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Cytochrome *c* Oxidase: Understanding Nature's Design of a Proton Pump†

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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 aa_3 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 2 or 3 subunits in the simplest bacterial systems and as many as 13 dissimilar subunits in mammals [for a review, see Wikström et al. (1981)].

All aa_3-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 with reducing equivalents derived from cytochrome *c*:

$$4\text{Cyt } c^{2+} + O_2 + 4H^+ \rightarrow 4\text{Cyt } c^{3+} + 2H_2O$$

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 5 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 mass, 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 which copurify with the electron transfer and dioxygen reduction activity. On the other hand, since 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.

Finally, cytochrome oxidase represents a distinct class of proton translocation devices whose principles of operation are 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

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small subset of these conformational states remains to be seen.

The purpose of this paper 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. Since 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 a_3 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. On the basis of 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 a_3, 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 copurified 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 a six-coordinate low-spin heme A, axially ligated by the nitrogens from two neutral imidazoles in both the oxidized and reduced states (Babcock & Callahan, 1983; Martin et al., 1985). This metal center is generally assumed to be the primary acceptor of electrons from ferrocytochrome *c*. However, this position is not unambiguous and must be reevaluated in light of the rapid electron equilibration between cytochrome *a* and Cu_A recently reported by Morgan et al. (1989). 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 the cytochrome *a* 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 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., 1986a).

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. It has been 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 which 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 infrared (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 (Assa 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 unpaired electron of Cu_A and various nuclei. The copper hyperfine interaction is unusually small and isotropic [Stevens et al. (1982) and references cited 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_2's 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 by 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 a_3 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 a_3 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 a_3 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).

Interactions among 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 a_3 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 a_3 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 a_3. When Cu_A is chemically modified, the iron–histidine stretching and formyl stretching modes of cytochrome a_3 change in frequency as observed by resonance Raman spectroscopy (Larsen et al., 1989). Also, the rate of cyanide binding to cytochrome a_3 is accelerated when Cu_A is modified by heat treatment (Li et al., 1988). Recently, spectroelectrochemical experiments have also uncovered an anticooperative interaction of $\sim$40 mV between cytochrome *a* and Cu_A which may be either electrostatic or conformational in nature (Blair et al., 1986a). In support of this, Brudvig et al. (1984) and Scholes et al. (1984) 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 1 shows our current view of the redox-active metal centers in cytochrome oxidase.

Subunits. Mammalian cytochrome oxidase has been shown to contain at least 13 inequivalent subunits: 3 are coded by the mitochondrial DNA and synthesized on the mitochondrial ribosomes (subunits I–III), and the remaining subunits (subunits IV–XIII) are coded for by the nuclear DNA and synthesized in the cytosol. Most eukaryotic organisms have multisubunit 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

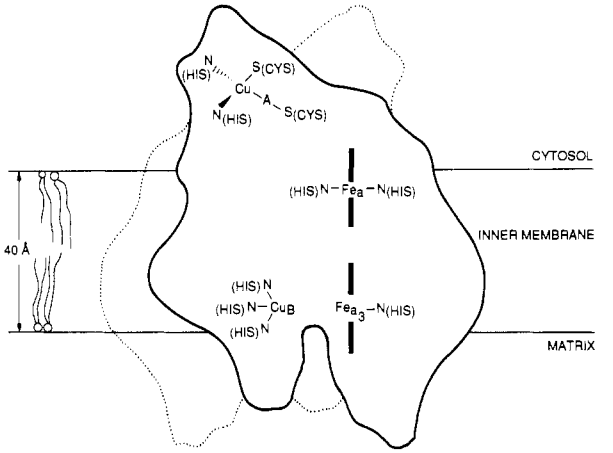

FIGURE 1: Pictorial representation of the cytochrome oxidase dimer in the inner mitochondrial membrane, including the approximate relative positions of the redox-active metal centers. The non-redox-active metal centers (Zn, Mg) are not shown. It should be noted that the intersite distances are not drawn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

I–III. The remainder of the subunits presumably perform a regulatory function.

Since 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 subunits III was proposed to be involved in the proton translocation process because it is a membrane-spanning polypeptide which is sensitive to the carboxyl reagent dicyclohexylcarbodiimide (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. Finel and Wikström (1988) recently suggested that subunit III plays a role in the oligomerization of the protein, which is perhaps important to the proton pumping function of the enzyme. However, Moody and Rich (1989) have recently carried out experiments which suggest that the monomer form of the enzyme is competent in proton translocation.

Several methods of subunit III removal have now been developed. These subunit III-less enzymes often display no proton pumping activity when assayed with 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 a 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 (1987) and 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 proteopliposomes 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 the 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 tech-

nologies, 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 aa_3-type cytochrome oxidases from *P. 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 which seem to be homologous to the three mitochondrially coded mammalian oxidases. Of these bacterial enzymes, the one from *P. denitrificans* is the most thoroughly studied. It also most closely resembles the mitochondrially coded bovine heart subunits, as does the *T. novellus* enzyme. The structure of the cytochrome oxidases from the thermophilic bacteria, however, seem to be different. The *T. 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 these bacterial aa_3-type oxidases exhibit similar functional characteristics and have spectroscopic properties almost indistinguishable from those of 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 oxidases 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 aa_3-type oxidases from *Pseudomonas* 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 *N. europa* strains capable of oxidizing ferrocytochrome *c* and reducing dioxygen. The cytochrome oxidase from *H. 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 & Chan, 1985). Unfortunately, it is not known whether these Cu_A-less enzymes are capable of pumping protons. Finally, a cytochrome oxidase from *T. thermophilus* has recently been purified which 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 new development in the bacterial oxidase field is the cloning of the cytochrome oxidase genes for *P. denitrificans* (Raito et al., 1987) and PS3 (Sone et al., 1988). These studies have already proven useful in identifying a subunit III in *P. 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 more clearly delineate structure–function questions that are not amenable to traditional biochemical and biophysical techniques.

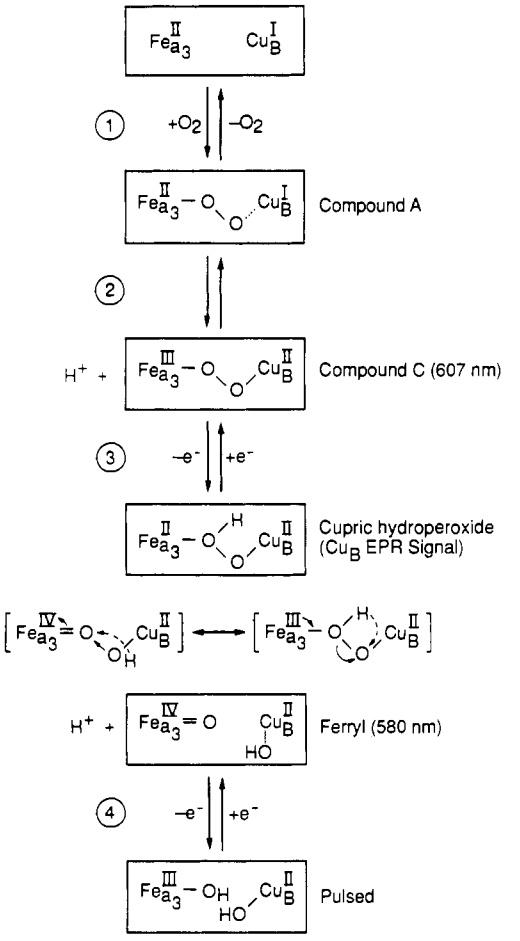

FIGURE 2: Scheme depicting the four electron reducing steps of the dioxygen reduction reaction on the basis of the available chemical and spectroscopic evidence. (See text for details.)

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 which 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., 1986; Chan et al., 1988b), 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 2. In step 1, dioxygen binds to ferrous cytochrome a_3, forming a dioxygen adduct called compound A (Chance et al., 1975a,b). This species is short-lived, lasting no 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 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 oxyferryl cytochrome a_3/cupric Cu_B species. It exhibits an optical spectrum with difference features (ferryl minus resting) at 580 and 428 nm. In addition, the EPR (Witt & Chan, 1987; Witt, 1988), resonance Raman (Witt, 1988), Mössbauer (Fee et al., 1988), and EXAFS (Chance & Powers, 1985; Kumar et al., 1988) experiments are consistent with an oxyferryl 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., 1988b). 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 with the two intermediates generated at the two- and three-electron-reduced levels of dioxygen reduction in the experiments which proceed in the forward direction. The first intermediate observed upon charging the membrane is a species with the 580-nm absorption band, spectroscopically identical with the oxyferryl species generated by Chan et al. (1988b) 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 compound 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 a_3, 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.

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 & Greenwood (1984a,b)]. In this section, we merely point out the major questions which 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, since 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 30–600 molecules of cytochrome *c* per second [see Wikström et al. (1981) and Brunori et al. (1987a) for a review]. The oxidation of ferrocytochrome *c* can be monitored with 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 by optical spectroscopy (Gibson & 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 nonhyperbolic kinetics [Malmstöm and Andrêasson (1985) and reference cited 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 & Andrêasson, 1985; Thörnström et al., 1988). Malmström and co-workers have proposed that the enzyme can pump protons only in one of these two conformations (E_2)

(Brzezinski & Malmström, 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 steady-state kinetics of cytochrome oxidase 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., 1988). 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 (nonpumping) 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, since 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 & Palmer, 1982; Andréasson et al., 1982), with some reports indicating that Cu_A may lag slightly (Wilson et al., 1975). To date, all of the experiments which 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} s^{-1} (Antalis & Palmer, 1982; Andréasson et al., 1982; 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 which 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 7000 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 and the dioxygen reduction site is based on the measurement of 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. On the basis of 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 that of the low-potential metal centers, and thus may not be relevant to the proton pumping forms of the enzyme.

The rate of electron transfer 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 equilibration 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 $a^{2+}/Cu_A{}^{2+} \rightleftharpoons$ cytochrome $a^{3+}/Cu_A{}^{1+}$). Thus, the electron equilibration is extremely rapid compared to the turnover rate of the enzyme (30–600 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, 1966). However, on the basis of 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 di-

rection to the electron transfers. Since that time, Wikström and many others have come to view cytochrome oxidase as a proton pump which 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 which 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 a hydroxide from the cytosol to the matrix (Mitchell, 1987). These schemes are ingenious. Unfortunately, ligand-based redox loops necessarily have unity H^+/e^- stoichiometry, and it has been shown that the H^+/e^- stoichiometry 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 which actively translocates a proton via a mechanism other than a substrate-based redox loop, i.e., the coupling between electron transfer and proton trannsfer 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 oxidiase 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.

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 by use of 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., 1986b; 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 which exists in the literature.

(*A*) *Linkage.* In order for electron transfer to be linked to proton transfer, one requirement is that these two activities 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 which includes the sidedness of the membrane, one can envision an eight-state "cubic" formalism as proposed by Wikström (1981) and shown in Figure 3.

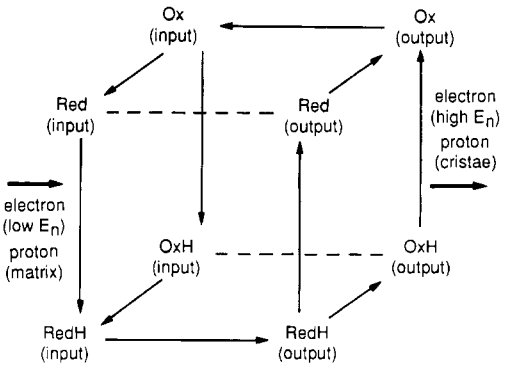

FIGURE 3: Eight-state cubic formalism which links the two states of the electron translocating element, the two states of the proton translocating element, and the two sides of the membrane, as proposed by Wikström (1981). (See text for a more detailed description.)

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. (1986b) and Krab and Wikström (1987), the eight-state model arises 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 16, and not 8 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., 1986b).

To rectify this, Krab and Wikström (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 coworkers. We assume that specificity of the first kind is implicitly built into the eight-state cubic scheme.

(*B*) *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. The other state is an "electron output state" with all of the associated proton movements completed, 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., 1986b), or specificity of the first kind (Krab & Wikström, 1987). If electron gating is absent, electron transfer may occur in states of the enzyme which 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 nonproductive 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 which 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 specificity 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 side may be I/O competent. Therefore, we feel that electron and proton gating together correspond to "specificity of the second kind".

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. Since 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/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 & 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*-(hydroxymercurio)benzoate (pHMB) to produce a structurally altered type 2 Cu_A site (Gelles & Chan, 1985). 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 nonionic 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 either displayed 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 by 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 linkages. 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, a model will have to be either 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.

(*A*) *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

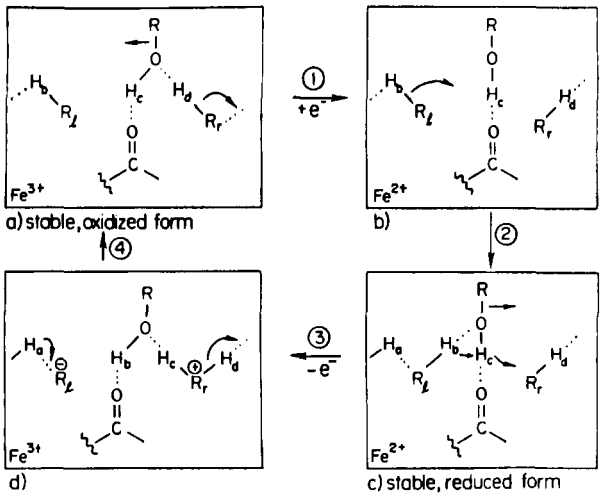

FIGURE 4: Babcock model for redox linkage based on cytochrome *a*. (See text for details.)

and Callahan (1983) are shown in Figure 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 strength 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 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 provisions for the gating of the electron flow to obviate futile cycles.

Since 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 have recently 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.

(*B*) *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

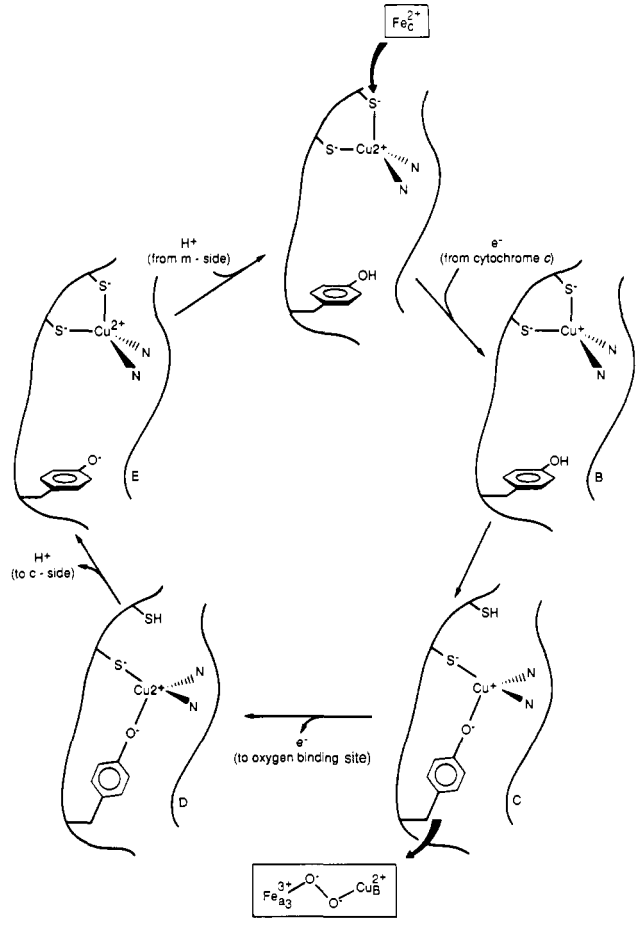

FIGURE 5: Chan model for redox linkage based on Cu_A. (See text for details.)

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 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., 1988a). 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_a's 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. Since 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, 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.

Identification of Redox Linkage Site: 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 mehanism 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 which 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 causing the electron carrier downstream to 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 noninteractive. If the redox element which 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.

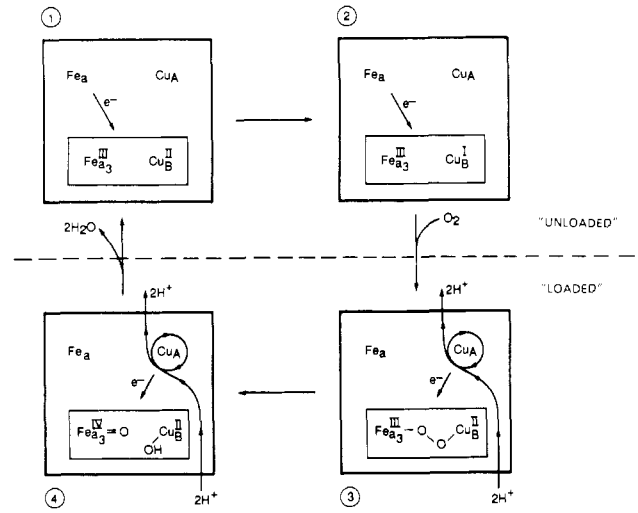

FIGURE 6: Proposal for the complete turnover cycle of cytochrome *c* oxidase which integrates the intramolecular electron transfer, dioxygen reduction, and proton pumping events. (See text for a detailed description.)

Complete Turnover Cycle: A Hypothesis

We conclude this paper with a proposal for the complete turnover cycle of the enzyme, wherein 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 which 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 (or cytochrome *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 which relies on two of the four electron transfer steps in the catalytic cycle does require a level of complexity which has not been considered in most models up until this point. In this section, we 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.

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), so we propose that Cu_A is the site of redox linkage.

As shown in Figure 6, the first two electrons enter the oxidized binuclear center in the absence of dioxygen. Since 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*.

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 which follow must 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 trigger a conformational change that directs the electron flow through the pumping site (Cu_A). In studies of the single turnover of the enzyme, Cu_A is always reoxidized 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 are 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 possible 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. 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 the 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 rate 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.

Registry No. H^+, 12408-02-5; cytochrome *c* oxidase, 9001-16-5.

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