV.1 and are displayed the same way.


10 nmol H+
A
B
pH
1 min.
Time

spectrum is complex, a significant portion of the copper has been converted to a form with an EPR signal very similar to that of the *p*HMB-modified enzyme. Furthermore, we obtain poorly coupled vesicles that display leaky behavior in the proton pumping assay when the heat-treated enzyme is reconstituted. The result that a leaky enzyme can be obtained without the use of *p*HMB strongly argues against covalent modification of thiols not coordinated to Cu_A being the cause for the leakiness observed in the present work.

Thus, it appears that the perturbation of the Cu_A site is responsible for the present results. This would support the proposal by Gelles et al. (1986) that Cu_A constitutes the site of redox-linked proton translocation in cytochrome *c* oxidase. It must be noted, however, that in the event proton translocation activity were unaffected by the modification, the protons ejected into the extravesicular medium would escape detection because of the leakiness of the modified enzyme. An alternative interpretation therefore is that the Cu_A-modification introduces an adventitious transmembrane leak while the proton translocating apparatus remains intact.

The disruption of the pumping site remains nevertheless an attractive explanation for the high leak rates observed. Clearly, the proton translocating site must be accessible to protons in the aqueous bulk phases on both sides of the membrane. Because the metal centers in cytochrome *c* oxidase are all located inside the protein, the enzyme must contain provisions to facilitate intramolecular proton transfers. It has thus been demonstrated that the site of oxygen binding and reduction, which requires the exchange of both water and protons with a bulk phase, is specifically connected to the matrix side of the membrane (Konstantinov et al., 1986). Although the mechanism for proton conduction remains obscure, similar proton pathways can be envisioned, linking the site of energy coupling to both sides of the membrane.

A minimum requirement for the pumping site is that a bound proton should not have access to both sides of the membrane simultaneously (Wikström et al. 1981; Tanford, 1983). A structural perturbation that removes this property, and consequently interconnects the two channels, would result in the kind of proton leak observed in the *p*HMB-modified cytochrome *c* oxidase described here. For example, in the model proposed by Gelles et. al. (1986), the cupric ion serves as an electrostatic barrier against adventitious proton transfers. A structural perturbation of the site which removes this function of the metal center can be easily imagined.

The finding that *p*HMB-modified cytochrome *c* oxidase "uncouples" co-reconstituted native enzyme in the respiratory control measurements suggests that the leak is capable of turning over at a rate approaching that of the electron transfer reaction in the native enzyme (~200 s^{-1}). The proton transfer mechanism involved is thus clearly kinetically competent for a role in the normal catalysis.

In conclusion, we have demonstrated that exhaustive modification of cytochrome *c* oxidase with *p*HMB is accompanied by the creation of a facile proton leak. This result is most likely due to the perturbation of the Cu_A site, and implies a role for the latter in the control of proton transfers in the enzyme. To qualify as a site for energy transduction a metal center must, however, be capable of controlling both proton and electron transfers (DeVault, 1971; Wikström et al., 1981; Blair et al., 1986). A plausible mechanism for the control of electron transfers by Cu_A has been put forward by Gelles et al. (1986). Here, it was shown how structural changes at the site can be exploited to "gate" electrons as required for energetic coupling. Although the proposed electronic structure of the Cu_A site indicates that it is conducive to redox-linked structural changes (Li, et al., 1988), little direct evidence for this exists at this time . Thus, although Cu_A is an attractive candidate for the proton translocating site in cytochrome *c* oxidase, further work is needed to place the proposal on a more solid

foundation. Specifically, the experimental demonstration of electron gating remains an important objective.

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V Heat Treatment of Cytochrome *c* Oxidase Perturbs the Cu_A Site and Affects Proton Pumping Activity

Abstract.

It has been previously reported that mild heat treatment (43°C for ca 60 min) abolishes the proton pumping activity of cytochrome *c* oxidase while leaving the oxidase activity and cytochromes *a* and a_3 unperturbed [Sone, N. & Nicholls, P. (1984) *Biochemistry 23,* 6550-6554]. We herein describe the effects of this heat treatment on the Electron Paramagnetic Resonance (EPR) and optical absorption signatures of the redox-active metal centers in the enzyme. We find that heat treatment of the oxidized enzyme causes a local structural perturbation at the Cu_A site. After heat treatment, the enzyme sample contains three subpopulations, each of which has a different structure at Cu_A. These include: (i) native Cu_A; (ii) a type 2 copper species similar to the one produced by chemical modification by *p*-(hydroxymercuri)benzoate (*p*HMB) [Gelles & Chan, (1985) *Biochemistry 24,* 3963-3972], and (iii) a novel type 1 copper species. In addition to changes at the Cu_A site, we find that heat treatment results in accelerated cyanide binding and the removal of subunit III. If the cytochrome *c* oxidase is heat treated while fully reduced, none of these changes are observed except for subunit III depletion. Furthermore, partial (CO-mixed valence derivative) reduction of the enzyme as well as ligand binding to cytochrome a_3 also protect the enzyme against the heat-induced changes, indicating that the oxygen binding site plays a role in stabilizing the Cu_A site against structural perturbations.

When the enzyme that was heat treated while fully oxidized is reconstituted into phospholipid vesicles, the resultant proteoliposomes behave similarly to vesicles containing the *p*HMB-modified enzyme [Nilsson et al. (1988) *Biochemistry 27,* 296-301]. These vesicles display low respiratory control despite activity and membrane orientations similar to vesicles containing the native enzyme. When assayed for proton

pumping activity, vesicles containing the heat-treated enzyme exhibit an unusually high permeability to protons. In contrast, if the heat treatment is carried out on the fully reduced enzyme under anaerobic conditions, the Cu_A site remains intact even though subunit III is lost. Reconstitution of this enzyme derivative with phospholipids produces vesicles with respiratory control ratio, membrane orientation, and activity comparable to those obtained with the native oxidase. These vesicles exhibit proton pumping activity with an apparent H^+/e^- stoichiometry of 0.4-0.5 and a faster rate of transmembrane ΔpH dissipation compared to vesicles containing the native enzyme. This behavior is consistent with proton pumping from a subunit III-depleted enzyme. Thus, protecting the Cu_A site from modification also protects the enzyme from forming a proton conducting pathway in the protein, suggesting that the Cu_A site plays a major role in the mechanism of proton pumping in cytochrome *c* oxidase.

Introduction.

It is now generally accepted that cytochrome *c* oxidase is a proton pump. This enzyme catalyzes the final electron transfer in the mitochondrial respiratory chain (from ferrocytochrome *c* to molecular oxygen) and uses part of the redox energy involved to pump protons from the mitochondrial matrix to the cytosol against a protomotive force. With the issue of proton pumping in cytochrome *c* oxidase now resolved, recent attention has been directed toward determining the site of redox-linked proton translocation. It is generally assumed that one of the four redox-active metal centers in the enzyme is the site where electron transfer is coupled to proton pumping. Because the chemistry at the oxygen binding site changes markedly during the stepwise reduction of molecular oxygen, the binuclear cytochrome a_3-Cu_B site seems ill suited for involvement in a process that translocates one proton per electron input into the enzyme. Indeed, recent proton pumping experiments (Wikström & Casey, 1985) seem to implicate the low potential centers, cytochrome *a* and Cu_A, as the most likely site of redox-linked proton pumping activity. These low potential redox centers shuttle electrons from ferrocytochrome *c* to the dioxygen molecule anchored at the reduction site. Since cytochrome *c* and the low potential centers have nearly the same reduction potential, it is unlikely that there is sufficient free energy in the intermolecular electron transfer to pump a proton against a protomotive force. It is therefore more likely that the conversion of redox energy to proton free energy is associated with the electron transfer from the low potential centers to the dioxygen binding site.

Cytochrome *a* has received attention as the probable site of redox-linked proton translocation (for a review, see Wikström et al., 1981) because it exhibits a pH-dependent midpoint potential of -30 mV/pH unit in mitochondria (Aratzabanov et al., 1978) and in the resting form of the enzyme (Blair et al, 1986). In fact, there has been a tendency to rule out Cu_A as the site of energy transduction because its midpoint

potential exhibits only a small pH dependence. Reduction of cytochrome *a*, however, is linked to the uptake of only 0.5 proton in the resting enzyme, and in the carbon monoxide-inhibited enzyme, its pH dependence decreases to ca. -9 mV/pH unit, a proton uptake of 0.15 proton (Blair et al., 1986). Often, it has been assumed that a protonation/deprotonation event should be thermodynamically linked to oxidoreduction of the proton pump site. As a result, the lack of unambiguous evidence for stoichiometric protonation/deprotonation associated with these two metal centers has been considered problematic in the identification of the site of redox-linked proton pumping in cytochrome *c* oxidase.

As recently shown by Gelles et al. (1986), a pH-dependent midpoint potential is not necessary for a viable and efficient proton pump in cytochrome *c* oxidase. Therefore, both cytochrome *a* and Cu_A deserve consideration as possible sites of proton pumping. Few structurally explicit models exist for the mechanism of proton pumping by either cytochrome *a* or Cu_A. Callahan and Babcock (1983) proposed that proton translocation is linked to electron transfer *via* a redox-dependent weakening of the hydrogen-bond between the formyl group of cytochrome *a* and a hydrogen-donating tyrosine. The atypical structure of the Cu_A site, on the other hand, has led Chan and co-workers to hypothesize a role for Cu_A in redox-linked proton translocation (Chan et al., 1979). More recently this group (Blair et al., 1986; Gelles et al., 1986; Chan et al., 1987) has proposed a novel mechanism by which a redox-linked ligand substitution or rearrangement at the Cu_A site is the basis for proton pumping in cytochrome *c* oxidase.

Despite these proposals, there are few experimental studies that address the mechanistic details of proton pumping or the site of redox linkage. Several investigators have approached the study of proton pumping by selectively inhibiting this part of the enzyme's activity. Binding of DCCD1 (Casey et al., 1980) and subunit III depletion

(see Prochaska et al., 1987 for a review) have both proved effective in lowering the proton to electron stoichiometry of the proton pump without seriously affecting the oxidase activity of the enzyme. Unfortunately, neither of these perturbations has lead to unambiguous structural information about the proton pump. In a recent report, Gelles & Chan (1985) described an attempt to obtain a variant of the enzyme in which both cysteines of the Cu_A site were displaced by p-(hydroxymercuri)benzoate (*p*HMB) treatment. Nilsson et al. (1988) subsequently showed that the *p*HMB-modified enzyme did not sustain proton pumping activity and suggested a role for Cu_A in proton translocation.

Sone & Nicholls (1984) reported earlier that heat treatment of cytochrome *c* oxidase at 43°C results in inhibition of the enzyme's proton pumping activity while leaving its dioxygen reduction activity intact. It was also demonstrated by resonance Raman spectroscopy that cytochromes *a* and a_3 suffer only minor perturbations upon heat treatment (Sone et al., 1986). To complement these earlier studies we have now examined this heat-treated enzyme for other structural perturbations that could account for the disruption of proton pumping. We find that incubation of cytochrome *c* oxidase at 43°C results in a dramatic alteration of the optical and EPR signatures of the Cu_A site. In addition, vesicles containing the enzyme derivative obtained from the oxidized heat-treatment (HTO enzyme) do not sustain proton pumping activity. Instead, the HTO enzyme displays an unusual permeability towards protons, similar to the *p*HMB-modified enzyme of Gelles & Chan (1985), suggesting that Cu_A modification creates a passive transmembrane proton-conduction pathway through the protein. In support of this conclusion, when the Cu_A site is protected from modification by reduction, reconstitution of the resultant subunit III-depleted enzyme derivative (HTR enzyme) produces vesicles capable of sustaining proton pumping activity.

Materials and Methods.

Materials. N-2-(Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (Hepes), tris(hydroxyethyl)aminomethane (Tris), asolectin (soybean type IIs), carbonylcyanide-m-chlorophenyl hydrazone (CCCP) and horse heart cytochrome *c* (Type VI) were purchased from Sigma and used without further purification. Dodecyl-β-D-maltoside and valinomycin were obtained from Cal-Biochem. Cholic acid from U.S. Biochemical was purified by three-fold recrystallization. Amberlite XAD-2 was obtained from Mallinkrodt and washed according to Müller et al. (1986) before use. Biogel P-100 was purchased from Bio-Rad, and Sephadex G-125 from Pharmacia. All materials used in the electrophoresis experiments were ultrapure electrophoresis grade unless otherwise specified. Argon for anaerobic work was made oxygen free by passing it through a manganese oxide catalyst column. Carbon monoxide from Matheson ("Matheson Purity") was used without further purification.

Cytochrome *c* oxidase was isolated by the method of Harzell and Beinert (1974). Enzyme concentrations were determined by using a reduced minus oxidized $\Delta\varepsilon$ of 24 $mM^{-1}cm^{-1}$ at 605 nm. Each enzyme preparation used was stored at -80°C until used.

Enzymatic activity was assayed by spectrophotometrically monitoring ferrocytochrome *c* oxidation (Smith, 1955). Comparison of the activities of the heat-treated and native enzymes were in general agreement with those reported by Sone & Nicholls (1984).

Reduced cytochrome *c* oxidase was prepared as follows: The sample was placed in a spectrometer cuvette fitted with a vacuum stopcock and de-oxygenated by exchanging the atmosphere with purified argon and then agitating the liquid to equilibrate it with the atmosphere. Five such cycles were usually used. Sodium dithionite (G. Fredrich Smith Company) was then added to the solution from a side

arm. The CO-mixed valence compound of cytochrome oxidase was prepared by first removing the oxygen from an enzyme sample using argon, as above, and then replacing the argon atmosphere with carbon monoxide. The sample was then left in the dark at room temperature for several hours. Formation of these reduced forms of the enzyme was verified from their visible spectra.

Heat treatment of cytochrome c oxidase. Fully oxidized cytochrome *c* oxidase samples were diluted to concentrations of 25-50 mM in 50 mM Tris, 50 mM NaCl, 0.5% dodecyl-β-D maltoside (pH 7.7 at 25°C) and incubated at 43°C for 60 min in a constant temperature water bath. Heat-treated cytochrome *c* oxidase samples were equilibrated to ice temperature before further treatment or characterization. Fully reduced and partially reduced cytochrome *c* oxidase samples were prepared in the same buffer using anaerobic optical cuvettes. Heat treatment of these samples was accomplished by immersion of the cuvette into constant temperature water bath.

Optical Spectroscopy. Optical spectra in the visible and near IR region (350-900 nm) were collected at 2°C on a Beckman Acta (Model C-III) dual beam spectrophotometer, the digital output of which was collected by a Spex Scamp computer. Baseline spectra were digitally subtracted.

EPR spectroscopy. EPR spectra were recorded on a Varian E-line Century Series X-Band Spectrophotometer equipped with a 12 bit analog to digital converter used for the computer digitization of the signal. Sample temperature was maintained at 77K by immersion of the sample in liquid nitrogen or at 7K by a liquid helium cryostat (Oxford Instruments). Oxygen was removed from EPR samples by a single equilibration with argon gas immediately prior to freezing the sample.

Cyanide Binding Assay. Cyanide binding to cytochrome a_3 was monitored at 414 nm using a Beckman Acta Model C-III dual beam spectrophotometer. All data were collected at 2°C with samples containing ca. 10 mM cytochrome *c* oxidase and an

approximately 50-fold excess of KCN (added as a concentrated stock solution). See Figure V.5 for details.

SDS-PAGE and gel filtration. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed using a gel system and conditions similar to that described by Rigell et al. (1987), except that samples were typically denatured for 4 hours at ice temperature before loading. The gels shown were stained with Coomassie blue. The enzyme samples were allowed to stand overnight before centrifugation in order to allow the subunit III to aggregate. Scanning densitometry data were collected using a Beckman Acta Model C-III dual beam spectrophotometer and gel scanning attachment.

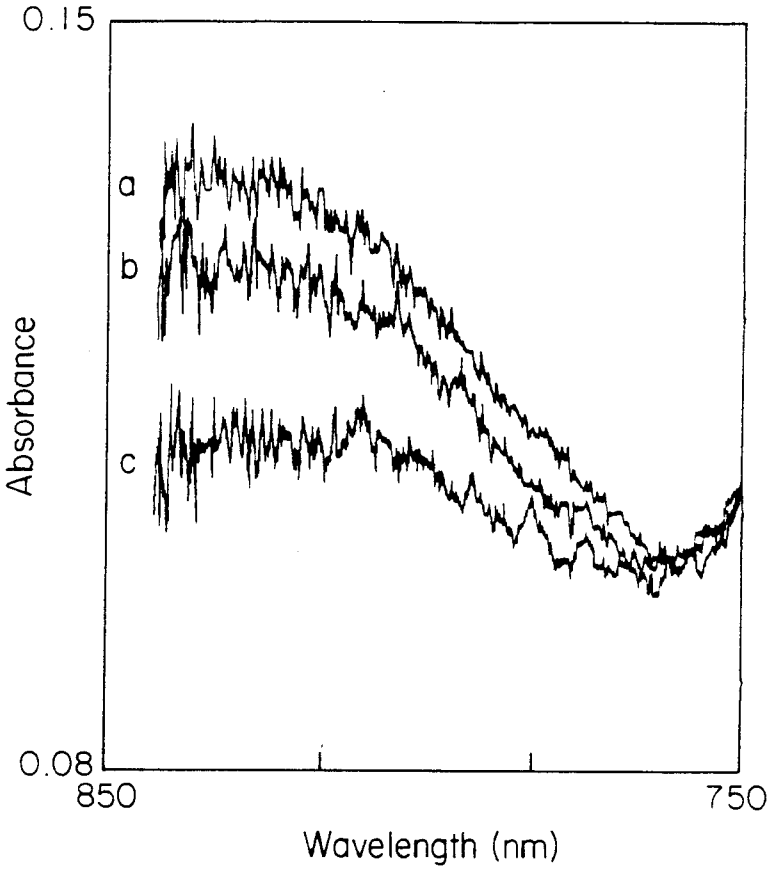
Gel filtration (Biogel P-100 or Sephadex G-125) was performed at room temperature (23°C) using a ca. 10 x 2 cm column. The enzyme was eluted with 50 mM NaCl, 50 mM Tris, 0.5% dodecyl-β-D-maltoside, pH 7.7.

Proton pumping measurements. Heat treated enzyme derivatives were reconstituted into phospholipid vesicles by the cholate dialysis method and characterized in terms of their respiratory control ratio (RCR), membrane orientation, as well as H+/e- stoichiometry as outlined in Nilsson et al. (1988) and Chapter III for the *p*HMB-modified enzyme. Proton pumping was assayed by monitoring the extravesicular pH changes following the addition of ferrocytochrome *c*. Assay conditions and buffers were the same as described in Nilsson et al. (1987).

Results.

Effects of heat treatment on the spectroscopic properties of Cu_A. Heat treatment of cytochrome *c* oxidase causes significant structural changes to Cu_A that are evident from both near IR and EPR spectra. In the near infrared, the native oxidized Cu_A center gives rise to a broad, weak ($\varepsilon = 2\ mM^{-1}cm^{-1}$) absorption at 830 nm (Figure V.1). This band has been attributed to a charge transfer transition between Cu^{2+} and one or more

Figure V.1: Near IR absorption spectra of: a) native resting cytochrome c oxidase; b) cytochrome *c* oxidase heat treated while reduced; and c) cytochrome *c* oxidase heat treated while oxidized.

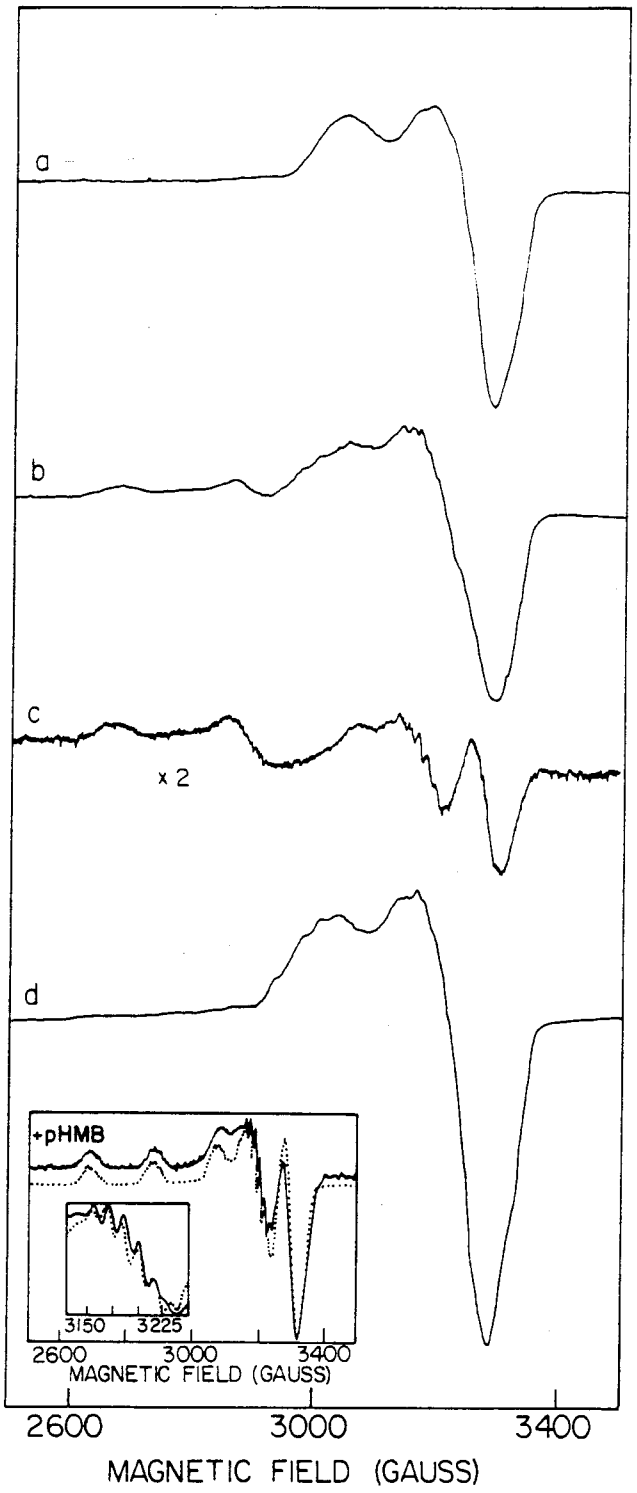

0.15
0.08
Absorbance
a
b
c
850
750
Wavelength (nm)

cysteine ligands (Beinert et al. 1980; Blair et al. 1983). Upon heat treatment the intensity of the 830 nm absorption diminishes by approximately 75%, indicating that a large fraction of the Cu_A centers in the sample has undergone a structural change.

The nature of this perturbation can be seen more clearly in the EPR spectrum. Figure V.2a shows the EPR spectrum of native oxidized cytochrome *c* oxidase at 77K. (Under these conditions, the spectrum arises exclusively from the Cu_A site.) This Cu_A EPR spectrum is unique among biological copper centers because it is unusually isotropic and does not exhibit resolved hyperfine splittings (Aasa & Vänngård, 1975; Hoffman et al. 1980; Stevens et al. 1982). As seen in Figure V.2b, heat treatment alters the Cu_A spectrum substantially. The most notable difference is the appearance of resolved copper hyperfine lines not present in the native Cu_A signal. Because Cu^{2+} EPR lineshapes reflect both the nature of the ligands and the ligation geometry of a copper center (Peisach and Blumberg, 1974), the observed spectral changes indicate that major structural alterations occur at the Cu_A site upon heat treatment.

The new EPR spectrum appears to contain contributions from at least two new copper species in addition to a signal from residual Cu_A. We have been able to resolve some of these spectral contributions by exploiting differences in the redox potentials of the species from which they arise. Reduction of heat treated cytochrome *c* oxidase under mild conditions (ca. 10-fold excess of ascorbate and a catalytic amount of cytochrome *c*), results in the disappearance of approximately 75% of the copper EPR signal. What remains is an EPR signal (Figure V.2c) closely resembling the type 2 species produced by the chemical modification of the Cu_A site by *p*HMB (Gelles & Chan, 1985). (For a review of copper protein classifications, see Gray and Solomon, (1986)). Although this type 2 copper species is not reduced by ferrocytochrome *c*, the enzyme's physiological reductant, it can be reduced by the addition of solid sodium dithionite (data not shown).

Figure V.2: EPR signal from the Cu_A site in cytochrome c oxidase. a) Native resting enzyme; b) Heat treated resting enzyme; c) Heat treated enzyme reduced with ascorbate and a catalytic amount of cytochrome c; d) Spectrum b minus spectrum c. Microwave frequency, 9.159 GHz; microwave power, 10 mW; modulation frequency, 100 KHz; modulation amplitude, 10.0 Gauss; sample temperature, 77K. Inset: (Solid line) *p*HMB-modified cytochrome c oxidase. (Dashed line) Simulated powder EPR spectrum calculated for *p*HMB-modified CuA. Data taken from Gelles & Chan (1985).


a
b
c
x 2
d
+pHMB
3150
3225
2600
3000
3400
MAGNETIC FIELD (GAUSS)
2600
3000
3400
MAGNETIC FIELD (GAUSS)

The EPR spectrum of the ferrocytochrome *c*-reducible component can be obtained by subtracting spectrum 2c from 2b. This difference spectrum, shown in Figure V.2d, appears to consist of two signals: native Cu_A and a new copper signal with a Cu^{2+} hyperfine coupling constant (0.004 cm^{-1}) characteristic of the type 1 copper centers of blue copper proteins. The fraction of EPR visible copper represented by this "blue" copper species can be estimated as follows: The type 2 species accounts for 25% of the total copper signal. Quantitation of the 830 band (Figure V.1) shows that 25% residual native Cu_A remains in the sample. Thus another 25% of the EPR signal intensity can be assigned to native Cu_A. The remaining 50% appears to be made up by the type 1 ("blue") copper species. Table I summarizes the measured g-values ($g_{||}$) and hyperfine coupling constants ($A_{||}$) for native Cu_A as well as the type 1 and type 2 copper species produced upon heat treatment of the enzyme.

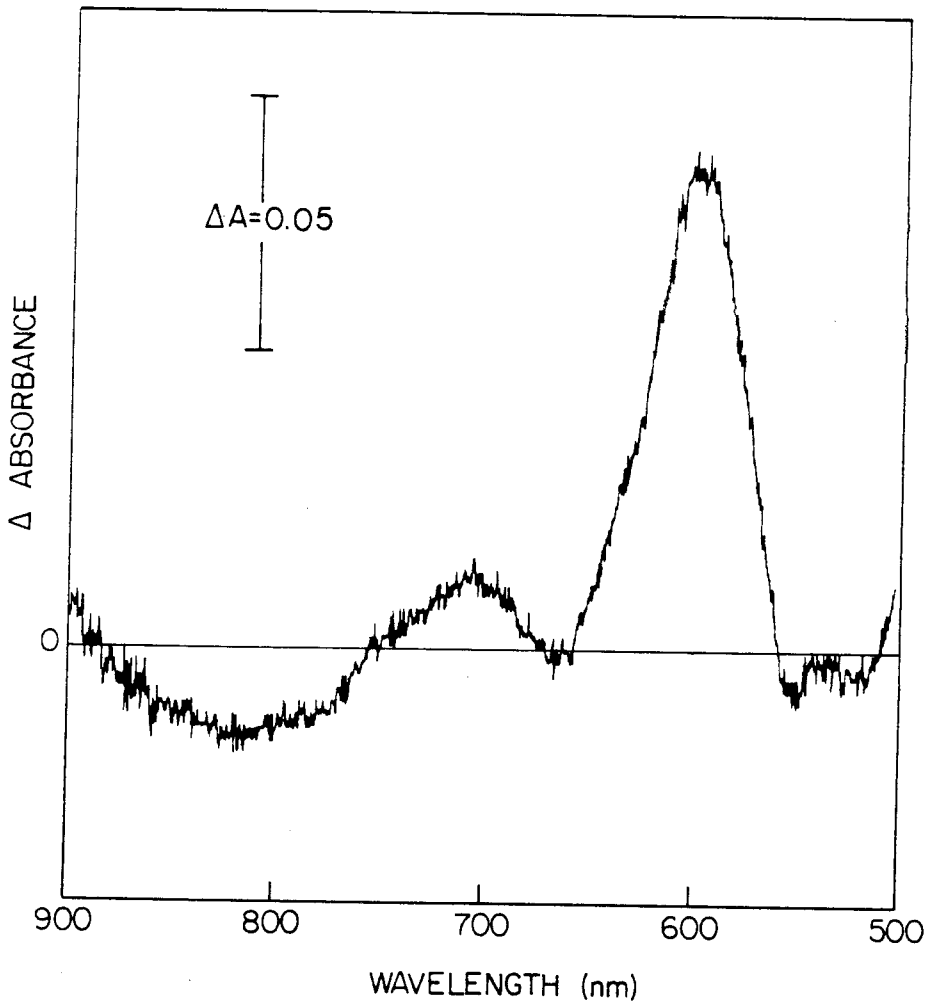
Additional evidence for the formation of a "blue" copper species during heat treatment comes from the visible absorption difference spectrum between the heat treated and native cytochrome *c* oxidase (Figure V.3), which reveals a broad band with ε ca. 1.5 $mM^{-1}cm^{-1}$ in the 610-615 nm region and a smaller feature at approximately 740 nm. These optical features are remarkably similar to the cysteine to copper charge transfer transitions of blue copper proteins that give them their characteristic blue color (see Gray and Solomon, 1986 for a review.) It should be noted, however, that this absorption difference lies directly beneath the heme A alpha band absorption. While the spectra of the hemes do not appear significantly altered upon heat treatment (see below), even small changes in a band of such large extinction (24 $mM^{-1}cm^{-1}$) may produce significant features in the difference spectrum. *p*HMB-modified cytochrome *c* oxidase (Gelles & Chan, 1985) also shows small changes in this spectral region compared to the native enzyme (P. Smith, personal communication).

Table V.1: EPR Parameters for Cu_A in Various Cytochrome *c* Oxidase Species

Copper species	$g_{\|\|}$	$A_{\|\|}$ (cm^{-1})
Native Cu_A	2.18	----
Type 2 (heat treatment)	2.19	0.020
Type 2 (*p*HMB-modification)	2.21	0.019
Type 1 (heat treatment)	2.16	0.004
Type 2 coppers[a]	2.18 - 2.25	0.017 - 0.020
Type 1 coppers[a]	2.19 - 2.87	0.0035 - 0.009

[a]Data for typical copper EPR parameters taken from Boas et al. (1978).

Figure V.3: Visible absorption difference spectrum of heat treated resting minus native cytochrome c oxidase in the 500-900 nm region.

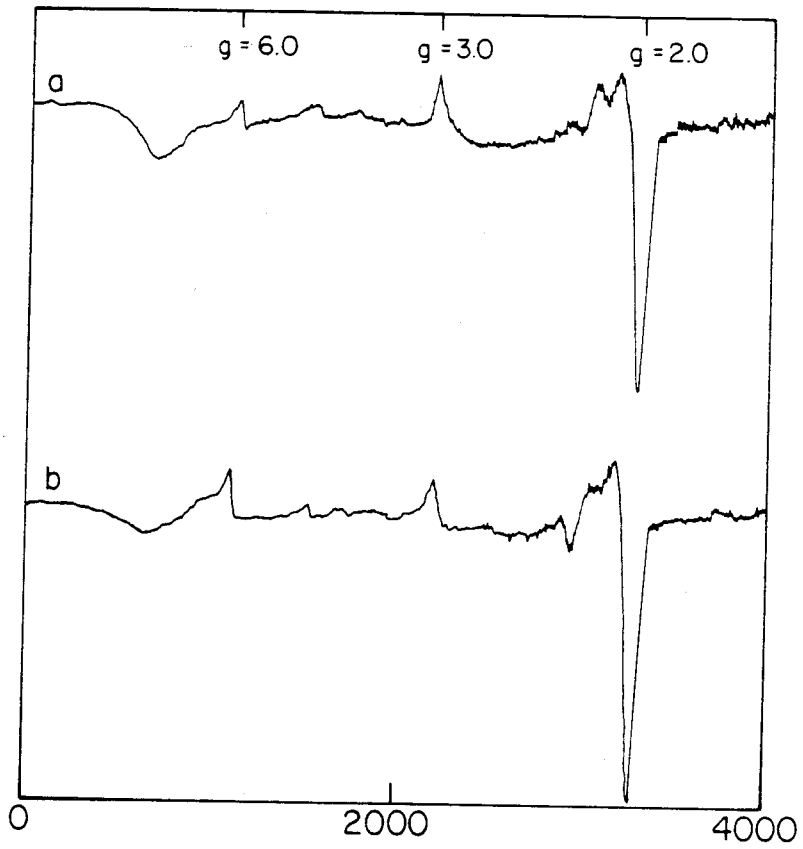

ΔA=0.05
Δ ABSORBANCE
0
900
800
700
600
500
WAVELENGTH (nm)

Effects of heat treatment on cytochromes a, a_3 and Cu_B. Resonance Raman studies of heat treated cytochrome *c* oxidase (Sone et al. 1986) have shown the hemes to be largely unperturbed. To verify these conclusions we have used low temperature EPR spectroscopy to monitor the effects of heat treatment on cytochromes *a* and a_3 and Cu_B. Spectra of native and heat treated cytochrome *c* oxidase are compared in Figure V.4. At 7K, only minor changes are observed in the EPR signatures for cytochromes *a* and a_3. The EPR signal at g=3.0, which has been assigned to the low spin ferric heme of cytochrome *a* shows no significant changes in spectral shape, intensity, or position. The broad signal at g=12, assigned to the antiferromagnetically coupled cytochrome a_3-Cu_B site, decreases in intensity by 10-30% compared to native enzyme. This signal, however, varies dramatically in intensity and shape from preparation to preparation. The sharp feature at g=6 arises from either partial reduction or uncoupling of the binuclear cytochrome a_3-Cu_B site, or from denaturation of cytochrome *a* (Brudvig et al. 1980). Quantitation of the g=6 signal shows that it arises from less than 2% of the heat treated enzyme molecules. Taken together, these observations indicate that following heat treatment, cytochrome *a* remains structurally intact and that the coupling between cytochrome a_3 and Cu_B remains unbroken.

Oxidase activity of the heat treated enzyme. Although the "blue" copper species can be reduced by ferrocytochrome *c*, the type 2 copper cannot be, and therefore, cannot be expected to participate in electron transfer during turnover. In fact, *p*HMB modified cytochrome *c* oxidase that has been shown to contain almost exclusively a type 2 copper A site, has only 20 to 30% of the activity of the native enzyme (Gelles & Chan, 1985). In contrast, 70 to 80% of native activity remains after heat treatment, suggesting that the type 1 copper is still active in electron transfer.

Cyanide binding to heat treated cytochrome c oxidase. Recently, it has been shown that reduction of Cu_A is involved in the closed-to-open transition of the oxygen

Figure V.4: EPR spectra of: a) native resting and b) heat treated cytochrome *c* oxidase. Microwave frequency, 9.16 GHz; microwave power, 10 mW; modulation frequency, 100 KHz; modulation amplitude, 10.0 Gauss; sample temperature, 10K. Spectra shown are normalized to enzyme concentration.


g = 6.0
g = 3.0
g = 2.0
a
b
0
2000
4000
MAGNETIC FIELD (GAUSS)

binding site, leading to rapid binding of cyanide (Scholes and Malmström, 1986; Copeland et al. 1987). These results suggest that the heat treatment, which causes a large perturbation at the Cu_A site, might also have consequences at the oxygen binding site. The kinetics of cyanide binding in the native and heat treated enzymes are compared in Figure V.5. Heat treatment of the enzyme accelerates the binding of cyanide more than ten-fold. Because the binding is not monophasic, the relative rates were estimated by comparing initial slopes of the curves.

Subunit III depletion in the heat treated enzyme. The heat treatment described also depletes subunit III from the enzyme. Figure V.6 shows densitometer scans from denaturing SDS-polyacrylamide electrophoretic gels of cytochrome *c* oxidase. Subunit III is clearly absent in the trace for the heat treated enzyme.

Heat treatment of fully reduced cytochrome c oxidase. The preceding results were all obtained by heat treating cytochrome *c* oxidase in its oxidized, resting state. The same heat treatment, carried out on the fully reduced enzyme, produces almost none of these effects. Subunit III is still lost (Figure V.6c), but otherwise, the reduced form of the enzyme is protected against all of the effects of heat treatment documented above. As shown in Figure V.1b, the 830 nm absorption, indicative of native Cu_A, retains 80% of its intensity when the enzyme is heat treated in its reduced form and then reoxidized. (Reoxidation is necessary because the 830 nm feature arises from oxidized Cu_A only.) The actual fraction of native Cu_A remaining was probably larger than 80% because the sample was centrifuged to remove traces of denatured enzyme before this spectrum was taken. Subsequent experiments with other batches of enzyme show as much as 97% protection of the Cu_A site from modification. Copper EPR spectra (data not shown) support the conclusion that Cu_A is largely unperturbed.

Heat treatment of reduced cytochrome *c* oxidase does not produce the accelerated cyanide binding observed with heat treatment of the oxidized enzyme. When

Figure V.5: Cyanide binding curves for: a) native resting enzyme; b) cytochrome *c* oxidase heat treated while reduced; and c) cytochrome *c* oxidase heat treated while oxidized. Cyanide binding was observed by monitoring the absorbance at 414 nm (A414). The quantity $\ln\Delta A_{414}$ is ln[A(final)-A(t)/A(initial)-A(final)].

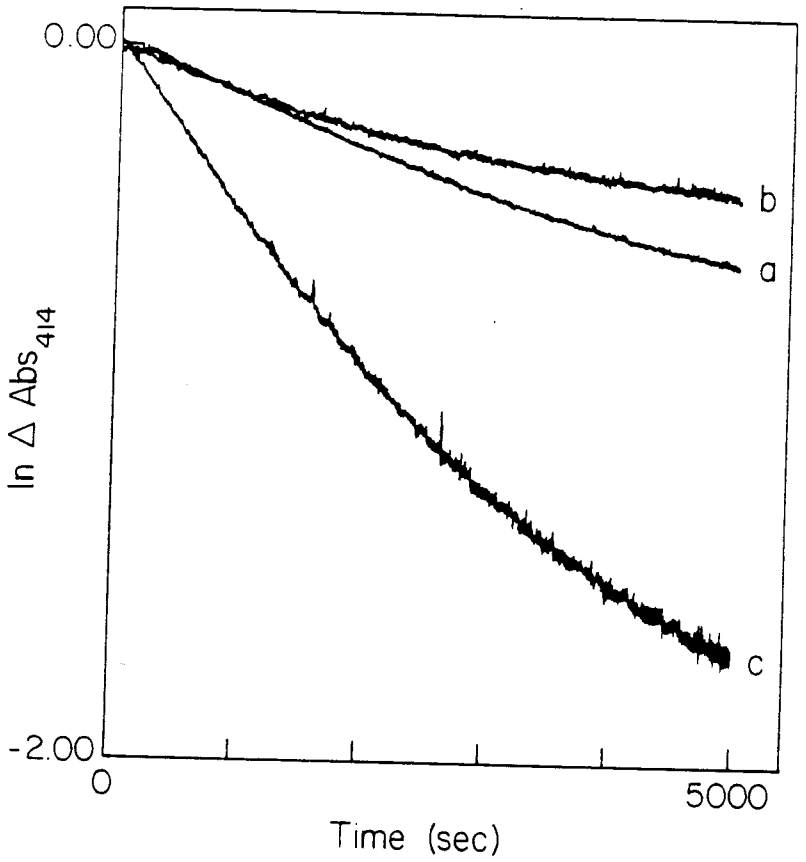
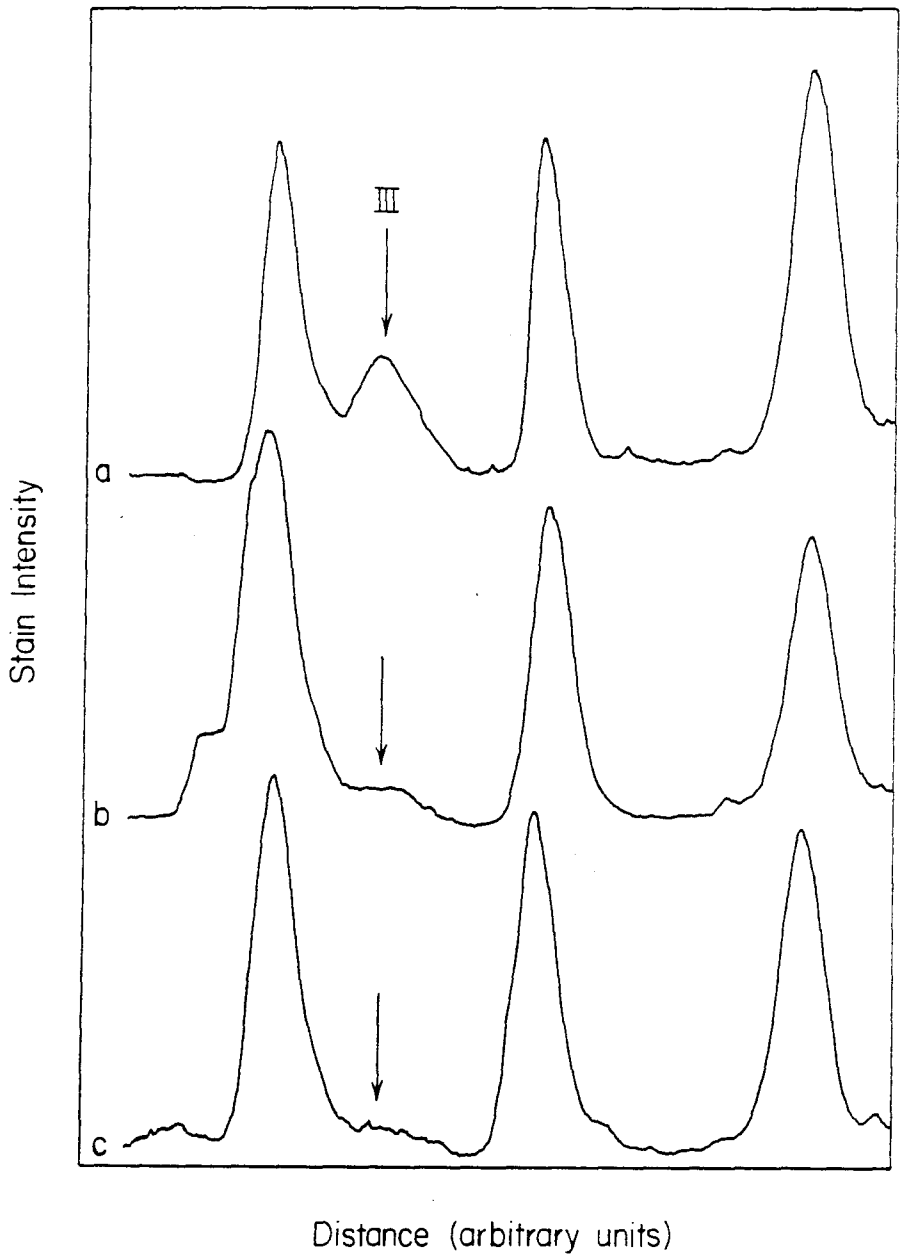

0.00
-2.00
0
5000
Time (sec)
ln Δ Abs414
b
a
c

Figure V.6: Densitometric traces of SDS-PAGE gels showing a) native enzyme, b) enzyme heat treated while oxidized; and c) enzyme heat treated while reduced. Subunit III is indicated by the arrow.


III
a
b
c
Stain Intensity
Distance (arbitrary units)

a sample of the reduced enzyme was heat treated and then reoxidized overnight at ice temperature to allow it to revert completely to the resting state, the kinetics of cyanide binding to this sample, observed the next day, were even slower than those of the resting enzyme (Fig. 5b). This discrepancy is probably explained by the fact that freshly prepared resting enzyme contains small subpopulations of more active forms of the enzyme, which could bind cyanide quickly (Brudvig et al., 1980). Presumably, the overnight incubation allowed the enzyme to assume a more homogeneous resting state.

Heat treatment of mixed-valence derivatives. Cytochrome *c* oxidase contains four redox-active metal centers each of which accepts an electron when the enzyme becomes reduced. The observed protection effects could arise from the reduction of any one of the metal centers or a combination thereof. In order to localize the protection phenomenon, we performed heat treatments on several mixed-valence and ligand-bound forms of the enzyme. Heat treatment of the carbon monoxide mixed valence compound (in which cytochrome a_3 and Cu_B are reduced and cytochrome *a* and Cu_A are oxidized) results in no greater loss of intensity at 830 nm than heat treatment of the reduced enzyme. This is strong evidence that it is the reduction of the oxygen binding site that is responsible for the protection observed in the fully reduced enzyme. Heat treatment of the cyanide-bound, fully oxidized enzyme results in some loss of intensity at 830 nm, but still much less than that in the case of the native enzyme. Thus, ligand binding to the oxygen binding site can confer some protection even in the absence of reduction. Finally, the cyanide-mixed valence compound of the enzyme (in which cytochrome *a* and Cu_A are reduced) is no more protected against heat treatment than the fully oxidized cyanide compound. This suggests that, surprisingly, reduction of Cu_A does not protect the Cu_A site from heat-induced modification.

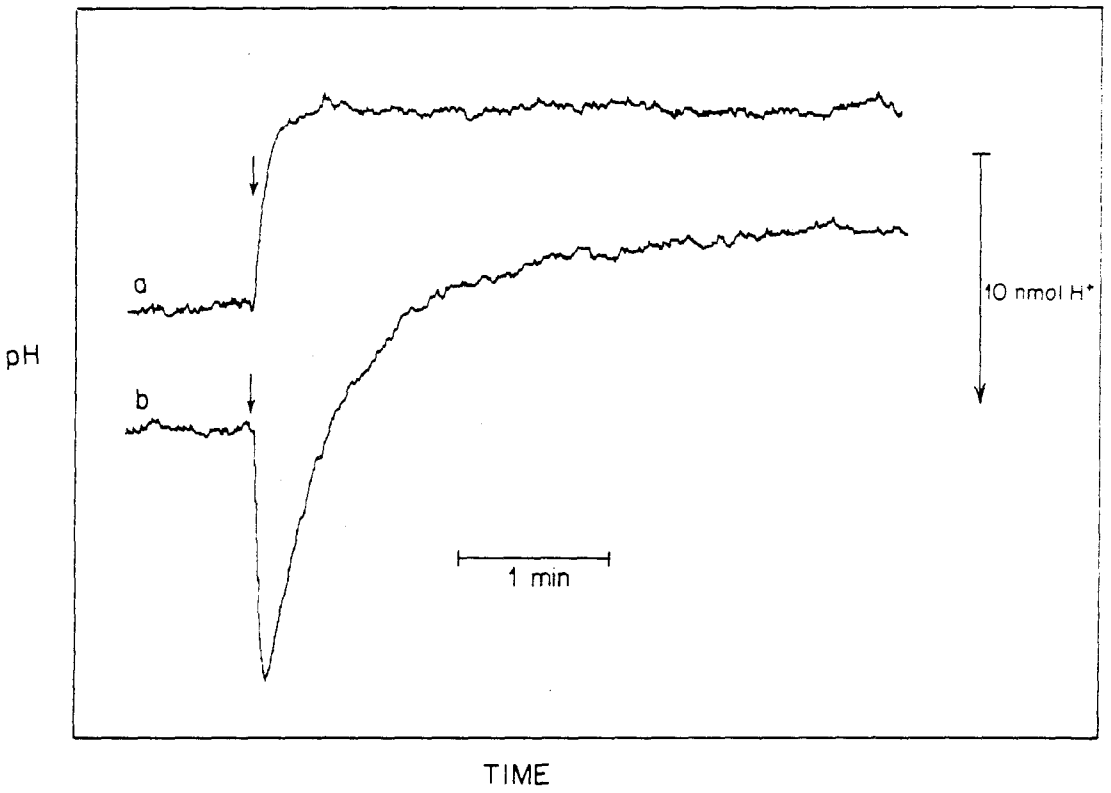
Gel filtration of the reduced heat treated enzyme. Heat treatment of both the oxidized and the fully reduced enzyme leads to the dissociation of subunit III. In the

experiments reported here, the dissociated subunit III polypeptide was removed from solution by incubating the sample overnight to allow the subunit to aggregate and then centrifuging the sample to pellet the aggregate. In our early experiments, we attempted to remove subunit III from the heat-treated enzyme solutions by gel filtration. Samples that had been heat treated in the reduced form and then reoxidized displayed an intact 830 nm absorption. However, when these samples were passed down a Biogel P-100 or Sephadex G-25 column, the 830 nm band largely disappeared. This result suggests that some aspect of the reduced heat treatment, perhaps the removal of subunit III, renders the enzyme more susceptible to structural perturbations.

Proton pumping in Heat-Treated-Oxidized (HTO) cytochrome c oxidase. Figure V.7 compares the proton pumping behavior of heat-treated-oxidized (HTO) and native enzymes that were reconstituted into phospholipid vesicles. We observe that rapid alkalinization accompanies the oxidation of ferrocytochrome *c* by the heat treated enzyme (Figure V.7a). In contrast, the addition of ferrocytochrome *c* to well coupled vesicles containing the native enzyme (Figure V.7b) results in a transient acidification followed by a slow decay of the transmembrane ΔpH to a new equilibrium value. The rapid rise in the extravesicular pH observed for vesicles containing the heat-treated enzyme is quite similar to the result obtained with cytochrome *c* oxidase modified by *p*HMB (Nilsson et al., 1988).

In contrast to the results of Sone & Nicholls (1984), our reconstitution of heat-treated cytochrome *c* oxidase produces vesicles with a low respiratory control ratio (RCR). Typically, RCR values of ca. 1.2-1.5 are obtained for the HTO enzyme while values of 4.0-6.0 are obtained for the native enzyme. As with the native enzyme, the orientation assay shows that between 75-85% of the HTO enzyme is reconstituted with the cytochrome *c* binding site facing the external medium.

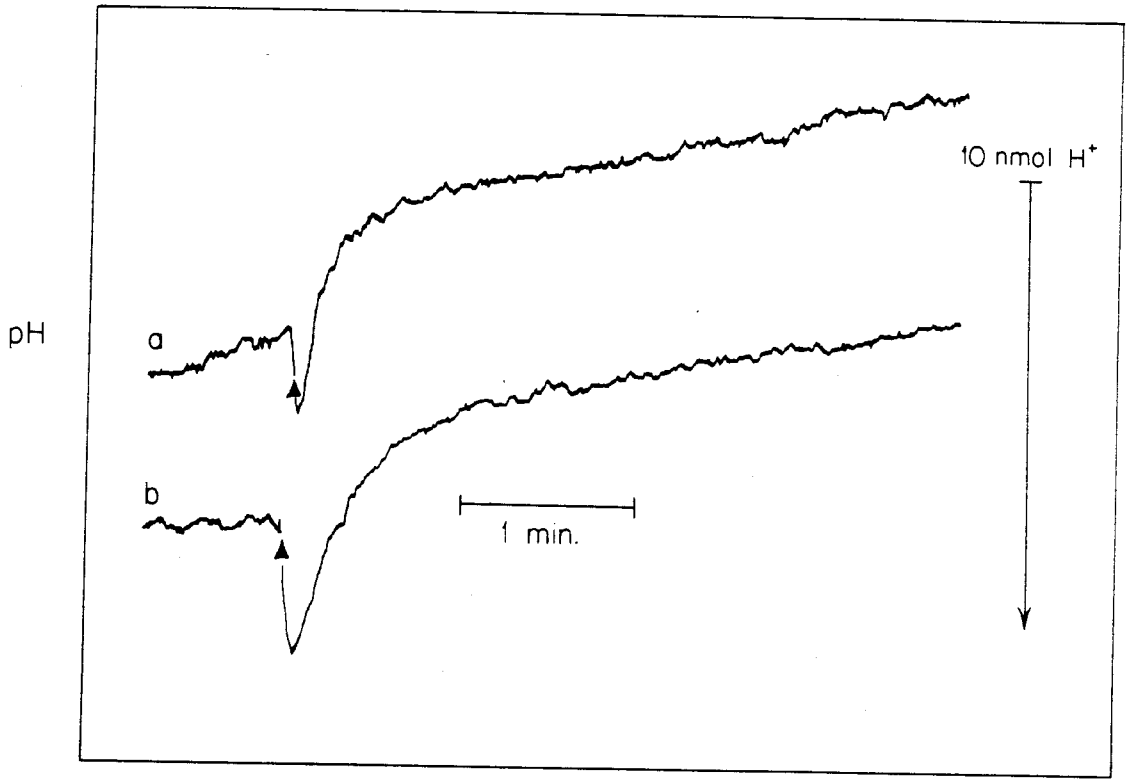
Figure V.7: Comparison of a) HTO and b) native cytochrome c oxidases in the proton pumping assay. The reaction mixtures in each case contained 0.25 mL vesicles (0.63 nmol enzyme), 1.1 mL medium (44mM KCl, 56 mM choline chloride) and valinomycin to a final concentration of 11 μM. The pH of the mixture was adjusted to that of the cytochrome *c* stock solution. At the times indicated by the arrow, 15.9 nmol of ferrocytochrome *c* was added.


pH
a
b
10 nmol H+
1 min
TIME

As discussed by Nilsson et al. (1988), the rapid alkalinization accompanying ferrocytochrome *c* oxidation by reconstituted *p*HMB-modified enzyme as well as the low RCR values may be accounted for either by scalar proton consumption by unreconstituted enzyme in the external medium or by a transmembrane proton conduction pathway in the modified enzyme. Because the HTO enzyme displays normal vesicle orientations, it is unlikely that unreconstituted enzyme is the cause of the observed low RCR values and the rapid alkalinization behavior. The most likely explanation for our observation is increased proton permeability in these vesicles resulting from creation of a transmembrane proton conductance through the enzyme during heat treatment.

Proton pumping in Heat-Treated-Reduced (HTR) cytochrome c oxidase. When reduced cytochrome *c* oxidase is heat-treated under anaerobic conditions, subunit III becomes dissociated, but the Cu_A site remains unperturbed. If the formation of a transmembrane proton conduction pathway in the heat-treated enzyme is linked to modification of the Cu_A site, vesicles containing enzyme that is heat-treated while in the reduced form should remain coupled and display proton pumping activity. Indeed, vesicles containing the HTR enzyme display RCR values of 2.5-3.0. For the enzyme preparation used in this experiment, native enzyme gives an RCR value of 2.4-3.0. As expected, the HTR enzyme is competent in the proton pumping assay (Figure V.8). However, in contrast to the native enzyme, H+/e- stoichiometries obtained for the HTR enzyme are 0.4-0.5. Typical stoichiometries obtained for this batch of native enzyme are 0.6-0.7. In addition, the acidification decay rate is slightly faster with the HTR enzyme than for the native enzyme. These results agree with proton pumping measurements on the reconstituted subunit III-depleted enzyme (see Prochaska, et al., 1987 for a review).

Figure V.8: Comparison of a) HTR and b) native cytochrome *c* oxidases in the proton pumping assay. Reaction conditions were the same as for Figure V.7 except that a different batch of enzyme was used.


pH
a
b
10 nmol H+
1 min.
TIME

Discussion.

Sone & Nicholls (1984) reported that incubation of cytochrome *c* oxidase at 41-43°C results in the inhibition of proton pumping activity with only minor perturbations of either the heme A centers or the oxidase activity. We have extended the earlier study to examine the metal centers of heat treated cytochrome *c* oxidase by EPR spectroscopy. We observe no significant changes in the spectra of the hemes but the EPR spectrum of Cu_A is appreciably altered, indicating pronounced structural changes at that site. Concomitant with the change in the Cu_A EPR spectrum is a 75% decrease in the intensity of the Cu_A 830 nm absorption band. Heat treatment of our enzyme preparation also results in a 20-30% loss of oxidase activity, a ten-fold acceleration of cyanide binding to cytochrome a_3, and the removal of subunit III. We further find that reduction of, or ligand binding to, the oxygen binding site of the enzyme prevents the effects of heat treatment except for the loss of subunit III.

Heat treatment of cytochrome *c* oxidase results in a dramatically altered Cu_A EPR spectrum. The new spectrum appears to consist of three components: a type 2 copper signal, a type 1 copper signal, and a contribution from residual native Cu_A. The type 2 copper signal corresponds to about 25% of the EPR-visible copper. Its g-values and hyperfine coupling constants are almost identical to those of the *p*HMB-modified Cu_A reported by Gelles & Chan (1985) (see Table V.1). Like the *p*HMB-modified copper, this species cannot be reduced by ferrocytochrome *c* but can be reduced by dithionite. It is therefore likely that these two type 2 modified Cu_A derivatives are similar in structure. We note that a similar type 2 copper signal is also observed upon treatment of cytochrome *c* oxidase with Ag^+ (Chan et al., 1978; Chan et al., 1979). The existence of such varied routes to this copper species suggests that the structure of the modified Cu_A center is not determined by the modification agent. Apparently, the tendency towards modification is an inherent property of the protein and ligand

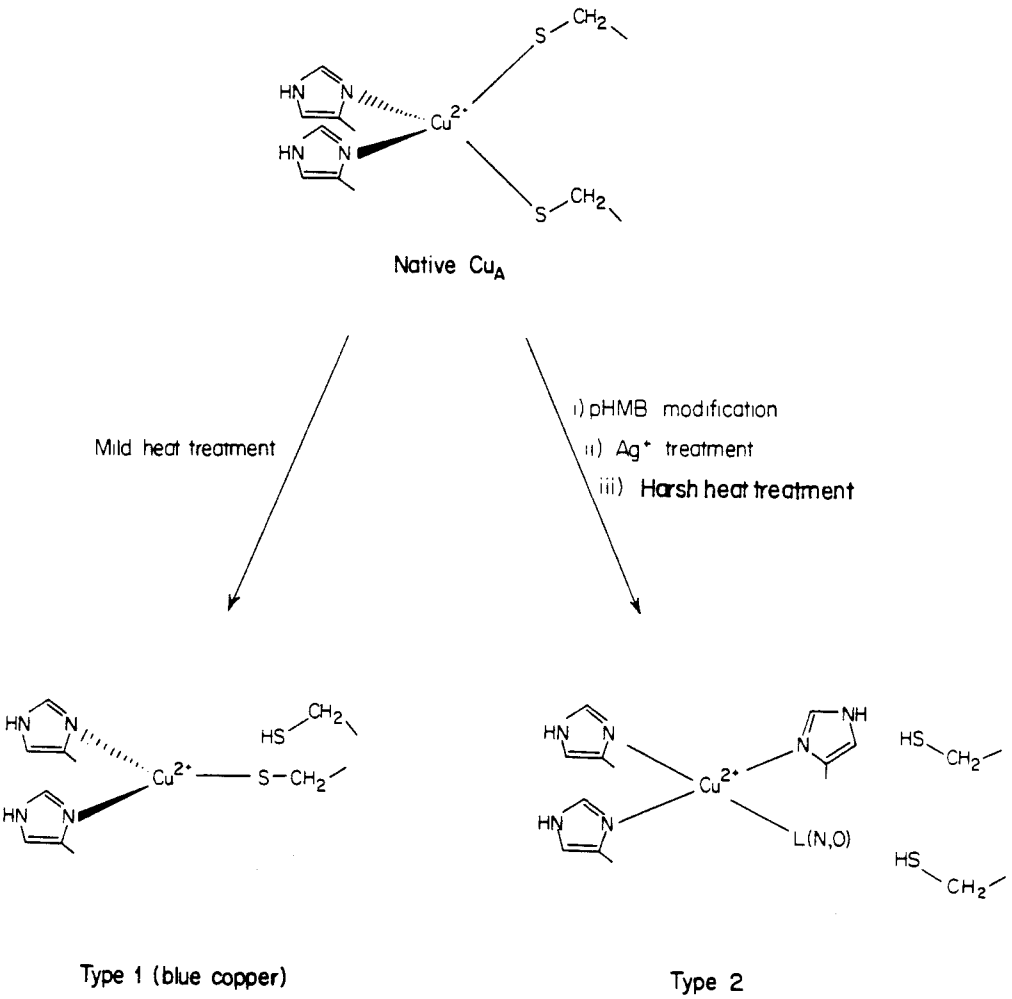
structure of the Cu_A site, and the same modification can be induced in several different ways.

The remainder of the copper EPR spectrum can be assigned to native Cu_A and a type 1 "blue" copper species. We estimate that 25% of Cu_A remains in its native form because the 830 nm absorption retains about 25% of its intensity after heat treatment. The remaining EPR contribution, which accounts for about 50% of the enzyme, displays a hyperfine coupling constant of 0.004 cm-1 (Figure V.2d, Table V.1) and bears a striking resemblance to the EPR signatures of the blue copper proteins.

Although our data do not allow for the unambiguous determination of structures, one possible model for the structures of these Cu_A derivatives and the routes to their formation is shown in Figure V.9. Native Cu_A has been shown to have at least one histidine ligand and two sulfur ligands, of which one is from a cysteine side chain (Stevens et al., 1982; Li et al., 1987; Martin et al., 1988). In our model, the displacement of one cysteine ligand would lead to the formation of a type 1 "blue" copper site and displacement of both cysteines would lead to the formation of a type 2 copper site. Gelles & Chan (1985) have shown that the type 2 copper EPR signal is likely to arise from a site with at least three nitrogenous ligands, and a recent EXAFS study of the *p*HMB-modified enzyme has implicated a fourth nitrogen or oxygen ligand (Li et al., 1987). We propose that the harsh conditions under which the type 2 copper signal is produced results in the displacement of both cysteine ligands. With milder conditions, such as incubation at 43°C, the displacement of one cysteine could predominate, leading to a type 1 copper species with one cysteine and two nitrogenous ligands.

Sone & Nicholls (1984) reported that the oxidase activity of their heat treated enzyme is the same as that of the native enzyme. We observe a 20 to 30% decrease in activity. By comparison, *p*HMB-modification of the enzyme results in an 80% loss of

Figure V.9: Proposed scheme for the production of observed forms of modified Cu_A. Displacement of one thiolate ligand during heat treatment as shown in the left pathway results in the formation of a species with spectroscopic properties similar to a type 1 (blue) copper center. Displacement of two thiolate ligands, as in *p*HMB-modification, Ag^+ treatment, and partially during heat treatment, results in the formation of a type 2 copper center as shown in the right pathway. In the case of chemical modification by *p*HMB or Ag^+, the free cysteine sulfhydryls shown for the type 2 copper may exist as thiolate ligands to Hg^+ or Ag^+, respectively.


Native Cu_A
Mild heat treatment
i) pHMB modification
ii) Ag^+ treatment
iii) Harsh heat treatment
Type 1 (blue copper)
Type 2
L(N,O)

activity (Gelles & Chan, 1985). The activity of our heat treated enzyme is therefore consistent with the presence of a 25% subpopulation bearing a type 2 copper having the same low level of turnover as the *p*HMB modified enzyme. This raises the possibility that Sone & Nicholls heat treatment, which was performed on a different enzyme preparation, produces the type 1 "blue" copper species without significant formation of a type 2 copper species.

Gelles & Chan (1985) explained the diminished activity of the *p*HMB-modified enzyme in terms of a decreased reduction potential of the type 2 copper center. They reasoned that because the type 2 copper cannot be reduced by ferrocytochrome *c*, a key step in the enzyme's electron transfer pathway is disrupted. Enzyme bearing the type 1 "blue" copper center appears to have nearly full activity, presumably because the type 1 copper can accept electrons from ferrocytochrome *c*.

Our data show that reduction of, or ligand binding to, the oxygen binding site stabilizes the Cu_A site with respect to heat treatment. This is clear evidence for a conformational link between the oxygen binding site and Cu_A. We have also demonstrated a correlation between Cu_A modification and the acceleration of cyanide binding. Copeland et al., (1987) recently demonstrated that reduction of Cu_A is associated with the triggering of the "closed-to-open" transition at the oxygen binding site. Although our heat treated enzyme does not have the extremely rapid cyanide binding properties of the "open" conformation, the parallel between these findings is of some interest because both involve communication between Cu_A and the oxygen binding site.

Recently, Hill & Robinson (1986) showed that cytochrome *c* oxidase that had been depleted of subunit III by extensive incubation in lauryl maltoside, displayed fast cyanide binding kinetics. In the present study, we find that enzyme that is heat treated while oxidized displays loss of subunit III as well as fast cyanide binding in addition to

a modified Cu_A site. In contrast, enzyme that is heat-treated while reduced exhibits normal Cu_A spectroscopic signatures and cyanide binding kinetics similar to that of the resting enzyme following reoxidation, even though subunit III is lost. Thus, using this method, subunit III removal alone causes neither the observed structural perturbations at the Cu_A site nor the elevated cyanide binding rates. However, the ease with which Cu_A is modified when heat treated enzyme is passed down a gel filtration column suggests that the enzyme is more susceptible to modification at the Cu_A site when subunit III is lost.

Protection of the Cu_A site from heat-induced modification also protects the enzyme from displaying a heat-induced proton conductance. This observation suggests that it is a perturbation of the Cu_A site that causes a transmembrane proton leak in the enzyme. Nilsson et al. (1988) have obtained evidence for a similar transmembrane leak in the *p*HMB-modified enzyme and suggested that it is linked to disruption of the Cu_A site. It is therefore likely that these two enzyme derivatives contain similar proton conduction pathways. Although the EPR spectrum for the HTO enzyme is complicated, nearly 25% of the copper appears to be converted to a form almost spectroscopically identical to *p*HMB-modified Cu_A. The similarity of these two copper sites suggests that it is the type 2 form of the Cu_A site that is associated with the transmembrane leak. As noted earlier (Nilsson et al., 1988), the presence of a small fraction of leaky enzymes may mask proton pumping activity from the remaining fraction of the enzyme in the vesicle preparation because a substantial portion of the reconstituted vesicles will contain both intact and leaky enzyme molecules in the same vesicle.

The type 2 copper sites produced by heat-treatment and *p*HMB modification do not participate in electron transfer from ferrocytochrome *c*. In contrast, the type 1 copper site, which is present in ca. 50% of the heat-treated enzyme molecules, is electron transfer competent. Because even 25% of the type 2 copper species is

sufficient to uncouple a majority of the reconstituted vesicles, whether the modified enzyme with the type 1 Cu_A is capable of redox-linked proton translocation or behaves as an uncoupler is an issue that cannot be addressed at present.

In addition to the present heat treatment and *p*HMB-modification (Gelles & Chan 1985), it also has been found recently that heating cytochrome *c* oxidase in the detergent Sulfobethane-12 yields a Cu_A-modified type 2 copper site in high yields (Nilsson et al., submitted to Biochemistry). Treatment of the enzyme with Ag^+ also modifies the Cu_A site in a similar manner (Chan et al., 1978, Chan et al., 1979). Such strikingly similar type 2 copper sites resulting from Cu_A modification by methods as diverse as chemical thiol reagents and localized heat denaturation suggests that the protein architecture about the Cu_A site is essential to its structural integrity. The fact that all Cu_A modifications we have observed [including heat-treatment in Sulfobethane 12 (Nilsson et al., 1988)] result in the same kind of transmembrane proton permeability strongly implicates a role for Cu_A in proton transport within the enzyme.

Gelles et al. (1986) have proposed a theoretical model for proton pumping based on Cu_A as the site of energy transduction and proton pumping. In this model the pump site was required to be in contact with protons from both the matrix and the cytosol *via* appropriate proton channels. Proton gating was achieved by allowing protonation-deprotonation steps to occur either on the matrix side or the cytosolic side of the pump, but not both sites at once. This is tantamount to a modified alternating access model (Wyman, 1979, Wikström, et al., 1981). One possible interpretation for the results observed here is that Cu_A is associated with proton gating via an alternating access mechanism. If Cu_A is the proton gate, disruption of the site may destroy the alternate access of the pump site to protons, forming a contiguous proton channel through the enzyme. Because protons must travel to and from the pump site on a time scale comparable to electron transfer within the enzyme, the fast alkalinizations

observed are consistent with such a disruption of proton gating. An alternate explanation for the observed proton permeability is that *p*HMB and heat modifications cause a protein conformational change that opens up a proton conduction pathway within the enzyme, stretching from the matrix side to the cytosolic side of the enzyme. This possibility seems less likely, however, because it is inconceivable that such a gross conformational change can take place without also disrupting cytochrome *a* or the oxygen binding site. In fact, removal of subunit III, a 30kD membrane-bound polypeptide, induces a large change in protein architecture without leading to significant uncoupling. The HTR enzyme species that has been protected from Cu_A modification by reduction, is also protected from the formation of a proton leak through the enzyme. These observations all argue for a picture in which a transmembrane proton leak is specifically associated with disruption of the Cu_A site.

The work described here suggests that Cu_A plays an important role in proton conduction through cytochrome *c* oxidase. While it appears that Cu_A may participate in proton gating, it is also enticing to speculate that Cu_A is the site of energy transduction because Cu_A functions as an electron shuttle between cytochrome *c* and the oxygen binding site. As discussed by Chan et al. (1987), the Cu_A site may be amenable to redox-linked structural changes that make it a good candidate for the site of energy transduction. This hypothesis proposes that the two cysteine sulfurs coordinated to oxidized Cu_A (Cu^{2+}) interact with one another in such a way that reduction of the site (to Cu^{1+}) may cause a ligand substitution or rearrangement. It is interesting to speculate that this ligand rearrangement actually involves a local protein conformational change that also gates protons by alternating access. This model predicts that the Cu_A-modified derivatives of cytochrome *c* oxidase are incapable of pumping protons. However, because the formation of an efficient proton leak in the Cu_A modified enzyme derivatives would effectively mask proton pumping activity, it is impossible to

determine whether the proton pumping machinery is intact in the Cu_A-modified enzymes. Finally, because in addition to proton gating, the free energy transducer must also gate electrons, it is important to experimentally demonstrate electron gating before Cu_A can be identified as the site of redox-linked proton translocation.

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VI A Conformational Change is Associated with Dioxygen Binding to Cytochrome *c* Oxidase: A Flow-Flash Fluorescence Study

Abstract.

Spectral changes in the tryptophan fluorescence spectrum of a protein are indicative of conformational changes that alter the environment of the tryptophan residues. Such spectral changes have been used to study the protein conformational changes associated with the light-harvesting protein bacteriorhodopsin [Jang, D. J., Corcoran, T. C., & El-Sayed, M. A. (1988) *Photochem. Photobiol., 48,* 209-217]. We have measured time-resolved changes in the tryptophan emission intensity during the reaction of dioxygen with the fully-reduced and mixed-valence forms of cytochrome *c* oxidase using the flow-flash method [Gibson, Q. H., & Greenwood, C. (1963) *Biochem. J., 86,* 541-554]. After initiation of the dioxygen reduction reaction by CO flash photolysis, we observe a rapid increase in the tryptophan emission intensity followed by a slow decay of 80 s^{-1} (for the fully reduced enzyme) and 120 s^{-1} (for the mixed-valence enzyme). This tryptophan emission intensity change does not occur in the absence of dioxygen, suggesting that a large-scale conformational change is associated with dioxygen binding and reduction at the reduced binuclear center.

Introduction.

Cytochrome oxidase is the terminal enzyme in the aerobic respiratory chain. In eukaryotes, the enzyme spans the inner mitochondrial membrane and catalyzes the reduction of dioxygen to water (Wikström et al., 1981). In this reaction, the electrons are derived from the oxidation of ferrocytochrome *c* on the cytosolic side of the membrane while the protons consumed during the dioxygen reduction reaction are taken up from the mitochondrial matrix. In addition to catalyzing the reduction of dioxygen to water, cytochrome oxidase also catalyzes the redox-linked translocation of up to 4 H^+ from the mitochondrial matrix to the cytosol for every molecule of dioxygen reduced (Wikström, 1977). Thus, the reaction catalyzed by cytochrome oxidase is electrogenic, and contributes to the proton electrochemical gradient across the inner mitochondrial membrane. Cytochrome oxidase contains four redox-active metal cofactors, two heme irons and two copper ions. Two of these centers (cytochrome a_3 and Cu_B) form the site of dioxygen binding and reduction on the matrix side of the mitochondrial membrane. When dioxygen and its reduction intermediates are bound, the reduction potential of the site is high (near that of dioxygen). The other two centers, cytochrome *a* and Cu_A, have lower midpoint potentials (near that of cytochrome *c*) and serve to shuttle electrons from ferrocytochrome *c* to the dioxygen binding site.

Cytochrome oxidase belongs to a distinct class of proton translocation enzymes that are not well understood. In simple terms, the enzyme can be thought of as a molecular machine which must undergo an ordered sequence of conformational changes in order to carry out its function as a redox-linked proton pump. These conformational changes must gate the intramolecular electron and proton transfers in such a way that kinetic competence may be achieved.

Several types of conformational changes, both large and small, have been studied in cytochrome oxidase. It has been shown that purified cytochrome oxidase can exist in several conformations which exhibit different reactivities toward reductant and externally added ligands [for a review see Colosimo et al. (1986) and references therein]. The metal centers in these different conformational "forms" also display distinctive spectroscopic properties (Brudvig et al., 1981). However, it should be noted that cytochrome oxidase appears to exist in far fewer conformational "forms" in the mitochondrion than in the isolated enzyme (Wikström et al., 1981).

Other lines of investigation have identified conformational changes associated with the binding of cytochrome *c* to cytochrome oxidase (Michel et al., 1989 and references therein). These studies suggest that the binding of cytochrome *c* induces a conformational change in cytochrome oxidase at the cytochrome *a* site.

There is also considerable evidence that the reduction of cytochrome *a* and Cu_A causes substantial conformational changes in cytochrome oxidase. Yamamoto and Okunuki (1970) have shown that the reduced enzyme is more susceptible to protease digestion than the oxidized form. Cabral and Love (1972) used sedimentation studies to show that reduction of the enzyme resulted in a ~3% increase in the protein volume. By comparing the volume change in the fully reduced and CO mixed-valence (only cytochrome a_3 and Cu_B reduced), these authors attributed this volume change to the reduction of cytochrome *a* and Cu_A. Using zinc cytochrome *c* fluorescence as a probe for conformational changes in cytochrome oxidase, several investigators have also shown that the reduction of cytochrome *a* and Cu_A triggers a conformational change in the enzyme (Dockter et al., 1978; Vanderkooi et al., 1977; Kornblatt & Luu, 1986; Geren & Millett, 1981; Miki & Takayoshi, 1984; Alleyne & Wilson, 1987).

In order to understand the mechanism of proton pumping, it has become important to identify those conformational changes which occur during turnover. Most of the experimental evidence for conformational changes of this kind have come from studying the steady-state kinetics of the enzyme. For example, the kinetic behavior of the onset of inhibition due to cyanide binding has led to the suggestion that the enzyme cycles between two conformations during turnover. Stopped flow experiments indicate that simultaneous reduction of cytochrome *a* and Cu_A converts the dioxygen binding site to an "open" conformation. This "open" state is characterized by a cyanide binding rate (to oxidized cytochrome a_3) that is at least five orders of magnitude faster than the "closed" conformation (Nicholls et al., 1972; Van Buuren et al., 1972; Jones et al., 1984; Jensen et al., 1984; Scholes & Malmström, 1986). Also, the analysis of the nonhyperbolic kinetics of cytochrome *c* oxidation has led to the suggestion that the reaction cycle for cytochrome oxidase includes two distinct conformational states in which the primary electron acceptor has different reduction potentials (Brzezinski & Malmström, 1986). These authors have argued that the existence of two distinct conformations is an inherent property of redox-linked proton pumps.

In addition to these experiments there is also circumstantial evidence that Cu_A and the binuclear center are in close conformational contact. Since it has been suggested that Cu_A is the site of redox linkage to proton translocation (Chan et al., 1989), an allosteric interaction amongst these metal centers may play an important role during the proton pumping cycle. Li et al. (1988) showed that heat-induced modification of the Cu_A site correlates with an increase in the rate of cyanide binding at cytochrome a_3 . Furthermore, ligand binding at cytochrome a_3 was shown to protect the Cu_A site from the heat treatment modifications. These results have been corroborated by resonance Raman measurements on the chemically and heat-modified cytochrome oxidases, which have shown that the cytochrome a_3 iron-histidine

stretching and formyl stretching vibrations decrease in intensity when the Cu_A site is modified (Larsen et al., 1989).

Although the available experimental evidence suggests an important role for conformational changes in the catalytic cycle of cytochrome oxidase, there have been few ways to directly observe conformational changes that occur during turnover. The use of tryptophan fluorescence spectroscopy to probe conformational changes in proton pumping systems was recently reported by Copeland et al. (1986) for cytochrome oxidase and by Jang et al. (1988) for bacteriorhodopsin. Unfortunately, the conclusions derived from the work on the oxidized and reduced forms of cytochrome oxidase must be re-evaluated to account for systematic artifacts in the data. However, the use of this technique to monitor protein conformational changes during turnover remains valid. In this paper, we report the use of time-resolved tryptophan fluorescence to observe a protein conformational change that occurs during the turnover cycle of cytochrome oxidase using the flow-flash technique. We report the measurement of the kinetics of this protein conformational change and find that it is associated with the reaction of dioxygen with the reduced binuclear center.

Materials and Methods.

Materials. Cytochrome *c* oxidase was isolated from beef hearts according to the method of Hartzell and Beinert (1974). The enzyme was solubilized in 50 mM MOPS buffer (pH 7.4) containing 0.5% Tween 20 (Pierce) and stored at -80°C until use. Cytochrome *c* (Sigma Type VI) was used without further purification.

Sample preparation. Enzyme samples for the flow flash experiments were made by diluting cytochrome *c* oxidase to 10 mM in 50 mM phosphate buffer (pH 7.4) containing 0.5% lauryl maltoside (Calbiochem) or 0.5% Tween-20 (Pierce ultrapure). These buffers were chosen to minimize the background fluorescence. The enzyme

solutions, typically 100 mL of 10 mM enzyme, were equilibrated with CO by exchanging the atmosphere of the flask with CO ("Matheson Purity") ten times. The COMV enzyme (cytochrome a_3 and Cu_B reduced, cytochrome *a* and Cu_A oxidized) was prepared as described in Morgan et al. (1989). To prepare the CO-inhibited, fully-reduced (COFR) enzyme (all four redox-active metal center reduced), a similarly CO-equilibrated solution of cytochrome *c* and ascorbate (three times recrystallized) was added to the cytochrome oxidase solution to give a final concentration of 200 nM cytochrome *c* and 600 μM ascorbate. After the addition of cytochrome *c* and ascorbate, the enzyme solution was exchanged with CO twice more. The samples were kept several hours at 4°C to ensure complete reduction. Formation of the COMV and COFR forms of the enzyme was verified by optical spectroscopy.

Oxygenated buffer was made by exchanging 150-200 mL of 50 mM phosphate buffer (pH 7.4) containing 0.5% lauryl maltoside or 0.5% Tween 20 with pure oxygen several times.

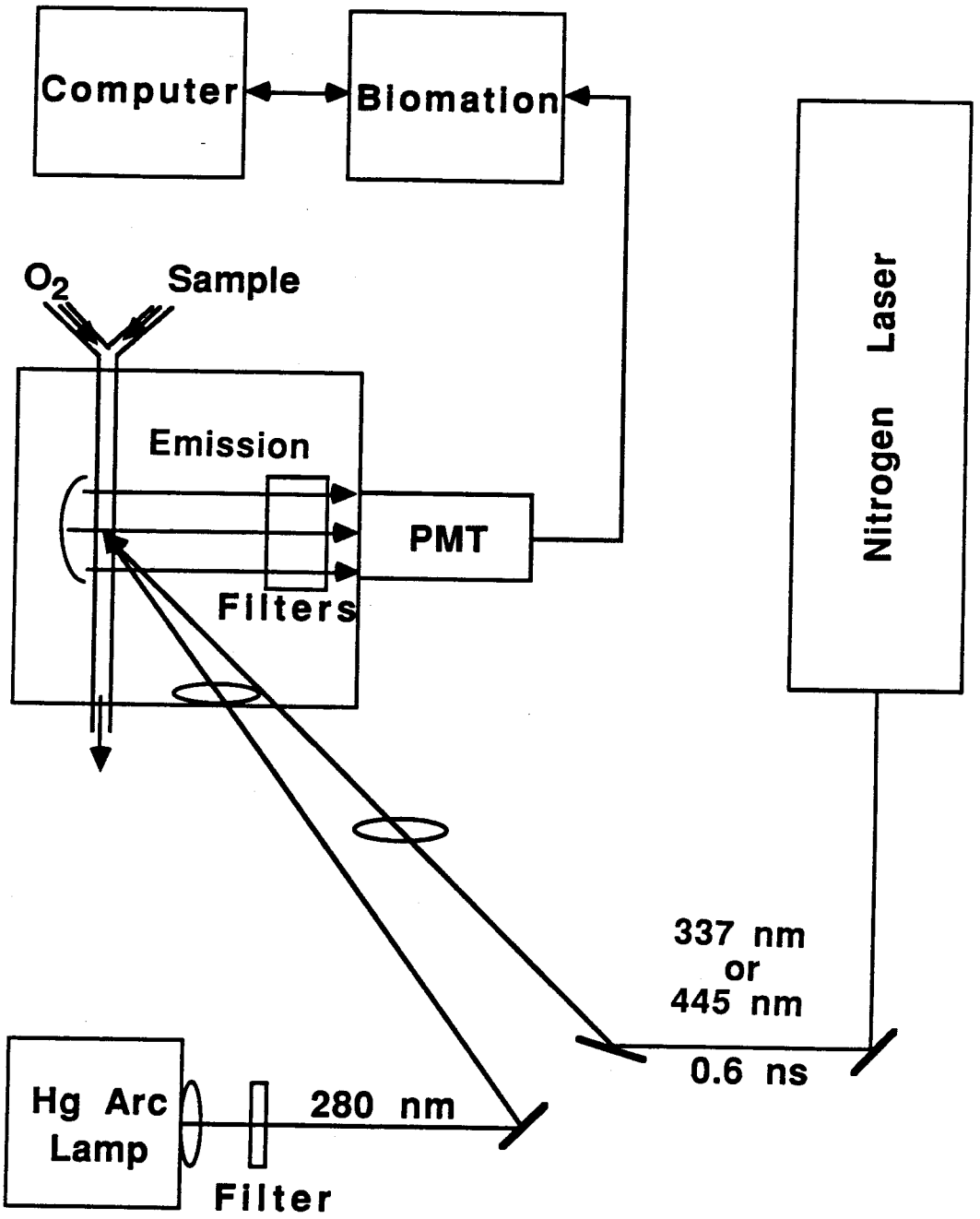
Flow-flash fluorescence spectrophotometry. The flow-flash experiment was performed essentially as described in Gibson & Greenwood (1963) except for the following differences. As prepared, the COFR samples contained excess of reductant (ascorbate). Since the samples were prepared using a nanomolar concentration of cytochrome *c*, the rate of re-reduction after one turnover is much slower than any of the events that accompany the reaction of the fully reduced enzyme with dioxygen. The mixing of the COFR and COMV forms of the enzyme with oxygenated buffer was accomplished using a continuous flow apparatus. Both the enzyme and oxygenated buffer solutions were pumped from their containers in the dark and anaerobically, using a dual-head peristaltic pump equipped with 1mm I.D. rubber tubing (Viton). The solutions were mixed in a black-masked Y-shaped glass tube, and the output was

immediately flowed into a 2mm x 4mm rectangular quartz cell. The flow rate was adjusted to approximately 5 mL/min.

The optical equipment used for the flow-flash fluorescence measurements is shown in Figure VI.1. The continuous wave excitation source was selected from a 100 W Hg arc lamp (Pek Labs 401) using a narrow band interference filter (280±5 nm). Photodissociation of the CO was accomplished by the 337 nm pulse from a nitrogen laser (PRA LN1000) or by the 445 nm pulse from a nitrogen pumped dye laser. Both photolyzing wavelengths yielded identical results. The photolyzing laser pulse had an energy of ~1 mJ (337 nm) or ~1μJ (445 nm) with a temporal pulse width of 0.6 ns and was focused to a ~2 mm spot size. The excitation beam was also focused to a 2mm diameter spot coincident with the laser beam. The fluorescent photons were collected using a concave mirror (F/C = 1.2) behind the sample, and detected by a gated photomultiplier tube (PMT). Wavelength discrimination was accomplished by a set of colored glass filters. Data was taken admitting several wavelength ranges. All of these experiments produced identical results.

Kinetic data was recorded with a Biomation 805 transient digitizer, which was interfaced to an Apple II+ computer. For a typical experiment, the laser repetition rate was set to 4 Hz, and was triggered to fire 150 channels after the Biomation began to collect data. The input capacitance of the detection system was measured at 120 pf, and the PMT termination was 10KΩ, resulting in a detection time constant of 100 ns. The data shown were an average of multiple scans (indicated in the Figure legends).

Figure VI.1: The flow-flash fluorescence experiment. See text for details.


Computer
Biomation
Nitrogen Laser
O_2
Sample
Emission
PMT
Filters
337 nm
or
445 nm
0.6 ns
Hg Arc
Lamp
280 nm
Filter

Results.

In these experiments, the reaction of dioxygen with either the fully-reduced or the mixed-valence forms of the enzyme was initiated using the flow flash method. This technique takes advantage of the stability of the COFR and COMV forms of the enzyme in the absence of light. When these CO-inhibited forms of the enzyme are mixed with oxyenated buffer in the dark, their reaction with the dissolved dioxygen is quite slow. After mixing, it is possible to initiate the reaction of fully reduced or mixed valence cytochrome oxidase with dioxygen. For a detailed description of this technique, see Gibson & Greenwood (1963) and Greenwood & Gibson (1967). In the flow-flash system used for these experiments, the oxygen reduction reaction was initiated by laser photodissociation of CO from the COFR and COMV forms of the enzyme in the presence of oxygen in a continuous flow apparatus.

Figure VI.2 shows the steady state fluorescence spectra of reduced and oxidized cytochrome *c* oxidase. The tryptophan emission maximum occurs at 330 nm for both the reduced and oxidized forms of the enzyme. For the 337 nm photolyzing pulse, the emission wavelength range monitored was between 355 and 400 nm. For the 445 nm photolyzing pulse, a larger window was chosen between 330 and 400 nm.

The COFR enzyme. The flow-flash tryptophan emission intensity change between 355 and 400 nm for the COFR enzyme is shown in Figure VI.3a. Following flash photolysis of the CO, there was an increase in emission intensity corresponding to a few percent (~2-5%) of the total fluorescence, followed by a return to the original emission level. We interpret the rising phase corresponds to the formation of a new conformational state. The decay phase then corresponds to the relaxation of this new conformational state back to the original conformation. The time resolution for the rate of formation was limited by the data collection apparatus used. By estimating half-times we can place a rough lower limit on the rate constant for this phase at ~10^6 s^{-1}.

Figure VI.2: Corrected steady state fluorescence spectra for a) oxidized and b) reduced cytochrome *c* oxidase. The oxidized and reduced forms of the enzyme have identical fluorescence spectra. Each spectrum consists of a sum of four consecutive scans.

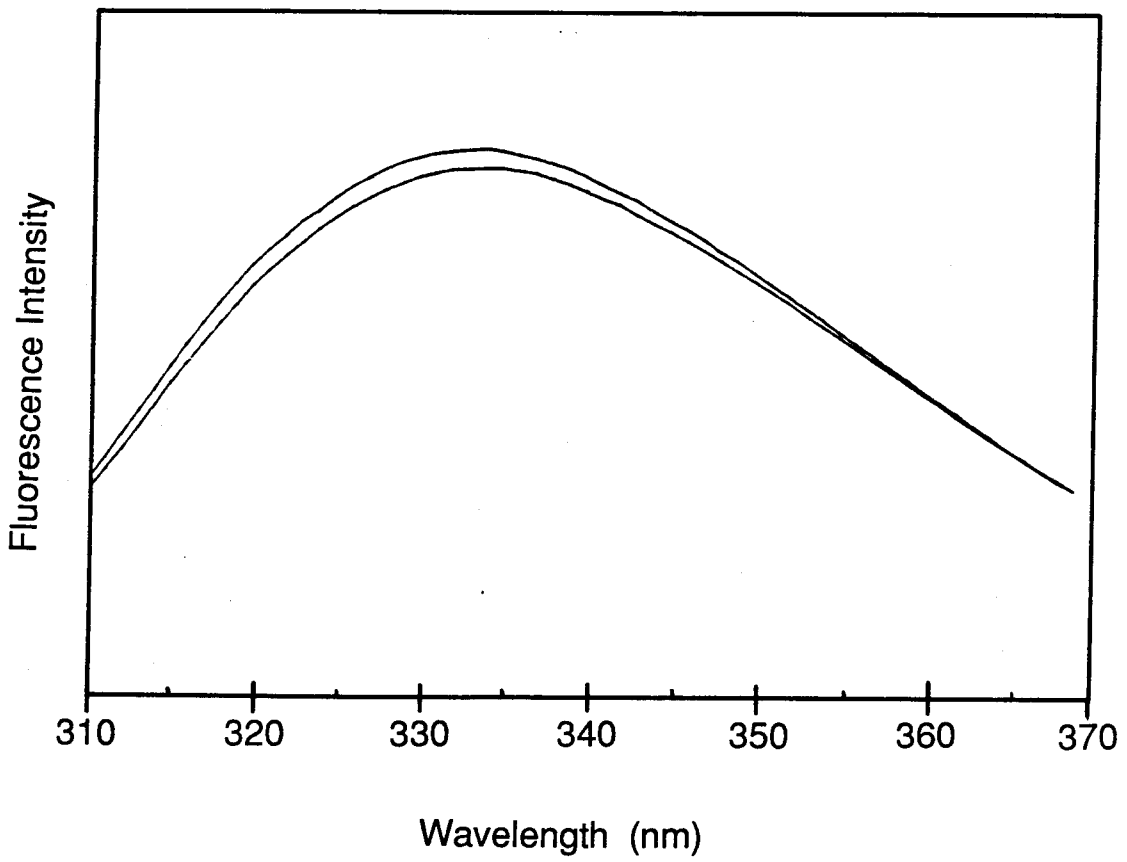
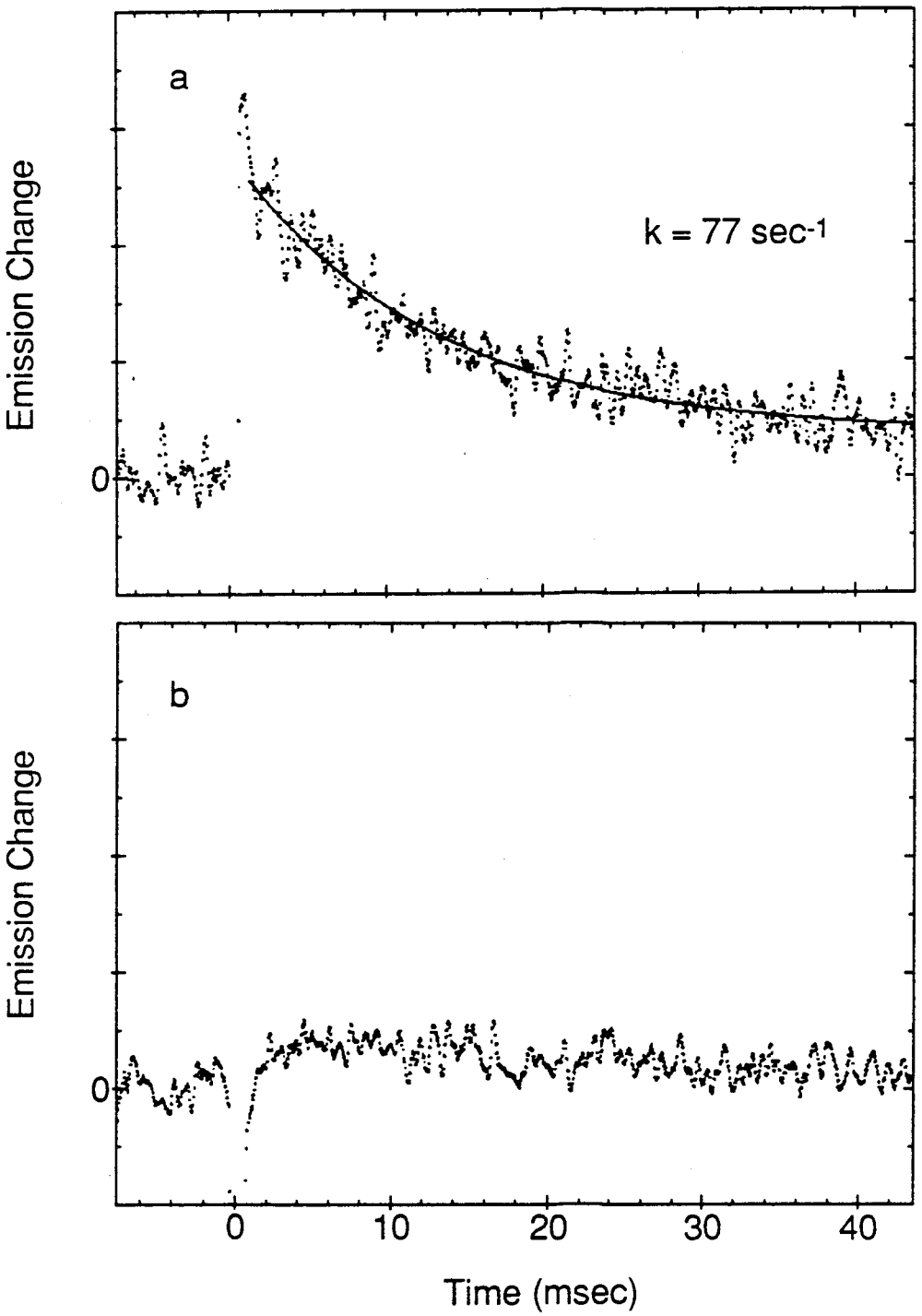

Fluorescence Intensity
310
320
330
340
350
360
370
Wavelength (nm)

Figure VI.3: Flow-flash fluorescence kinetics for a) CO-inhibited, fully reduced enzyme in the presence of oxygen and b) CO-inhibited, fully reduced enzyme in the absence of oxygen. The concentration of enzyme used for trace b) was roughly twofold higher than that for trace a). Each trace shown is an average of 8192 scans.

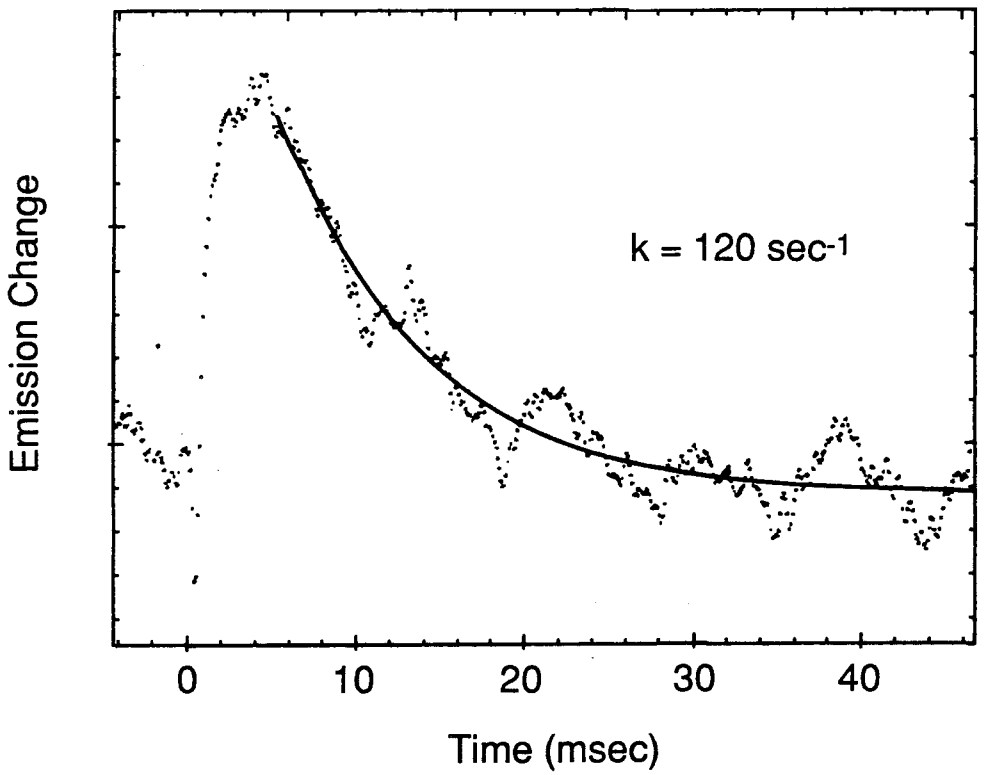

a
k = 77 sec-1
Emission Change
0
b
Emission Change
0
0
10
20
30
40
Time (msec)

The rate constant for the relaxation of the new conformation was fit using a non-linear least squares theoretical fit to both the raw and 5-point smoothed data, yielding a first order rate constant of 80 and 77 s^{-1}, respectively. The 5-point smoothed data are shown.

Because the observed rate constant for the relaxation kinetics is quite similar in magnitude to that for CO recombination in the fully reduced enzyme (ca. 100 s^{-1}), an identical data set was collected for the fully reduced, CO-inhibited enzyme in the absence of oxygen. As shown in Figure VI.3b, photodissociation of CO in the absence of oxygen causes no change in tryptophan fluorescence in the wavelength region monitored. Therefore, the fluorescence change observed in Figure VI.2a can only be associated with the dioxygen reduction cycle of the enzyme.

The COMV enzyme. Because the open-to-closed transition reported by Scholes & Malmström (1986) involves the reduction of cytochrome *a* and Cu_A, the time-resolved tryptophan fluorescence change was also monitored in the COMV enzyme. Figure VI.4 shows the fluorescence emission change for the COMV flow-flash experiment. The data were collected in the 330-400 nm region, with CO photolysis accomplished by 445 nm laser light. The results here were identical to those obtained for data collection in the 355-400 nm region with a 337 nm photolysis beam (data not shown). With cytochrome *a* and Cu_A oxidized, the tryptophan emission intensity changes were quite similar to the changes observed for the COFR enzyme, except that the rate of formation for the conformational change was slower, with an estimated lower limit for the rate constant of 10^5 s^{-1}. However, the relaxation phase was fit to a single exponential with a rate constant of 110 s^{-1}, a value quite similar to the decay for the COFR enzyme. Again, the photodissociation of CO in the absence of dioxygen caused no changes in the fluorescence emission intensity (data not shown).

Figure VI.4: Flow-flash fluorescence kinetics of the CO mixed-valence enzyme. The data shown were an average of 2048 scans.


Emission Change
k = 120 sec-1
0
10
20
30
40
Time (msec)

Discussion.

The origin of the intrinsic fluorescence of bovine cytochrome c oxidase. Recently, Hill et al. (1986) presented a detailed investigation on the nature of the intrinsic fluorescence of bovine cytochrome oxidase. Using a series of ionic and non-ionic quenchers, these authors were able to assign the observed emission spectrum to tryptophan residues buried in a hydrophobic environment. Because cytochrome oxidase contains two heme groups that are good energy transfer acceptors, Hill et al. (1986) also applied Förster's theory of resonance energy transfer to cytochrome oxidase. They found that the average distance between the hemes and tryptophans in the protein was 30Å with an error within 10% of this value (Haas et al., 1978).

We have studied the tryptophan emission lifetimes in cytochrome oxidase using a 290 nm pulsed laser and monitoring the emission intensity over time by photon counting. These results show a multiexponential decay, which can be fit well by the sum of three single exponential functions. This decay varies slightly with the emission wavelength. However, there was essentially no difference in the lifetimes between the oxidized and reduced forms of the enzyme (P. Smith, unpublished data). As mentioned earlier, Copeland et al. (1986) reported a shift in the tryptophan fluorescence spectrum upon reduction of Cu_A. Unfortunately, these results must be re-interpreted because of a systematic artifact in the data. It appears at this point that the fluorescence spectra for the oxidized and reduced enzymes at equilibrium are nearly identical. We have also measured the tryptophan fluorescence spectrum for the oxidized form of the cytochrome *c* oxidase from *Paracoccus denitrificans*. Despite the fact that the bacterial enzyme contains far fewer tryptophan residues, the fluorescence spectrum appeared nearly identical (P. M. Li, unpublished results).

Taken together, these results indicate that the observed fluorescence spectrum probably arises from a small group (at least 3) of the 52 tryptophan residues found in

the bovine enzyme. These tryptophan residues probably are buried deeply in the protein, and have similar hydrophobic environments in both the oxidized and reduced forms of the enzyme.

Changes in the tryptophan fluorescence spectrum are indicative of conformational changes that alter the environment of tryptophan residues in the protein matrix (Lackowiz, 1983). Although the fact that the oxidized and reduced form of the enzyme exhibit identical fluorescence spectra, transient conformational changes can occur during the turnover cycle. Jang et al. (1988) have monitored similar transient fluorescence intensity changes during the turnover cycle of bacteriorhodopsin. We attribute the increase in emission intensity during the reaction of dioxygen with the fully-reduced and COMV forms of the enzyme to a transient change in the environments of a small group (at least 3) of tryptophan residues buried in a hydrophobic section of the protein. Furthermore, based on the work of Hill et al. (1986) it appears that these residues reside at least 30Å away from the heme groups and the cytochrome *c* binding site. Therefore, we feel that these results indicate that a conformational change on the global scale is associated with the binding and reduction of dioxygen at the binuclear center.

It is important to note that an inner filter effect due to a changing absorbance spectrum in the wavelength region of the observed emission spectrum could also cause the fluorescence changes observed here. We have taken precautions to alleviate this possibility in two ways. First, the experimental setup used excitation and photolysis light focused at the surface of the flow cell to minimize inner filter effects. This kind of measurement protocol has proven effective for the measurement of fluorescence changes in the bacteriorhodopsin system (Jang et al., 1988). Second, we have observed that the emission intensity occurs independently of the observation wavelength.

The rising phase in the flow-flash fluorescence experiment. For the COFR form of the enzyme, two phases were observed in the time-resolved tryptophan emission intensity. We interpret the first phase to correspond to the rapid formation of a new conformational state (within 3-4 μs), which is specific to dioxygen binding and reduction at the binuclear center. This conformational change is clearly absent in the control experiments in which ligand dissociation occurs, in the absence of dioxygen. Based on the time constant of the instrument and the rate of data collection, a conservative estimate (lower limit) for rate constant of formation is at least 10^6 s^{-1}.

In the COMV form of the enzyme, the rate constant for formation is estimated at 10^5 s^{-1}. Therefore, it appears that the redox state of cytochrome *a* and/or Cu_A can affect the rate of the observed conformational change. This effect may be related to the allosteric interaction between Cu_A and the binuclear center alluded to by Li et al. (1988) or the interaction between cytochrome *a* and the binuclear center reported by Malmström and coworkers (Nilsson et al., 1989). In particular, it appears that, in order for the conformational change to proceed rapidly, Cu_A and/or cytochrome *a* must be reduced. It should also be noted that the "closed to open" conformational change has been suggested to require two electrons in the low potential centers (Scholes & Malmström, 1986).

The decay phase in the flow-flash fluorescence experiment. Interestingly, the decay phase for the observed conformational changes for both forms of the enzyme were well fit by a single exponential. Both the fully reduced and COMV forms of the enzyme exhibited similar decay rates. Computer fits to a three parameter single exponential were sufficient to describe the decay curves to a high degree of confidence. These results indicate that the conformational change induced by dioxygen binding and reduction at the binuclear center can be described by a conversion between two conformations. Furthermore, because the decay rates are so similar for both the

fully-reduced and COMV forms of the enzyme, it appears that the decay of the flow-flash induced "new" conformation may depend only on the redox state of the low potential centers and not on the nature of the intermediates at the binuclear center.

The nature of the observed conformational change. The flow-flash technique primarily has been used to study the electron transfers from the low potential metal centers to the oxygen binding site during turnover. In room temperature flow flash experiments, Hill and Greenwood (1983) reported several phases of electron transfer from cytochrome *a* and Cu_A to the oxygen binding site during the oxygen reduction reaction. At high oxygen concentrations (680 mM), three kinetic phases were observed at 605 nm. At the time, these phases were assigned to electron transfers from cytochrome *a* to the oxygen binding site and exhibited rate constants of $2.5x10^4$ s^{-1}, 10^4 s^{-1}, and 850 s^{-1}. It must be noted that the peroxy- intermediate (Compund C), which is formed immediately upon the reaction of dioxygen with the reduced binuclear center, has an intense absorption band at 607 nm, and that the ferryl intermediate (at the three-electron level of dioxygen reduction) has an absorption band at 580 nm. Therefore, the kinetic phases observed at 605 nm are likely to correspond to a convoluted sum of contributions from the reduction of Compund C or the ferryl, and electron transfer from cytochrome *a* rather than three independent electron transfers from cytochrome *a* to Cu_A or the binuclear center. We favor the interpretation that the first two rapid phases correspond to the reduction of Compund C by Cu_A as a consequence of electron transfers from Cu_A or cytochrome *a* . The last phase, which corresponds to a small absorbance change, can be assigned to a contribution from the reduction of the ferryl at 605 nm. The kinetics of electron transfer from Cu_A to the oxygen binding site (measured at 830 nm) are consistent with this interpretation. The first phase corresponds to the reduction of Compound C by Cu_A. The second phase then corresponds to electron transfer from Cu_A to the ferryl intermediate. These results

are not inconsistent with an electron transfer pathway in which cytochrome *a* donates electrons to Cu_A, which is the primary electron donor to both Compund C and the ferryl intermediate (at the three-electron level of reduction).

Compared to the reoxidation rates for Cu_A, the conformational change observed here is at least as fast as first phase of reoxidation which can be assigned to reduction of Compound C. It is likely that this conformational change preceeds the electron transfer from the low potential centers to Compound C at the oxygen binding site.

In contrast to the flow-flash measurements, several studies on the activity of cytochrome *c* oxidase in both purified and mitochondrial systems have reported turnover numbers close to an order of magnitude lower than the slowest electron transfer rates observed by flow-flash techniques. Most detergent solubilized enzyme systems show oxygen turnover numbers varying from 30 to 150 s-1 (Nicholls & Kimelberg, 1972) while enzyme in lipid-reconstituted systems or the detergent lauryl maltoside have oxygen turnover numbers near 400 s-1 (Rosevear, et al., 1980). (The enzyme used in this experiment exhibits a turnover number of ca. 30 s^{-1} in Tween 80.) [For comparison to electron transfer rates, these numbers must be multiplied by four.]

Several proposals have been offered for the apparent discrepancy between electron transfer rates measured by flow flash and derived from oxygen turnover number. It has been proposed that the rate-limiting step in the catalytic cycle is product dissociation of ferricytochrome *c* from the catalytic site (Speck et al., 1984, Sinjoro et al., 1984), a process that is not observed in the flow-flash experiment. Alternatively, Brzezinski et al., 1985 suggested that the concept of a single rate-limiting step in such a complex reaction is meaningless because even steps with relatively large rate constants may limit the rate if enough steps exist. It is interesting to note that the decay

rate for the conformational change observed in the flow-flash experiment is quite similar to the turnover rate of the enzyme. These results are consistent with facile electron transfer steps, and a conformational change being the rate-limiting step in the turnover of the enzyme.

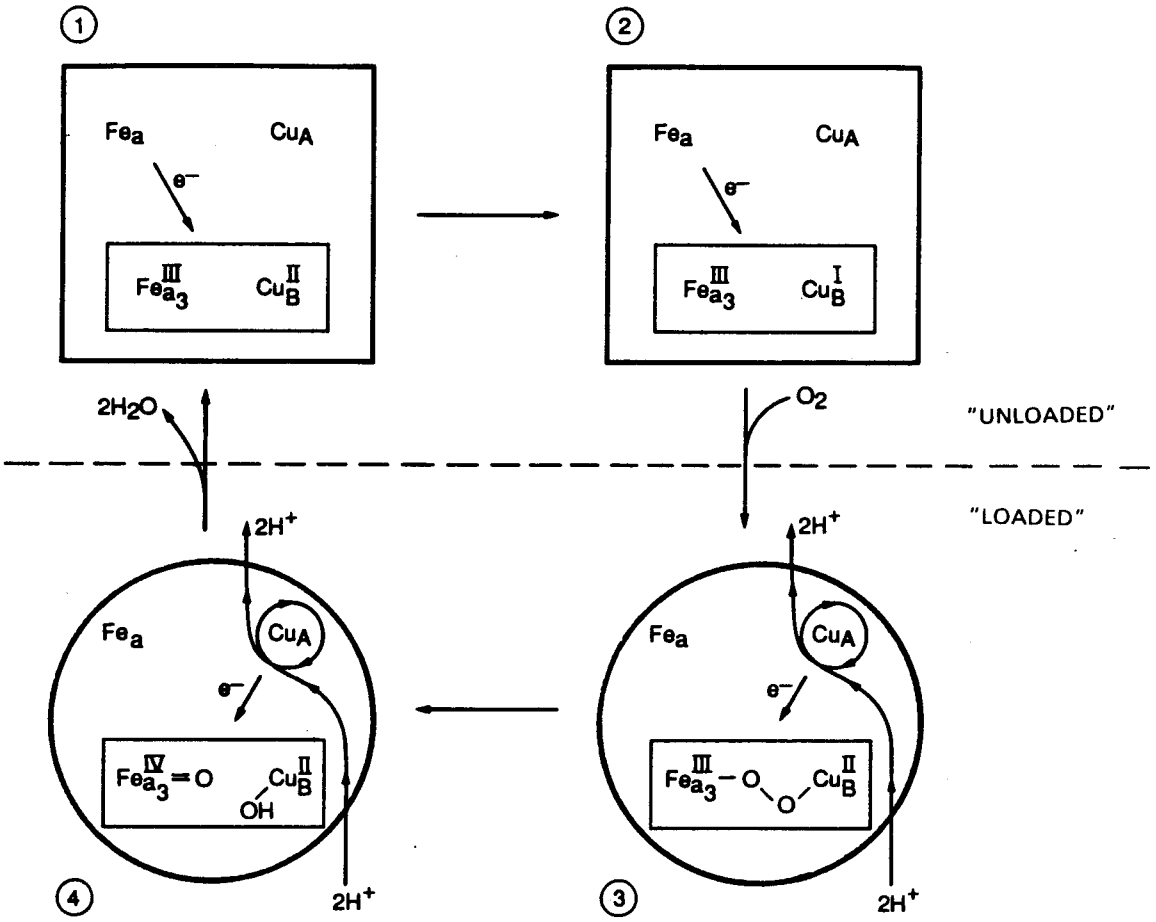
Implications for the catalytic cycle of cytochrome c oxidase. At this time, there is no concrete evidence for the role of this conformational change in the catalytic cycle. However, the results here are consistent with the a fast conformational change, followed by the electron transfers from cytochrome *a* to Cu_A, and Cu_A to the dioxygen intermediates. It also appears that the rate for formation for this conformational change is dependent on the redox state of cytochrome *a* and/or Cu_A. When these centers are oxidized, the conformational change occurs at least ten times more slowly than when these centers are oxidized. It is interesting to note that in the COFR enzyme, the observed conformational change is likely to preceed most of the electron transfers from cytochrome *a* and Cu_A to compound C at the dioxygen binding site. However, when the electrons are not present in the low potential centers, the conformational change does not occur with such facility. The importance of these results is twofold. First, these results indicate that the observed conformational change is linked to electron transfer. Second, they suggest that there is cooperativity between the low potential centers and the binuclear center in causing this conformational change. Recently, it has been suggested that protons are pumped only during electron transfer steps to the peroxy- and oxyferryl- intermediates of the dioxyen reduction cycle (Chan & Li, 1989). Implicit in this proposal is that the enzyme must distinguish between the pumping and non-pumping parts of the dioxygen reduction reaction. Specifically, the first two electron transfer steps must be non-pumping, while the second two steps must be coupled to the translocation of four protons from the mitochondrial matrix to the cytosol. The results presented here suggest that a rapid

conformational change occurs when two conditions are satisfied. An electron must be in cytochrome *a* or Cu_A *and* a dioxygen reduction intermediate must be at the binuclear center in order to induce the rapid conformational change. Ligand binding to the reduced binuclear center alone does not appear to cause any changes in the protein conformational state. Therefore, it is attractive to assign the function of this rapid conformational change to a switch that converts the enzyme from a non-pumping state to a pumping state.

In terms of a simple model for the catalytic cycle based on a proton pump that involves redox linkage at the Cu_A site, the conformational change observed during the flow-flash reaction could correspond to a switching scheme as shown in Figure VI.5. The first two electron transfers to the "unloaded" binuclear center occur in one conformation, denoted as a square. In this conformation, the electron transfer rate is maximized, because the pump is effectively disengaged. When dioxygen binds and becomes reduced at the binuclear center and electrons are present in the low potential centers, a conformational change to a new state is induced, corresponding to a circle. In this conformation, the intramolecular electron transfer occurs from the pump site (Cu_A) to the dioxygen intermediates (Compound C and the oxyferryl), thereby engaging the pumping element. This is tantamount to a conformational "clutch".

The model outlined above is by necessity speculative. However, the results presented here show that this conformational change in cytochrome *c* oxidase occurs rapidly enough to be involved in turnover. Although more work is needed to show that this conformational change is involved in switching the electron transfer pathways, we feel that kinetic studies of this kind offer a new way in which to study changes in the protein matrix to cytochrome *c* oxidase during the turnover cycle.

Figure VI.5: A simple model for the turnover cycle of cytochrome *c* oxidase using Cu_A as the site of redox linkage. The square conformations represent the "unloaded" form of the enzyme in which dioxygen has yet to bind to the binuclear center. The round conformation represents the "loaded" form of the enzyme, with dioxygen or its reduction intermediates bound at the binuclear center. As shown, the flow-flash reaction corresponds to the conversion from the "unloaded" to the "loaded" forms, inducing a conformational change. See text for further details.


1
2
Fea
CuA
e⁻
Fe$^{III}_{a_3}$
Cu$^{II}_{B}$
Cu$^{I}_{B}$
2H2O
O2
"UNLOADED"
"LOADED"
2H+
Fe$^{IV}_{a_3}$=O
OH
Fe$^{III}_{a_3}$–O
O
3
4

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VII A Proposal for the Complete Turnover Cycle of Cytochrome *c* Oxidase

We conclude this thesis with a proposal for the complete turnover cycle of the enzyme based on the experimental results presented in Chapters II-VI. In this proposal, we attempt to integrate both the electron transfer and proton pumping events with the dioxygen chemistry.

Any chemically acceptable proton pumping model must be consistent with all of the available biochemical evidence at hand. Hence, one of the challenges behind the model-building exercise is integrating the proton pump into an already complicated turnover cycle that involves four electron transfers to at least four different dioxygen reduction intermediates. Once again, we emphasize the crucial role that dioxygen plays in proton pumping because it is the reduction of dioxygen to water that provides the free energy for proton pumping. Accordingly, we assert that it is the electron transfer from Cu_A to the dioxygen intermediates bound at the binuclear center that is linked to the translocation of the protons. Such a model is consistent with the recent results of Wikström (1989) that suggest that protons are pumped during only two of the four electron transfer steps. However, a proton pump that relies on two of the four electron transfer steps in the catalytic cycle does require a level of complexity that has not been considered in most models up until this point. In this section, we herein describe how a model based on Cu_A as the site of redox linkage can be integrated into a pumping cycle that derives energy from only two of the four electron transfer steps.

In Chapter II, we presented EXAFS measurements on *p*HMB-modified, Cu_A-depleted and native forms of cytochrome oxidase, which suggest that Cu_A is indeed coordinated by two cysteine ligands in addition to two histidine nitrogen ligands. Chapter III presented a continuation of this work, which refined the EXAFS-derived structure for the copper sites and presented new evidence for the coordination environment of the reduced copper sites. This work suggested that Cu_A undergoes a

ligand rearrangement upon reduction while the ligand structure of Cu_B remains constant. Based on these results, we proposed a model whereby a ligand exchange reaction is involved in the redox-linkage of electron transfer to proton translocation (Chapter I; Gelles et al., 1985). Experimental evidence for the involvement of the Cu_A site in proton pumping was provided in Chapter IV and Chapter V. These chapters described chemical and gentle heating procedures that specifically modified the Cu_A site, leaving the three other redox-active metal centers essentially unperturbed. When the *p*HMB-modified enzyme was reconstituted into phospholipid vesicles, and assayed for proton pumping activity, we observed a proton leak attributable to an increased proton conductance through the protein (Nilsson et al., 1987; Chapter IV). When the heat-treated form of the enzyme was reconstituted into vesicles, we observed identical behavior in the proton pumping assay (Li et al., 1988; Chapter V). Based on these results we proposed that the Cu_A site is intimately involved either in proton transport within the protein or in redox linkage.

In Chapter V, we also presented spectroscopic evidence that showed that the Cu_A site can be rendered into two different ligand environments upon heat treatment (Li et al., 1988). The first is a type 2 copper ligand structure similar to the *p*HMB-modified Cu_A site. The second is a "blue" copper-like ligand structure. These results suggested that the Cu_A site can be forced to undergo a ligand exchange reaction that involves a change in the sulfur coordination shell. Furthermore, it was shown that reduction of the enzyme and ligand binding at the dioxygen reduction site protected the Cu_A site against these kinds of modifications. Taken together, these results indicated that there is a strong allosteric interaction between the dioxygen reduction site that controls the lability of the sulfur coordination at the Cu_A site.

Based on the idea that the Cu_A site and the binuclear center were in allosteric contact, and that a ligand exchange reaction occurs upon reduction of Cu_A, we probed

the protein matrix for conformational changes that could be involved in this kind of allosteric interaction. In Chapter VI we presented time resolved fluorescence work that identified a protein conformational change that accompanies dioxygen binding and reduction in the flow-flash reaction.

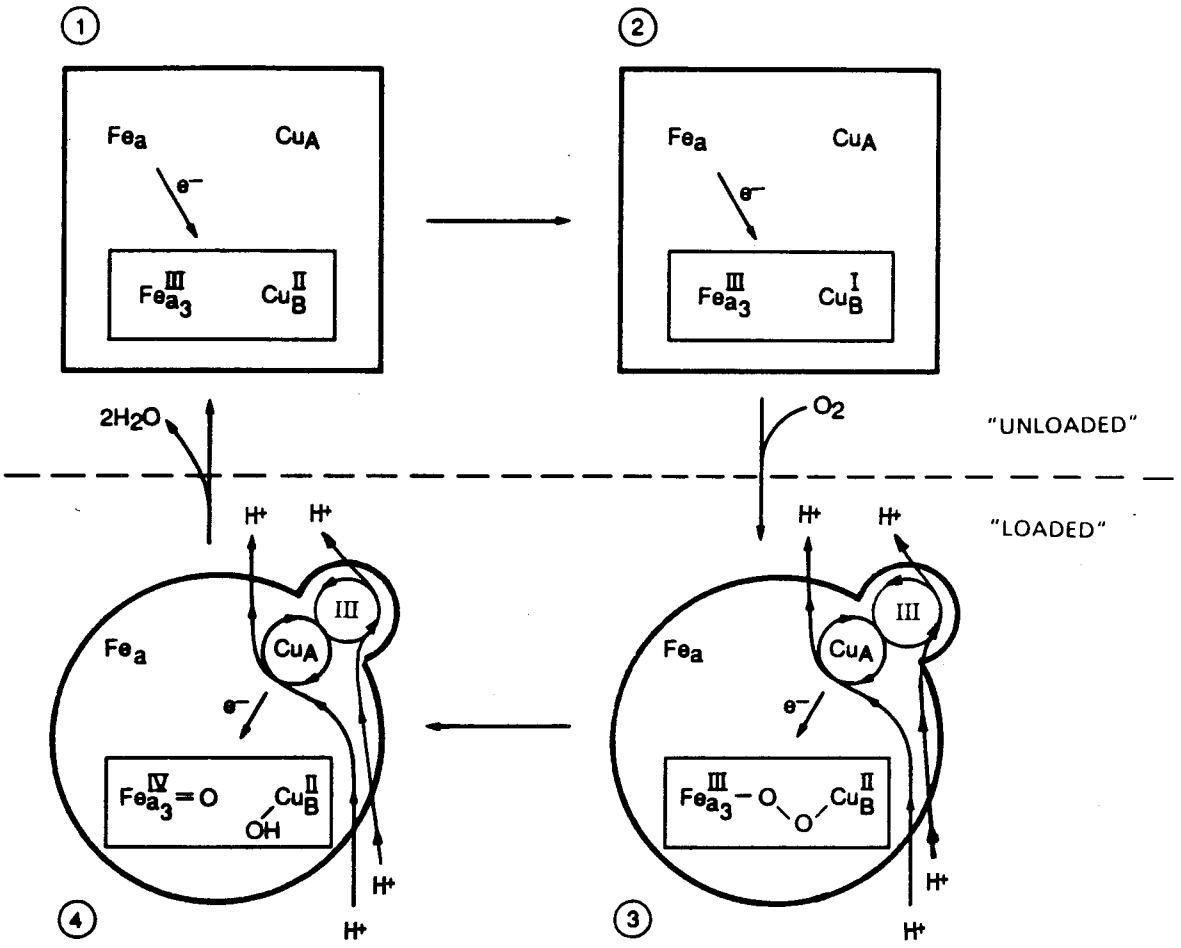
Based on this work we now propose a model for the complete turnover cycle of the enzyme. This model uses the Cu_A site as the site of redox linkage, and relies on a conformational change initiated by dioxygen binding and reduction to trigger the proton pumping cycle.

If the proton pump derives energy from only two of the four electron transfers to dioxygen, the pumping site (or the site of linkage) must be able to distinguish between the energetically productive and nonproductive electron transfers *or,* alternatively, electrons must pass through the pump site only when protons are pumped. We propose that allosteric coupling between the binuclear center and the low potential centers modulates the downhill electron transfer pathways. There is some circumstantial evidence for an allosteric interaction between Cu_A and the binuclear center (Li et al., 1988; Chapter V), so we propose that Cu_A is the site of redox linkage.

As shown in Figure VII.1, the first two electrons enter the oxidized binuclear center in the absence of dioxygen. Because cytochrome *a* and the "unloaded" binuclear center have similar potentials, these electron transfer steps do not contain sufficient free energy to pump a proton. However, these electron transfer events initiate the dioxygen chemistry, and therefore must occur with reasonable rapidity. We suggest that these two electron transfers occur *via* cytochrome *a*. This "unloaded" conformation is denoted by the square shapes.

When dioxygen binds to the reduced binuclear center and becomes reduced to the peroxy- intermediate, the driving force for the electron transfers from the low potential centers increases dramatically. The electron transfer steps that follow must

Figure VII.1: A proposal for the complete turnover cycle of cytochrome *c* oxidase, that integrates the intramolecular electron transfer, dioxygen reduction, and proton pumping events. (See text for a detailed description.)


1
2
Fea
CuA
e⁻
Fe$^{III}_{a_3}$
Cu$^{II}_{B}$
Cu$^{I}_{B}$
2H2O
O2
"UNLOADED"
"LOADED"
H+
III
Fe$^{IV}_{a_3}$=O
OH
Fe$^{III}_{a_3}$–O–O–Cu$^{II}_{B}$
4
3

be linked to proton pumping, and we propose that they occur *via* the Cu_A site. The suggestion is that dioxygen binding and its subsequent reduction to the peroxy-intermediate triggers a conformational change that directs the electron flow through the pumping site (Cu_A). The new "loaded" conformation is depicted by the circles. In studies of the single turnover of the enzyme, Cu_A is always re-oxidized before cytochrome *a*, suggesting that electron transfer from Cu_A to the binuclear center is more facile than from cytochrome *a* when the dioxygen reduction site is activated.

One consequence of having two distinct electron transfer paths is that the maximum level of reduction at steady state for cytochrome *a* and Cu_A cannot exceed 50%. Assuming that the highly driven electron transfer steps from Cu_A to the dioxygen intermediates is fast, the enzyme population at steady state will always contain a large contribution from states of the enzyme in which both cytochrome *a* and Cu_A are oxidized. This is consistent with the long-standing observation that the steady state levels of reduction for cytochrome *a* and Cu_A never exceed 50%. (Moroney et al., 1984; Gregory & Ferguson-Miller, 1989).

An important associated question is how the transfer of one electron can be coupled to two proton transfers. Two mechanisms are possible. The pump site may transport two directly linked protons per electron, or alternatively, one proton may be translocated *via* a directly linked process while a second proton is translocated *via* an indirect mechanism at another site. Our model of redox linkage proposes that the Cu_A site pumps one proton for every electron transfer that passes through the pump site. Accordingly, we favor a picture that includes two sites for proton translocation. It should be noted that the alternate site could reside in subunit III, where DCCD binding and subunit III depletion consistently diminish proton pumping activity by 50%. These results are consistent with subunit III being one of two proton pumping sites. It should be noted that if this model is correct, Cu_A and subunit III must be in conformational

contact. Finally, the possibility of two distinct electron transfer pathways to the "unloaded" and "loaded" dioxygen reduction site makes the issue of electron transfer pathways especially significant. Specifically, it becomes important to determine whether two pathways exist, and if so, how the enzyme switches between the two. Two possible switching mechanisms exist. First, the enzyme may control the equilibration between cytochrome *a* and Cu_A such that electron transfer through the pump site (presumably Cu_A) does not occur in species of the enzyme with an "unloaded" dioxygen reduction site. Second, the enzyme may directly control the rates of electron transfer from the low potential centers to the dioxygen reduction site, depending on the state of the enzyme. In this case, the cytochrome *a* to Cu_A electron equilibration rate may remain fast in all forms of the enzyme. These possibilities provide an exciting framework for the next generation of experiments on this fascinating enzyme.

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