Vectorial Chemistry in Bioenergetics: Cytochrome *c* Oxidase as a Redox-Linked Proton Pump

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Chemical reactions normally have directions in time but not in space. This is, however, not true in living systems, where many key processes occur in compartments which are limited by biological membranes. Cells, for example, have a high concentration of K^+, whereas the concentration is low in extracellular fluids; the reverse holds for Na^+. These concentration gradients are maintained by active transport of the cations. Several important physiological processes, such as nerve impulses or muscle contraction, involve changes in concentration gradients across membranes.

The energy necessary to create and maintain concentration gradients across biological membranes is generally derived from the hydrolysis of adenosine triphosphate (ATP), the universal energy currency in living cells. This reaction is catalyzed by membrane-bound ATPases, which are not just enzymes but molecular machines, having evolved the ability to couple the spontaneous hydrolytic reaction to the uphill transport of chemical substances against concentration gradients. This could not go on indefinitely, however, if there did not exist means for de novo synthesis of ATP. Most of this synthesis takes place in light-driven photophosphorylation and oxidative phosphorylation in photosynthesis and cell respiration, the two key processes of bioenergetics.

During the 1950s and 1960s a large number of biochemists were searching for a high-energy phosphorylated intermediate in the ATP synthesis of respiration and photosynthesis, but all these efforts were in vain. Peter Mitchell[1] suggested in 1961 that the simple reason for these failures was that such intermediates do not exist. Instead he formulated his chemiosmotic theory for oxidative and photosynthetic phosphorylation. According to this concept the key intermediate is an electrochemical gradient across the inner mitochondrial or thylakoid membrane. This was proposed to be formed because the electron-transfer reactions between the electron-transport complexes of these membranes were supposed to be coupled to the translocation of protons across the membranes.

Mitchell's idea was not entirely new. In 1945 the Swedish physiologist Lundegårdh[2] had suggested that the redox reactions in the electron-transport chains are anisotropically organized across a membrane, so that protons would be liberated on one side and consumed on the other. The idea that proton gradients drive the synthesis of ATP was new, however. The chemiosmotic theory was initially received with little enthusiasm by biochemists, and the search for the covalent intermediate continued during the 1960s. The situation changed gradually during the early 1970s, as evidence accumulated in favor of the hypothesis. By the middle of the decade most investigators in the field had become convinced that Mitchell was right in principle, if not in detail, and he was awarded the Nobel Prize for chemistry in 1978.

Redox Loops and Proton Pumps

Much of the current research in bioenergetics is concerned with the mechanism of proton translocation in the electron-transport chains, and this will also be the main theme of the present Account. Mitchell's original suggestion[3] was the redox loop, in which there are alternate hydrogen and electron carriers arranged in such a way that hydrogen atoms are transported one way and electrons the other way across the membrane. When the hydrogen atom has been transported to the other side of the membrane, it is split into a proton and an electron. The proton is released, and the electron is transported back to the side on which the hydrogen atom was originally taken up. In this way a net translocation of protons is achieved.

A difficulty with the redox loop as the sole mechanism of proton translocation is that it would lead to a low efficiency of electron-transport-driven ATP synthesis. The H^+/e^- stoichiometry of a redox loop can only be 1, which would mean that only part of the driving force available from the electron-transfer reactions would be utilized for proton translocation. For example, the cytochrome oxidase reaction has a driving force of 0.5 V, whereas the maximum electrochemical potential created in mitochondrial respiration is slightly more than 0.2 V. Thus, with a stoichiometry of 1, less than half of the available energy would be converted into an electrochemical potential. It was consequently gratifying when Wikström[4] established in 1977 that cytochrome oxidase is a redox-driven proton pump, translocating on the average one vectorial proton per electron from the inside (matrix) to the outside (cytosol) of the

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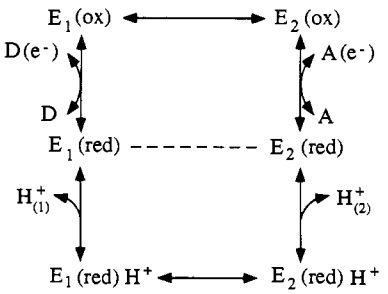

Figure 1. A simple reaction scheme for a redox-linked proton pump. For explanations, see the text.

mitochondrial membrane. He later also showed that the four protons consumed in dioxygen reduction (scalar protons) are specifically taken up from the matrix side,[5] so that an effective H^+/e^- stoichiometry of 2 is achieved.

The purpose of this Account is to describe some current experimental approaches used and results obtained in the elucidation of the mechanism of cytochrome oxidase as a redox-linked proton pump. The focus will be on recent work of my own research group. A key feature in the operation of this type of pump is a structural control of the rates of the electron and proton transfers, and consequently our approach has been mainly kinetic. My students and I have, in particular, studied the kinetics of internal electron transfer, including the reactions involved in dioxygen reduction. The effect of pH and D_2O on the rates has been investigated in attempts to identify those particular electron-transfer steps that are directly coupled to proton translocation.

In order to make my presentation understandable for the general chemical reader, I must first briefly summarize some established knowledge about cytochrome oxidase. This will be done without much documentation, since recent comprehensive reviews are available.[6-8]

Principles of Redox-Linked Proton Pumps

The general principles to which all redox-linked proton pumps must conform can be illustrated with the pump cycle shown in Figure 1. There must be an input state E_1 and an output state E_2, which provide an alternating access of the proton-translocating group to the two sides of the membrane. The driving reaction is an exergonic electron transfer from a donor (D) to an acceptor (A):

$$D(e^-) + A \rightarrow D + A(e^-) \quad (1)$$

If the donor can only react with E_1 and the acceptor with E_2, then the transducer must undergo the transition from E_1 to E_2 for the electron-transfer reaction in eq 1 to be completed. In this way the coupling between the redox reaction and proton translocation is achieved, provided that the reaction along the dashed line in Figure 1 is much slower than that at the bottom of the scheme. If the unprotonated reduced enzyme could undergo the input–output transition (dashed line), then the electron-transfer reaction would take place without proton translocation.

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Cytochrome Oxidase Structure

Mitochondrial cytochrome oxidase contains 12–13 different polypeptides. The functional unit appears to consist of the three heaviest subunits (I–III), and many bacterial oxidases contain these polypeptides only. The four redox centers of the oxidase, Cu_A, cytochrome *a*, and cytochrome a_3–Cu_B, are associated with subunits I and II. Cu_A is bound to subunit II, whereas the other centers are found in subunit I. From hydropathy plots it has been concluded that both polypeptides contain a number of transmembrane helices. Subunit II is anchored to the membrane by two such helices, but the Cu_A site is located outside the membrane. On the basis of spectroscopic properties as well as conserved amino acids in sequences available for several species, it has been suggested that this is a mononuclear site with two cysteine sulfurs and two imidazole nitrogens as ligands. There is an increasing amount of evidence, however, that it is a binuclear Cu(II)–Cu(I) site, in which one unpaired electron is shared equally between the two copper nuclei.[9]

Subunit I is believed to have no fewer than 12 transmembrane helices, but the distribution of six histidines conserved in 25 species suggests that the ligands to all three metals are associated with four of these (II, VI, VII, and X), as shown in Figure 2. Spectroscopic data indicate that cytochrome *a* is coordinated to two histidines, cytochrome a_3 to one histidine, and Cu_B to two or three histidines. A probable identification of these has been made by site-directed mutagenesis.[10] The structure in Figure 2 is far from a unique solution within the constraints of the data. Unfortunately such model building is the best one can do at present, since there are no crystals available allowing the determination of a high-resolution X-ray structure. Both hemes are embedded in the membrane, cytochrome *a* somewhat closer to the outer (cytosol) surface compared to cytochrome a_3, this being the side on which cytochrome *c* interacts with the oxidase. The two heme planes are perpendicular to the membrane plane, but not parallel with each other. The distance between the heme center of cytochrome *a* and Cu_B is 10 Å, whereas that between cytochrome a_3 and the same copper is only 3.5 Å. The distance between the two heme planes is about 12 Å.

The Dioxygen Reduction Cycle

Our knowledge about the mechanism of dioxygen reduction is derived from a number of experimental techniques. Gibson and Greenwood[11] pioneered the flow–flash technique, and this has subsequently been applied extensively. Here the reaction is initiated by photolysis of the CO complex of the fully or partially reduced enzyme in the presence of O_2. The time resolution has in recent years been greatly improved with the introduction of fast lasers. The progress of the reaction has generally been followed by optical absorption measurements, but additional information on the structure of the intermediates has been obtained

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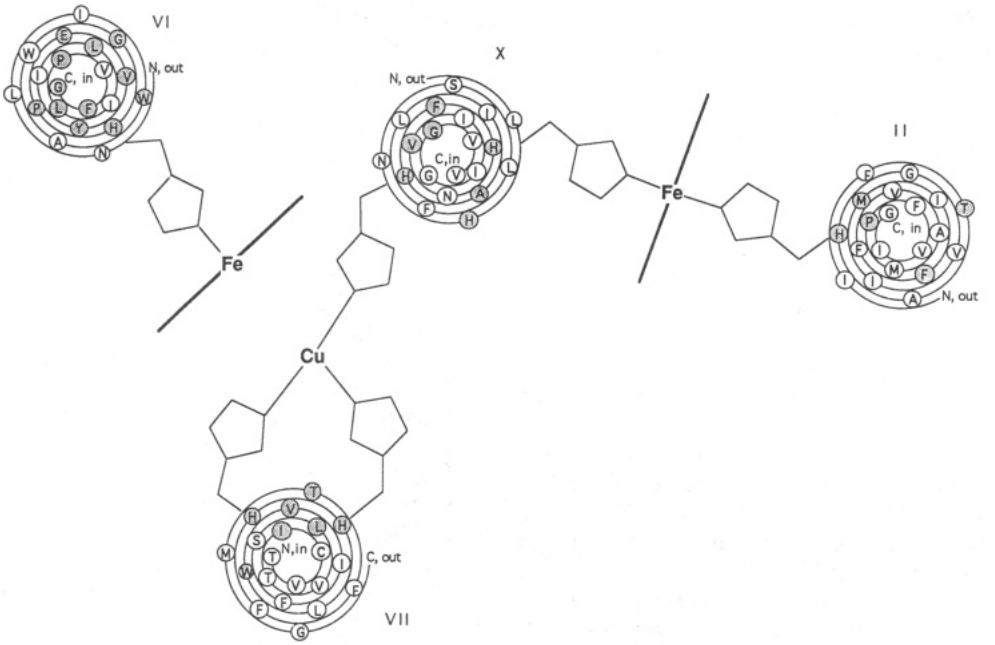

Figure 2. A structural model for the metal-binding sites in subunit I of cytochrome oxidase, showing the transmembrane helices VI, VII, X, and II, which contain the conserved ligand residues. Other conserved amino acids are indicated by shading. For further explanations, see the text. The model was constructed by Dr. Thomas Nilsson on the basis of data provided by Shapleigh et al.[10] and is reproduced with his permission.

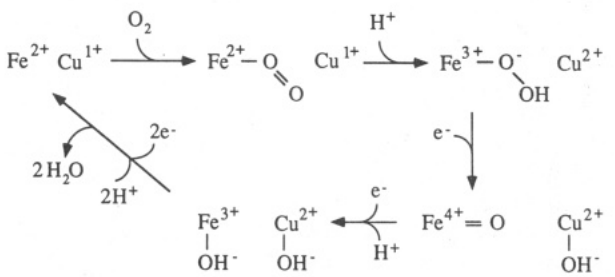

Figure 3. A reaction cycle for the reduction of dioxygen at the binuclear cytochrome a_3–Cu_B site.

by the application of time-resolved resonance-Raman spectroscopy. Another important technique has been low-temperature trapping of intermediates, which allows characterization of these by EPR. An unusual EPR signal was, for example, detected in the three-electron-reduced intermediate, and this was assigned to oxidized Cu_B interacting magnetically with cytochrome a_3 in the ferryl ion form.[12] Finally, significant information has been derived by partial reversal of the reaction in isolated mitochondria.[13]

A proposed reaction cycle for dioxygen reduction is given in Figure 3. A key structural feature is the presence of a binuclear oxygen-reducing site, which is present in all oxidases reducing both atoms of dioxygen to water.[14] It is notable that the closely related quinol oxidases have a binuclear site as well as a second heme but lack Cu_A.[15] One function of the binuclear site is to reduce dioxygen directly to peroxide, thereby overcoming the thermodynamic barrier to a one-electron reduction by a high-potential electron donor. It is generally considered that dioxygen only reacts with the two-electron-reduced site.

As shown in Figure 3, the initial oxygen adduct is converted to the peroxide by electron transfer from both cytochrome a_3 and Cu_B with the concomitant uptake of a proton. This is depicted as involving not a μ-peroxo bridge but binding to cytochrome a_3 only. There is some recent evidence from CO dissociation and recombination experiments that there is a protein ligand which can switch between cytochrome a_3 and Cu_B,[16] and this may prevent the binding of other ligands. In the next step an electron is transferred from cytochrome a with the formation of the ferryl intermediate. EPR experiments with oxygen isotopes[12] have shown that an oxygen atom derived from dioxygen interacts with Cu_B in this intermediate. With the addition of the fourth electron and the uptake of a proton, the binuclear site is reoxidized with the formation of two metal-coordinated hydroxyl ions. Finally, the cycle is completed by the donation of two electrons from cytochrome a/Cu_A, with the uptake of two protons and the release of two molecules of water.

The protons shown in Figure 3 are those involved in water formation (scalar protons) but not the protons translocated in the vectorial reactions. Wikström[17] has provided evidence that proton translocation occurs in two of the four electron-transfer steps only, namely, in going from the peroxide to the ferryl intermediate and from this state to the oxidized binuclear site. Thus, even if there is an average of one vectorial proton per electron, the stoichiometry in those steps that really translocate protons is two protons per electron.

Experimental Approach: Laser Flash Photolysis

The experimental systems used in our flow–flash studies of internal electron-transfer reactions in cyto-

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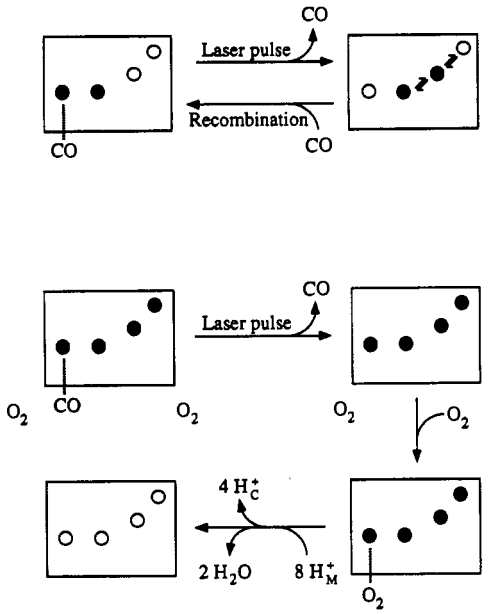

Figure 4. Two types of laser-induced flow-flash experiments. The circles in the boxes represent the four redox centers, from left to right, cytochrome a_3, Cu_B, cytochrome a, and Cu_A. An open circle represents an oxidized center and a filled one a reduced center. The experiment illustrated at the top involves the half-reduced enzyme in the absence of oxygen, whereas the one at the bottom is the reoxidation of the fully reduced oxidase. For further explanations, see the text.

chrome oxidase have been constructed by two of my former graduate students, Peter Brzezinski and Mikael Oliveberg, and are described in detail in their theses.[18,19] Essentially they consist of three sets of components: the laser, a mixing device, and a detection system. Initially we used a flash-lamp pumped dye laser,[20] but more recently an Nd:YAG laser has been employed.[21] We also had access to a detection device at 830 nm, consisting of a diode laser with corresponding photodiode, constructed by Örjan Hansson.[22] This has an unusually good signal-to-noise ratio, which allowed us to detect a new intermediate and also to collect data of a quality forcing a reinterpretation of some earlier results.[23] The laser pulses used to initiate reactions usually had a duration of 9 ns, and the detection devices operated in the microsecond range.

Our experimental systems allow two types of experiments, as illustrated in Figure 4. In the first one CO is flashed off the binuclear site in the partially reduced oxidase (2–3.5 electrons/2 hemes) in the absence of O_2. This initiates a backflow of electrons from the binuclear site, because the potential of cytochrome a_3 drops on CO dissociation. There is substantial negative cooperativity between the redox sites of cytochrome oxidase,[24] and this causes further electron redistribution following the initial backflow.

In the second type of experiment the photolysis is done in the presence of O_2 with the partially or fully reduced oxidase. This initiates progressive oxidation of the redox sites with the concomitant formation of oxygen intermediates. In this type of experiment it is also possible to measure the uptake and release of protons with the solubilized enzyme[25] or with enzyme reconstituted into phospholipid vesicles.[26]

Internal Electron Transfer in the Absence of Oxygen

Boelens et al.[27] have shown that there is a backflow of electrons on photolysis of the CO compound of the half-reduced oxidase, and this was later confirmed by Brzezinski and me.[20] We observed two phases with rate constants of 1.5×10^4 and 500 s^{-1}, respectively, and assigned the first one to electron transfer from the binuclear site to Cu_A and the second one to subsequent electron transfer to cytochrome a. A later investigation,[23] with the faster laser and the improved detection system in the near infrared, led to a reinterpretation, however. A new phase with a rate constant of 2.7×10^5 s^{-1} was found and assigned to electron transfer from the binuclear site to cytochrome a. The previously identified fast phase was reassigned to an equilibrium between Cu_A and cytochrome a. This assignment was strongly supported by the kinetic traces obtained at 830 nm, which had their maximum amplitude with the three-electron-reduced enzyme, because in this form cytochrome a/Cu_A is half-reduced. The slowest phase was suggested to represent a structural rearrangement.

The finding that the redox equilibrium between cytochrome a and Cu_A is slower than that between cytochrome a and the binuclear site suggests that the normal electron-transfer sequence is $c \rightarrow Cu_A \rightarrow a \rightarrow (a_3, Cu_B)$. This agrees with some recent work of Hill[28] and is also supported by studies showing that Cu_A accepts electrons from some external donors faster than cytochrome a.[29,30]

The rates of the two fast phases, which represent reactions that do not pump protons, show little dependence on pH.[20,23] The slowest reaction, on the other hand, does display a considerable pH dependence,[20] and it has been suggested to be the output–input transition.[23] The rate of the equilibrium between Cu_A and cytochrome a has a very small temperature dependence, corresponding to an enthalpy of activation as low as 2.1 kJ mol^{-1}.[20] This is favorable for the function of these two centers, which is to get electrons from cytochrome c quickly into the enzyme.

According to electron-transfer theory,[31] the rate constant for electron transfer (k_{ET}) is given by the

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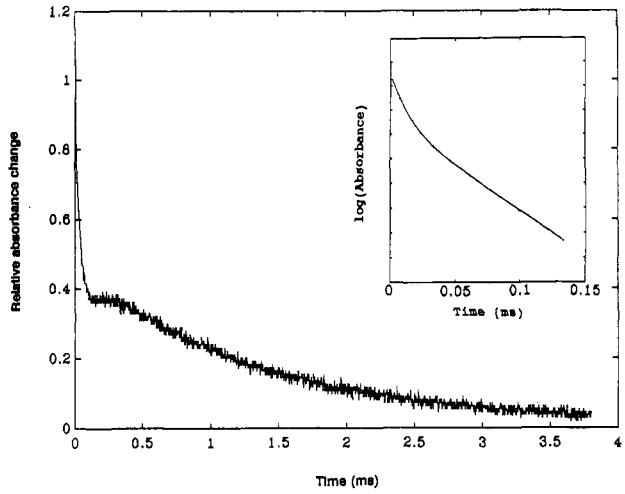

Figure 5. Absorbance changes at 445 nm on photolysis of the fully reduced cytochrome oxidase–CO complex in the presence of 1 mM O_2. Reprinted with permission from ref 21. Copyright 1989 Elsevier Science Publishers BV.

following expression:

$$k_{ET} = \nu \exp[-\beta(d - d_0)] \exp[-(\Delta G° + \lambda)^2/4\lambda RT] \quad (2)$$

In eq 2, ν is a frequency factor, d the distance between the redox centers, d_0 the van der Waals distance, and λ the nuclear reorganization energy. The constant β, which expresses the decrease in electronic coupling with distance, has experimentally been found to have a value of 0.7 Å^{-1} for redox centers in a protein.[32] If one applies eq 2 to the rate constant for the electron transfer between cytochrome *a* and the binuclear center, assuming a value for ν of 10^9 s^{-1}, and with a driving force ($\Delta G°$) of 0.15 eV and a distance of 10 Å, then the reorganization energy becomes 0.6 eV, which is a reasonable value.[32] If the same λ applies to the slow phase, which has a driving force close to 0, k_{ET} becomes 2×10^4 s^{-1}, which is not far from the experimental value (1.3×10^4 s^{-1}). Thus, the observed rates are in good agreement with the enzyme structure and electron-transfer theory, so that our data can be used with confidence for describing the pump mechanism.

The Reactions with Oxygen

Figure 5 shows the absorbance changes at 445 nm, when fully reduced cytochrome oxidase reacts with dioxygen.[21] The initial change is biphasic, as shown in the insert, but the two phases can only be resolved at high concentrations of O_2 (1 mM). Thus, the most rapid reaction is second-order and represents the formation of the initial oxygen adduct (Figure 3). Its pseudo-first-order rate constant is 9×10^4 s^{-1}, corresponding to a second-order rate constant of 10^8 M^{-1} s^{-1}. The three subsequent phases have rate constants of 2.5×10^4, 1.0×10^4, and 800 s^{-1}, respectively, and have been assigned to the successive formation of peroxide, ferryl ion intermediate, and fully oxidized enzyme. The rate constant of the slowest step (800 s^{-1}) is of the same order of magnitude as the maximum turnover (k_{cat}) of the enzyme.

The first three phases can also be observed at 830 nm, as shown in Figure 6.[33] The formation of the oxygen adduct is associated with an absorbance increase at this wavelength. Oliveberg and I have suggested on the basis of this observation that the oxygen molecule first binds to Cu_B and then shifts to cytochrome a_3,[33] which would be in analogy with the events on CO binding.[16] The absorbance decrease in the second phase, which can be observed also in the half-reduced enzyme (curve A), would then represent the shift. In the third phase, Cu_A is oxidized, in agreement with the observation that the amplitude is maximal with the fully reduced enzyme and zero with the half-reduced form (Figure 6).

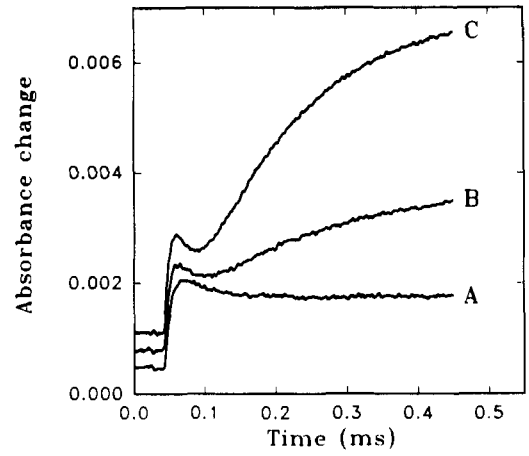

Figure 6. Absorbance changes at 830 nm associated with the same reaction as illustrated in Figure 5. Reprinted with permission from ref 33. Copyright 1992 American Chemical Society.

Transient Kinetics of Proton Pumping

Nilsson et al.[26] have followed the reoxidation of the fully reduced enzyme, which had been reconstituted into phospholipid vesicles. They included a pH indicator (phenol red) in the aqueous phase outside the vesicles and could in this way also measure the rate of proton release, as shown in Figure 7. The proton release is seen to be synchronous with the final step in the reoxidation reaction, which is in agreement with the observation that this is one of the proton-pumping steps.[17] The rate of electron transfer in this phase decreases considerably above pH 8,[21] and it also displays the largest solvent isotope effect of the three electron-transfer reactions, the rate in D_2O being 2.5 times lower than that in H_2O.[34] These results are consistent with this step coupling electron and proton transfers.

No proton release was found in the transition from the peroxide to the ferryl ion intermediate, which is also a proton-pumping reaction. The reason for this could be that the fully reduced enzyme is an artificial species not formed during normal turnover with both reducing substrate and oxygen present. In this situation, the peroxide is likely to be formed before cytochrome *a* is rereduced, whereas with the fully reduced form it is already reduced in the peroxide intermediate. This may allow rapid electron transfer to the binuclear site before the enzyme has picked up

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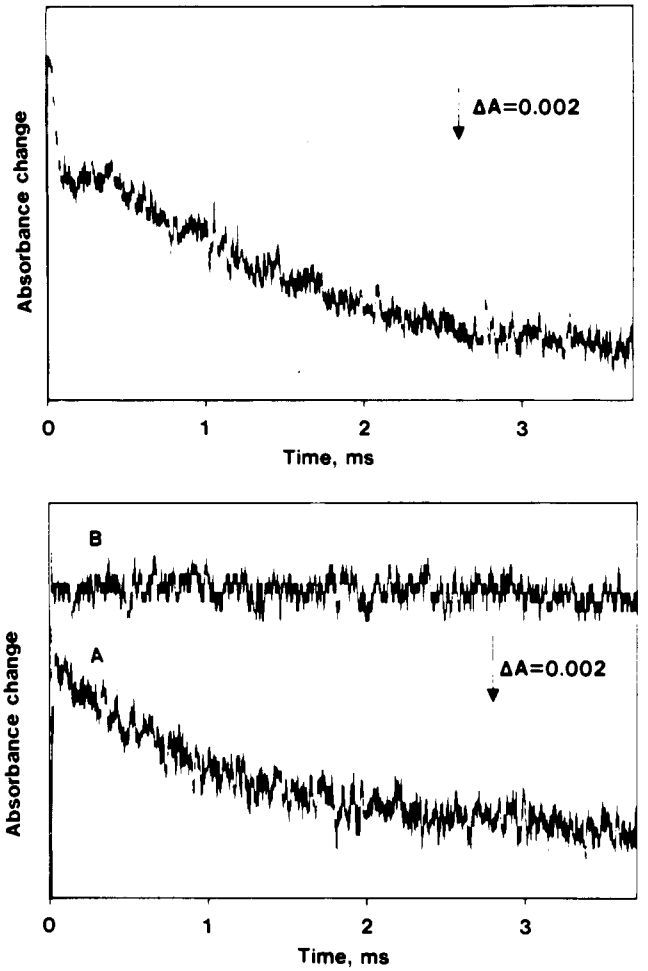

Figure 7. Absorbance changes associated with the reoxidation of the fully reduced oxidase reconstituted into phospholipid vesicles. The top trace was recorded at 605 nm and represents heme oxidation (cf. Figure 5), whereas the bottom traces were measured at 556 nm in the presence (A) and absence (B) of the indicator phenol red outside the vesicles. For further information, see the text. Reprinted with permission from ref 26. Copyright 1990 Elsevier Science Publishers BV.

two protons in the input state, and in this way bypass the proton pump.

A Reaction Sequence for Cytochrome Oxidase as a Redox-Linked Proton Pump

On the basis of our results on internal electron transfer in cytochrome oxidase in various states, Oliveberg and I[33] formulated a turnover cycle for cytochrome oxidase as a redox-linked proton pump, and this is reproduced in a slightly modified form in Figure 8. A central feature of our reaction cycle is that the observed redox interactions between the metal sites[24] are an integral part of the pump mechanism. I had first suggested this in 1985,[35] and later, together with Brzezinski, I proposed that this can also explain the nonhyperbolic steady-state kinetics of cytochrome oxidase.[36] In the input state (*i*) cytochrome *a* has a high potential, whereas that of cytochrome a_3 is low. Thus, cytochrome *a* can receive an electron from cytochrome *c*, but this will not be transferred to the binuclear site. On reduction of Cu_B, following the addition of the second electron from cytochrome *c*, the enzyme switches to the output state (o). In this, cytochrome *a* has a low potential and cannot be reduced by cytochrome *c*, when the concentrations of the two cytochromes are comparable. On the other hand, if the concentration of cytochrome *c* is much higher than that of cytochrome *a*, then electron donation can occur in the output state as well. In this way a second phase, characterized by a high K_m value, is obtained in the steady-state kinetics.

The cycle in Figure 8 starts on the left with the fully oxidized enzyme in the input state. This receives two electrons consecutively from cytochrome *c*, and these electons enter the oxidase via Cu_A, which is in rapid redox equilibrium with cytochrome *a*. These electrons are then transferred to the binuclear site, and the enzyme switches to the output state; the reduction state of Cu_B is assumed to control the input–output transitions.[8] Because the first two electrons are transferred to the binuclear site in rapid succession, the enzyme does not have time to pick up protons before switching to the output state. Thus, no protons are pumped in these reactions.[17]

The reduced binuclear site reacts rapidly with dioxygen. On formation of the peroxide intermediate, the oxidase returns to the input state. It now receives the third electron from cytochrome *c* and picks up two protons. When the electron is transferred to Cu_B, there is again a switch to the output state, and the two protons are released on the cytosol side of the membrane. In the peroxide intermediate there are no electrons on cytochrome *a* and Cu_A, whereas these sites are both reduced when starting from the fully reduced enzyme. This again has the effect that the transition to the output state takes place before the vectorial protons have been picked up, so that with the fully reduced enzyme one of the proton-pumping steps is bypassed.

The steps starting with the ferryl intermediate in the input state are essentially a repeat of the reactions following the formation of the peroxide intermediate, except that the oxygen chemistry at the binuclear site is different (Figure 8). The rate of the final step is not very much higher than k_{cat}. If the previous input–output transition (right-hand side of Figure 8) also has a rate constant of 800 s^{-1}, then k_{cat} would be 400 s^{-1}, provided all other steps are more rapid. The flash-photolysis experiments reviewed earlier show that the other internal electron-transfer steps have rate constants $>10^4$ s^{-1}, so it is reasonable that the two pumping steps provide the rate limitation. Even if the two rate-limiting steps do involve internal electron transfer, which is a common assumption,[7] these would in fact be limited by the rates of the conformational changes in the input–output transitions.

Concluding Remarks

Hopefully this Account has demonstrated that we now know a good deal about the vectorial chemistry of one important reaction in bioenergetics, the oxidation of cytochrome *c* by O_2, catalyzed by cytochrome *c* oxidase. In many ways the situation is disappointing, however. The mechanism I have presented (Figure 8) deals only with the formal kinetics and the chemical nature of the catalytic intermediates, but it provides no information on the molecular mechanism of the proton-translocating reactions. There are several spec-

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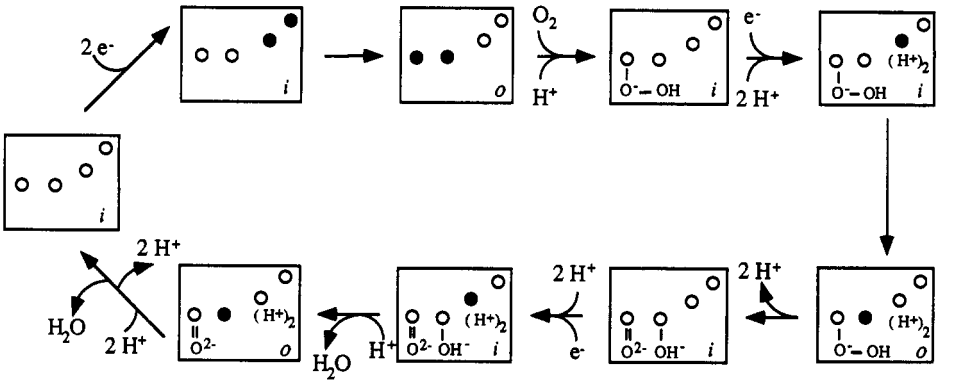

Figure 8. A reaction cycle for cytochrome oxidase as a redox-linked proton pump. The symbols used are the same as in Figure 4. For explanations, see the text.

ulations in the literature, but I will not discuss these, since they lack concrete experimental support. Most authors assume a direct coupling between electron transfer and proton translocation, i.e., that the redox elements also provide the proton-binding groups. The coupling may as well be indirect, however, since the substrates of the catalytic reaction and the species transported, the proton, do not exchange matter, as in a chemical reaction, but free energy only. In this case the proton and the electron would be bound at distant sites, as the substrate and effector in an allosteric enzyme. Indirect coupling definitely occurs in some ATP-driven pumps.[37] We could then only hope to delineate the mechanism, if much more detailed structural information became available.

In conclusion, an even more serious complication must be mentioned. I have assumed here that the species transported is really the proton. It is known, however, that active transport or ATP formation can in some systems be driven by concentration gradients of Na^+, and there are even bioenergetic processes in which either Na^+ or H^+ can operate. This has led Boyer[38] to suggest that the species translocated is actually the hydronium ion and not the proton. In such a case we should not be searching for acid–base groups but for structures of the crown ether type. Again it is apparent that the key to further progress in our understanding of cytochrome oxidase and other redox-linked pumps is real advances in our knowledge of the detailed structures of the proteins involved.

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