Control of Electron Delivery to the Oxygen Reduction Site of Cytochrome *c* Oxidase: A Role for Protons†

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ABSTRACT: We have studied the reaction of oxidized "pulsed" cytochrome *c* oxidase with reduced cytochrome *c* and with ruthenium(II) hexaammine using stopped-flow mixing. The rate of reduction of Fe_{a3} (the oxygen-binding heme) is not a linear function of the population of reduced Fe_a (the low-spin heme), as would be expected if electron transfer between these sites is rate-limiting. Instead, the rate can be increased significantly by increasing the driving force of the reductant (lowering of E_h) even after Fe_a is almost completely reduced. Reduction of Fe_{a3} becomes slower as the pH is raised, and consumption of protons can be seen simultaneously with electron entry into Fe_{a3}. Both the reduction of Fe_{a3} and the proton uptake are biphasic. To explain these findings, we propose a model in which (1) intramolecular heme−heme electron transfer is fast, and has an essentially constant rate; (2) when reduction begins, the midpoint potentials of Fe_{a3} and Cu_B are initially low, and only a small fraction of these centers become reduced; and (3) this reduced population is then stabilized by the uptake of protons. Thus, net reduction of Fe_{a3} and Cu_B is controlled by the amount of the low-potential population which becomes reduced together with the rate of proton uptake by this reduced low-potential species. Important consequences of this mechanism for the function of the enzyme and for the respiratory chain as a whole are discussed.

Cytochrome *c* oxidase[1] is the site at the end of the mitochondrial respiratory chain where oxygen is reduced to water. The energy from this reaction is conserved as an electrochemical proton gradient across the inner mitochondrial membrane, and thus becomes part of the driving force for the formation of ATP. Protons have a central role in this energy conservation mechanism. Formation of water from oxygen requires protons as well as electrons, and the enzyme accomplishes half of all its energy conservation simply by ensuring that these positively and negatively charged species are taken up from opposite sides of the membrane. Pumping of protons accounts for the remaining half of the energy. In all, for every oxygen molecule reduced to water, four protons are consumed from the mitochondrial matrix, and an additional four are pumped from the matrix to the cytosol (Babcock & Wikström, 1992).

While the enzyme accepts electrons from cytochrome *c* at the membrane surface, the chemistry by which oxygen is reduced to water takes place at a site within the membrane (a bimetallic center consisting of a heme and a copper: Fe_{a3} and Cu_B).[2] Movement of electrons from cytochrome *c* to this oxygen reduction site is managed by two additional metal centers, a copper (Cu_A) and a low-spin heme (Fe_a) (Wikström et al., 1981). Cu_A and Fe_a appear to be important in keeping the point to point distances for electron tunnelling workably small. (They may have additional control fucntions, perhaps related to the strong metal−metal redox interactions in the enzyme.) Cu_A lies close to the membrane surface (Rich et al., 1988), and is the point of entry for electrons from cytochrome *c* (Pan et al., 1991). Fe_a is buried in the membrane at about the same depth as the bimetallic oxygen reduction site (Hosler et al., 1993).

The process by which electrons are delivered to the oxygen-binding site has been the subject of a great deal of study (Andréasson et al., 1972; Wilson et al., 1975; Beinert et al., 1976; Antalis & Palmer, 1982; Malatesta et al., 1990; Hendler et al., 1993), both because electron transfer plays such an important role in the enzyme and because these redox processes are spectroscopically visible, and thus more accessible to experiment than, for example, proton movements. The pathway for this electron flow has been relatively well characterized (Hill, 1991); electrons enter the enzyme through Cu_A and move from there to Fe_a, and then to the oxygen reduction site. This is illustrated in Figure 1, in the context of a structural model of the enzyme.

Electron transfer from Cu_A to Fe_a appears to have roughly the same rate under all conditions. Measurements under anaerobic conditions have given (relaxation) time constants of between 35 μs and 60 μs for electron exchange between these two centers (Kobayashi et al., 1989; Morgan et al., 1989; Nilsson, 1992). A time constant of 45 μs has been measured for this step when electrons flow through the enzyme from a photoreductant to a ferryl intermediate at Fe_{a3} (Zaslavsky et al., 1993). Even the 170 μs measured for this

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[1] EC 1.9.3.1; a good introduction can be found in a collection of "mini reviews" edited by S. Ferguson-Miller (Ferguson-Miller, 1993).

[2] Abbreviations: E_h, redox potential; E_m, midpoint redox potential; Fe_a, low-spin heme; Fe_{a3}, oxygen-binding heme; Fe_c, heme of cytochrome *c*; k_{app}, apparent rate constant; SVD, singular value decomposition; τ, kinetic time constant, $\tau = t_{1/e} = t_{1/2}/\ln 2$; TMPD,*N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine.

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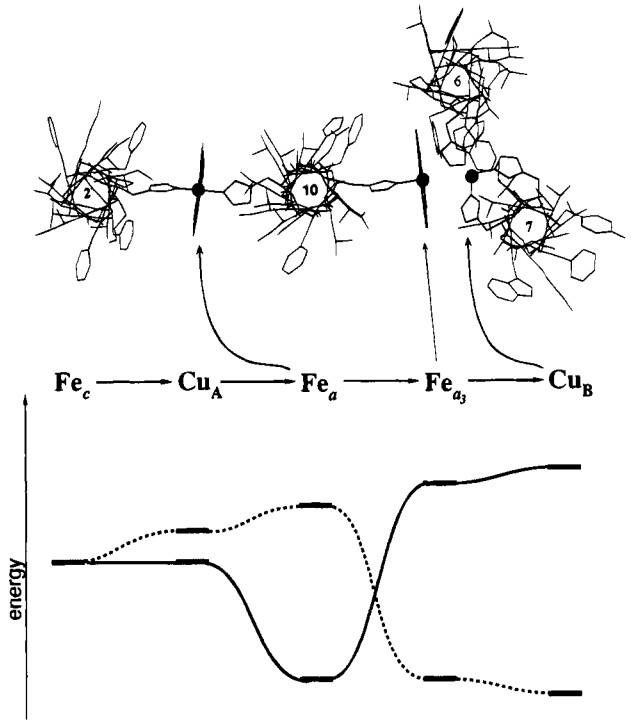

FIGURE 1: Structural and energetic models for electron movement in cytochrome *c* oxidase. The model at the top of the figure is derived from the primary structure of subunit I from the family of heme−copper oxidases. The four transmembrane helices (numbers 2, 6, 7, and 10) which bind Fe_a, Fe_{a3}, and Cu_B (black dots) are indicated, as seen from the top of the membrane. A schematic of the sequence of electron transfer carriers is shown in the center of the figure. (Fe_c and Cu_A which are shown here do not appear in the structural diagram since neither one is part of subunit I.) The lower part of the figure illustrates the energetic relationships among the redox carriers at two different stages of the reduction process. The solid line represents the enzyme at the beginning of the reduction process; Fe_a has the highest E_m in the system while the E_m's of Fe_{a3} and Cu_B are low, prior to proton uptake. The dashed line corresponds to the enzyme after Fe_{a3} and Cu_B have become reduced, and stabilized by proton uptake. Since the *y*-axis quantity is "energy," lower E_m's will appear higher in the figure.

electron transfer when the fully-reduced enzyme reacts with oxygen (Hill, 1991) is not dramatically different.

In contrast, the observed rates for electron redistribution between Fe_a and Fe_{a3} can vary dramatically depending on the circumstances under which the rate is measured. When fully reduced cytochrome *c* oxidase reacts with oxygen (an approximation of the physiological reaction), initial electron movement from Fe_a to Fe_{a3} is observed at 3×10^4/s (Oliveberg et al., 1989; Verkhovsky et al., 1994). Under other experimental conditions, rates have been measured from as slow as 1/s [when electrons enter the oxidized enzyme from cytochrome *c* (Antalis & Palmer, 1982; Sarti et al., 1990)] up to 3×10^5/s [when electron redistribution in the partially reduced enzyme is initiated photochemically (Oliveberg & Malmström, 1991; Verkhovsky et al., 1992)].

One explanation which has been widely accepted [see Malatesta et al. (1990) and references cited therein; Brunori et al., 1994] is that the rate of electron transfer (i.e., of the actual electron tunnelling event) between Fe_a and Fe_{a3} is different at different points in the catalytic cycle and in different forms of the enzyme. It has been suggested that this variability in rate is used by the enzyme to control the availability of electrons to oxygen, and thus to couple electron flow to proton translocation. However, a new generation of structural models, based on site-directed mutagenesis work, places Fe_a and Fe_{a3} at an essentially fixed distance—they are bound by histidine ligands which branch off of the same trans-membrane helix (see Figure 1). This, together with the fact that apparently similar states of the enzyme can show large variations in the rate of electron redistribution between Fe_a and Fe_{a3}, raises questions about the role of electron tunnelling in this variability.

Electron tunnelling rates are influenced by distance, driving force, rearrangement energy, and the nature of the intervening medium (Moser, 1992). The distance and chemical structure between the two hemes appear to be fixed [unless chemical bonds are broken; see Brunori et al. (1994) and Woodruff (1993)]. Large changes in the rate of electron redistribution between Fe_a and Fe_{a3} appear also to take place without corresponding changes in driving force or rearrangement energy; the "pulsed" enzyme, where rates of a few per second are observed (Sarti et al., 1990), does not appear to differ very much in terms of the redox potentials, or the spin and ligation states of the heme irons, from the photolyzed CO mixed-valence enzyme, where a rate of 3×10^5 s^{-1} is seen (Oliveberg & Malmström, 1991; Verkhovsky et al., 1992).

In this paper, we will explore the possibility that variability in the rate of electron redistribution between Fe_a and Fe_{a3} has a different cause. We will attempt to show that the slow reduction of Fe_{a3} by Fe_a, when electrons enter the oxidized enzyme from cytochrome *c*, is not caused by limiting electron tunnelling rates. Instead, it represents a slow electron redistribution within a much faster redox equilibrium. We will propose that net reduction of Fe_{a3} is controlled by a redox potential shift which takes place when the enzyme takes up protons during the reduction process, and that it is the proton uptake which actually limits the rate.

MATERIALS AND METHODS

Enzymes and Reagents. Cytochrome *c* oxidase was isolated from bovine hearts by the procedure of Hartzell-Beinert (1974), modified as described previously (Verkhovsky et al., 1994). Cytochrome *c* (prepared without trichloroacetic acid) was obtained from Sigma and used without further purification. Ruthenium hexaammine [hexaammineruthenium(III) chloride] was obtained from Aldrich and used without further purification.

Stopped-Flow Experiments. Measurements were made using a Unisoku stopped-flow system equipped with the "anaerobic" version of the mixing apparatus. The light detector was a Hamamatsu diode array capable of recording 512 points covering a spectral width of 208 nm, with a minimum interval of 1 ms per spectrum. With an applied air pressure of 5 bars, the mixing dead time was 3−4 ms. When needed, the head space of each syringe reservoir could be flushed either with oxygen-free, water-saturated nitrogen gas or with pure oxygen. The syringe housing itself was flushed with oxygen-free nitrogen, as provided for in the original design of the instrument.

Studies on the re-reduction of cytochrome *c* oxidase were made by mixing a buffer containing oxygen with a solution containing cytochrome *c* oxidase and a reductant, using stopped-flow methods. In addition to the primary electron donor (cytochrome *c* or ruthenium hexaammine), the enzyme samples always included an ultimate reductant (ascorbate or dithionite) which served to maintain the enzyme and the

primary donor in their reduced form prior to mixing. In the case of pH measurements, a substoichiometric amount of NADH was used as the ultimate reductant to reduce the ruthenium hexaammine.

The experiments were designed so that the enzyme would re-oxidize immediately upon mixing, and thereafter re-reduction would take place under anaerobic conditions. This was achieved in one of two different ways: (1) In some experiments (Figures 3, 4, and 7), the amount of oxygen added was smaller than the amount of enzyme. Under these conditions, not all of the enzyme will become oxidized, but at the end of the reaction, no oxygen will remain. Because the concentrations of enzyme and oxygen are similar, the reaction will take longer to come to completion than it would under pseudo-first-order conditions. Given a second-order rate constant of 1.38×10^8 M^{-1} s^{-1} (Orii, 1984), the reaction of 3 μM oxygen with 5 μM cytochrome *c* oxidase (the conditions of Figure 4) will be 90% complete in 5 ms, and 99% complete in 13 ms. (2) In experiments where dithionite was used as the ultimate reductant (Figures 5, 6), an excess of oxygen could be added since the dithionite will consume any oxygen which does not react with the enzyme, rendering the reaction mixture anaerobic within 3−4 ms (Petersen & Cox, 1980). With oxygen in excess, re-oxidation of the enzyme will be extremely fast. For these experiments, air-saturated buffer ($[O_2]$ = 256 μM) was mixed with the dithionite-containing enzyme solution.

In these experiments, the reference levels (zero absorbance) were set by recording the spectrum of the reaction mixture with all constituents in the fully reduced state. In this way, all oxidized components appear as deviations from the zero base line, and their amounts can be determined in spite of the fact that part of the reaction is lost in the mixing time. This fully reduced reference state could be obtained simply by waiting for some minutes after mixing since the samples contain an excess of a reductant, such as ascorbate, which will eventually re-reduce everything. On the time scale of the experiment, however, this final re-reduction is slow. After mixing, there will be a "window" of time in which the re-reduction of the enzyme by the immediate electron donor (for example, cytochrome *c*) can take place with little interference.

Data Analysis. The data from the stopped-flow instrument are a surface of absorbance values covering a time/wavelength plane. These large matrices (512 × 512) were manipulated using Matlab (The Mathworks, South Natick, MA). Three methods were used to decompose these matrices: (a) known spectra of light-absorbing components in the reaction (enzyme chromophores, dyes, etc.) were used as a basis set; (b) a global kinetic fit program (independent executable program run under a Matlab front-end) was used, which assumes that kinetic behavior at all wavelengths can be described in terms of a small number (2−4) of exponential components (Provincer & Vogel, 1983); (c) singular value decomposition (SVD). For the present work where the main task was to extract the time course associated with known components, the first method was primarily used. The basis set spectra for (reduced-minus-oxidized) cytochrome *c*, Fe_a, and Fe_{a3} are shown in Figure 2. They were obtained by routine ligand inhibition methods [see Morgan et al. (1993) and references cited therein]. In the interest of graphical clarity, the spectrum of phenol red is not shown.

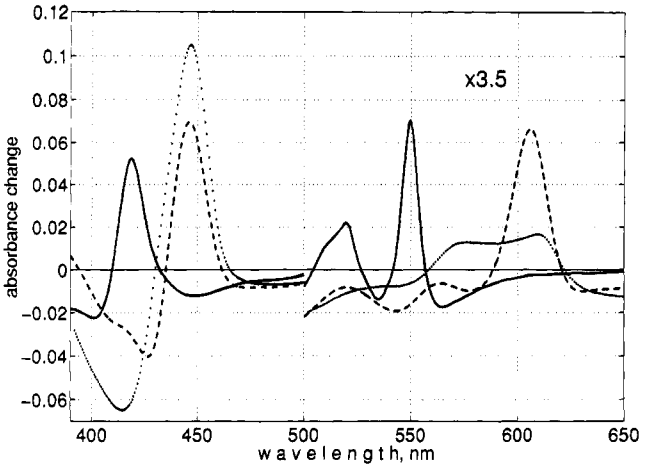

FIGURE 2: Reduced-minus-oxidized spectra used as a basis set for factor analysis of kinetic data. Cytochrome *c*, solid line; Fe_a, dashed line; Fe_{a3}, dotted line; spectra have been normalized to a concentration of 1 μM. (The spectrum of phenol red, used for data decompositions, is not shown.)

Computer Modeling. The structural model of part of subunit I (Figure 1) was made using HyperChem (release 2, Autodesk, Inc.)

RESULTS AND DISCUSSION

The reaction of reduced cytochrome *c* with oxidized cytochrome *c* oxidase is an almost classical experiment in this field. This experiment can be done in the absence of oxygen so that electron flow from the natural donor into the enzyme can be followed. Two innovations have helped us to reexamine this process:

First, we have used a stopped-flow instrument which records the complete spectrum of the system at each (millisecond) time point. With multiple wavelength data, the kinetic behavior of the various components in a complicated system can be separated much more reliably than when only a few wavelengths are measured. In most of the experiments presented here, we have made a factor analysis of the raw absorbance data using a basis set of known component spectra (i.e., the extinction coefficients at each wavelength; Figure 2). The results thus take the form of concentrations of chemical species as a function of time (see Materials and Methods).

Second, these experiments use the active "pulsed" form of the enzyme instead of the less active "resting" form. The "pulsed" enzyme is a fully-oxidized form of the enzyme which occurs transiently after reduction and re-oxidation, as originally discovered by Antonini and co-workers (Antonini et al., 1977). The same research group is also responsible for the elegant experimental technique which allows the "pulsed" enzyme to be used as a starting material for anaerobic re-reduction experiments (Malatesta et al., 1990).

In this method, a sample containing the reduced enzyme *together with* reductant is mixed, using a stopped-flow system, with a substoichiometric amount of oxygen. Roughly within the time of mixing, the oxygen oxidizes an equivalent amount of cytochrome *c* oxidase, resulting in a population of oxidized "pulsed" enzyme. At this point, the reductant, which has been present from the start, can begin to re-reduce the enzyme, filling the holes left behind after the reaction with oxygen. Unlike the initial reaction with oxygen, this re-reduction takes place, at least in part, on a millisecond

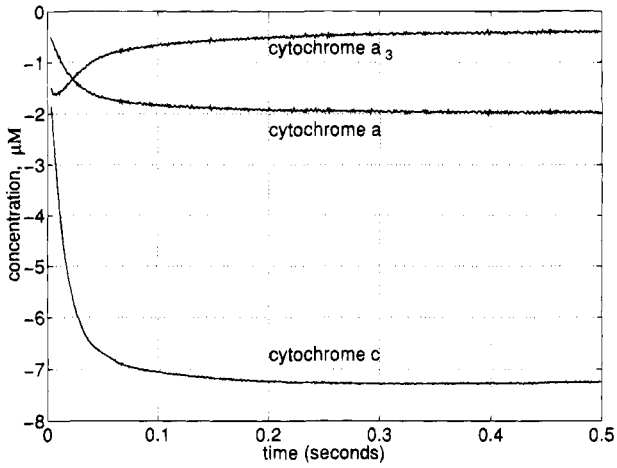

FIGURE 3: Re-reduction of cytochrome *c* oxidase by cytochrome *c*. Time courses for redox states of individual components. Conditions (all concentrations are for after mixing): MES (pH 6.5), 50 mM; KCl, 50 mM; *n*-dodecyl β-D-maltoside, 0.1%; cytochrome *c* oxidase, 5 μM; sodium ascorbate, 1 mM; oxygen, 2 μM; cytochrome *c*, 25 μM.

time scale, and can be observed in the stopped-flow instrument. Three important details of this technique should be pointed out: (1) After the initial reaction of the enzyme with oxygen, the system will be anaerobic, and no further turnover cycles will be possible (see Materials and Methods). (2) The reaction of fully-reduced cytochrome *c* oxidase with oxygen is an all-or-nothing process. The enzyme molecules which do not react with oxygen to become fully oxidized (and "pulsed") will still be fully reduced. There should be no population of partially reduced enzyme. (3) Any fully-reduced enzyme which remains after the oxygen has been consumed is a potential source of electrons as the complete system moves toward redox equilibrium. In fact, this population of enzyme is a relatively powerful reductant. The redox midpoint potential of Fe_a in the fully-reduced enzyme (the "lower-asymptotic" midpoint potential) is about 220 mV (Gibson & Greenwood, 1964), significantly lower than the 260 mV midpoint potential of the enzyme's physiological electron donor, cytochrome *c* (Dutton et al., 1970). This population of low-potential Fe_a can thus play an important role in the redox chemistry these experiments observe, a point we will return to shortly.

Experiments with Cytochrome c as Reductant. We began by studying the re-reduction of cytochrome *c* oxidase by cytochrome *c*. This stopped-flow experiment begins with the enzyme and the cytochrome *c* together in the same syringe, both maintained in their reduced state by a small amount of ascorbate. The other syringe is filled with buffer containing a substoichiometric amount of oxygen, enough to oxidize a part of the enzyme, but insufficient to allow multiple turnovers. Figure 3 shows the results of this experiment (given in terms of concentrations of oxidized Fe_c, Fe_a, and Fe_{a3}; see Materials and Methods). Upon mixing, oxygen oxidizes a fraction of the cytochrome *c* oxidase (approximately 2 μM out of 5 μM). Re-reduction of the enzyme by cytochrome *c* begins immediately. Re-reduction of Fe_a is very rapid: at the end of the dead-time, the major part of Fe_a appears reduced, although a small amount of oxidized Fe_a can be seen. Reduction of Fe_{a3} takes place more slowly. (A small phase in which Fe_{a3} becomes more oxidized can be seen at the beginning of the experiment. This is presumably the tail of the oxygen reaction, which extends beyond the dead-time because the concentrations of cytochrome *c* oxidase and oxygen are similar; see Materials and Methods.) As the reaction proceeds, electrons flow from cytochrome *c* into the enzyme and Fe_{a3} becomes reduced, but at the same time, somewhat surprisingly, Fe_a becomes more oxidized.

The presence of two different populations of enzyme, one oxidized (A) and one reduced (B), after mixing has important consequences for the interpretation of the data. Electron equilibration among Fe_c, Cu_A, and Fe_a is so fast that these centers should reach redox equilibrium in little more than the dead-time (Antalis & Palmer, 1982; Wilms et al., 1981). In the population of enzyme which has just been re-oxidized (A), the redox midpoint potential of Fe_a will be about 390 mV (Blair et al., 1986a), approximately 130 mV higher than that of cytochrome *c* (Dutton et al., 1970). Within the mixing time, electrons from cytochrome *c* should reduce this high-potential Fe_a almost completely. The appearance of 2 μM oxidized Fe_c on this time scale is consistent this interpretation. In the population of enzyme which was not oxidized by oxygen (B), the redox midpoint potential of Fe_a will be about 220 mV. Since this low-potential Fe_a (B) is also in fast redox equilibrium with cytochrome *c*, it can be expected to become partially oxidized by donating electrons to cytochrome *c*, and hence to the other population of Fe_a (A). Hence, at the end of the dead-time, Fe_a in the oxidized population (A) will be largely re-reduced, and essentially all of the oxidized Fe_a seen in the spectrum at this point will be from the low-potential population (B).

As the reaction proceeds, however, Fe_a in the re-reduced population (A) will also begin to reoxidize: Electrons from cytochrome *c* will flow through Cu_A and Fe_a into Fe_{a3} and Cu_B. As Fe_{a3} and Cu_B become reduced, the midpoint potential of Fe_a will drop, due to the strong anticooperative redox interactions between Fe_a and the binuclear center (Fe_{a3} and Cu_B). Reduction of Fe_{a3} will thus cause oxidation of Fe_a in this population of the enzyme (A). (Once Fe_{a3} becomes re-reduced and Fe_a has a low midpoint potential, enzyme from population A should be indistinguishable from population B.) Meanwhile, as Fe_c continues to become more oxidized, the overall E_h in the system will rise, and the low-potential Fe_a in both populations of enzyme will become oxidized, although this effect will be less dramatic that the oxidation caused by the shift of midpoint potential of Fe_a of the re-oxidized population (A).

Focusing only on the population of enzyme which reacts with oxygen, we can describe the following sequence of events: During the mixing time, oxygen reacts with, and oxidizes, this enzyme. (The tail of this reaction can be seen, as described above.) Also, roughly within the mixing time, Fe_a is re-reduced, essentially to completion, by electrons from cytochrome *c*. Now, Fe_{a3} (and presumably Cu_B) begins to become reduced, as electrons flow into the enzyme through Cu_A and Fe_a. Simultaneously, Fe_a begins to re-oxidize because reduction of the binuclear site metal centers brings the anticooperative redox interactions of the enzyme into play.

Reduction of Fe_{a3} is slow compared to reduction of Fe_a. The conventional explanation of this is that electron tunnelling between these centers is slow; if Fe_{a3} is a good electron acceptor, only a slow rate of electron transfer from Fe_a should limit its reduction. However, increasing the concentration of cytochrome *c* in these experiments causes Fe_{a3} to become

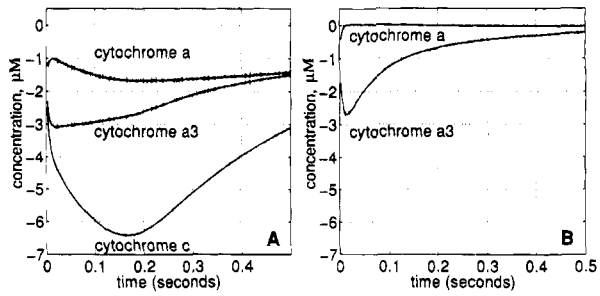

FIGURE 4: Re-reduction of cytochrome *c* oxidase by cytochrome *c* (left panel) and ruthenium hexaammine (right panel). Time courses for redox states of individual components. Conditions (all concentrations are for after mixing): Tricine (pH 8.5), 50 mM; KCl, 50 mM; *n*-dodecyl β-D-maltoside, 0.1%; cytochrome *c* oxidase, 5 μM; sodium ascorbate, 20 mM; oxygen, 3 μM; cytochrome *c*, 25 μM (left panel); ruthenium hexaammine, 2 mM (right panel).

reduced significantly faster [data not shown; see also Sarti et al. (1992)]. This already suggests that the conventional descriptions of the system may be inadequate because, as discussed above (in the relevant population, A), Fe_a is almost fully reduced even at the original, lower, cytochrome *c* concentration.

The key to investigating this question appeared to lie in raising the redox driving force applied. There are practical limitations to the driving force that can be obtained by using cytochrome *c* in these experiments; the redox midpoint potential of cytochrome *c* is 260 mV, which is higher than the lower-asymptotic midpoint potential of Fe_a. Even at fairly high concentrations of cytochrome *c*, as soon as any significant fraction of the donor becomes oxidized, there will not be enough driving force to reduce cytochrome oxidase to completion. We thus needed a reductant which could deliver a higher sustained driving force. Ruthenium hexaammine is an artificial donor which can replace cytochrome *c*. It exhibits slower bimolecular kinetics with cytochrome *c* oxidase than does cytochrome *c* (Scott & Grey, 1980), but has a much lower E_m.

Comparison of Experiments with Cytochrome c and Ruthenium Hexaammine. In Figure 4, parallel experiments with cytochrome *c* and ruthenium hexaammine as donors are compared. These measurements have been made at a higher pH (8.5 instead of 6.5 for Figure 3) which has the effect of slowing down the reduction of Fe_{a3}. The results with cytochrome *c* (Figure 4A) are essentially the same as in the previous figure. Re-reduction of Fe_{a3} is extremely slow, in spite of the fact that Fe_a is substantially reduced. However, the ascorbate concentration in this experiment is high enough for re-reduction of cytochrome *c* to begin, starting at about 180 ms. This lowers the E_h for the first time since the beginning of the experiment. Fe_{a3} begins to be reduced more quickly, and even Fe_a starts to be re-reduced.

When ruthenium hexaammine is used, the results are dramatically different (Figure 4B). Because the bimolecular reaction between ruthenium hexaammine and the enzyme is slower than in the case of cytochrome *c* (Scott & Grey, 1980), initial re-reduction of Fe_a is slower, but with the higher driving force, this process goes essentially to completion, and Fe_a is not re-oxidized once reduced. On the other hand, re-reduction of Fe_{a3} is much faster, than with cytochrome *c*, although Fe_{a3} still becomes reduced more slowly than Fe_a. Here too, the process goes to completion.

These results cannot be reconciled with the idea that reduction of Fe_{a3} is controlled by the rate of electron tunnelling from Fe_a. If this were true, the rate of reduction of Fe_{a3} would be proportional to the population of reduced Fe_a, which is clearly not the case. According to the analysis given above, Fe_a, in the population of enzyme which was oxidized by oxygen (A), will be almost completely re-reduced, both in the cytochrome *c* and in the ruthenium hexaammine experiments. If the actual electron transfer were rate-limiting, the rates of re-reduction of Fe_{a3} with the two different reductants should be almost the same. In fact, they differ by a factor of nearly 25. Even ignoring the different populations of enzyme and taking the spectroscopic data at face value, the total population of Fe_a is more than half-reduced in both systems, and thus these rates should not differ by more than a factor of 2. The fact that such a large difference can be brought about by a relatively small difference in the fraction of Fe_a reduced suggests that the redox potential (E_h) exerts its influence at some other point than in the population of reduced Fe_a.

A Thermodynamic Model for the Reduction of Cytochrome c Oxidase. To explain these findings, we now propose that the slow appearance of reduced Fe_{a3} in the reaction with cytochrome *c* is not due to a rate limitation of electron transfer between Fe_a and Fe_{a3}, but instead reflects a slow shift in the redox equilibrium between Fe_a and Fe_{a3} as the enzyme takes up protons upon reduction.

In this model, the rate of electron transfer (tunnelling) between Fe_a and Fe_{a3} is always fast—fast enough to account for the 3×10^5 s^{-1} rate measured in the "backflow" experiment. However, in the oxidized enzyme, the redox potential of Fe_{a3} is initially very low. When electrons enter the oxidized enzyme, they distribute throughout the enzyme, reaching equilibrium within microseconds. In this equilibrium system, some fraction of these electrons will reside on Fe_{a3}, but because of the low E_m, the subpopulation of reduced Fe_{a3} will be small, perhaps too small to measure. The redox behavior of Cu_B in this system is presumably similar to that of Fe_{a3}. This part of the model is similar to a proposal by Bickar and co-workers (Bickar et al., 1986).

It has been observed that reduction of Fe_{a3} and Cu_B is accompanied by the uptake of protons (Mitchell & Rich, 1994; Hallén et al., 1994). If the small population of enzyme in which low-potential Fe_{a3} is reduced now begins to take up protons, this will have the effect of raising the midpoint potential of Fe_{a3}, and trapping an electron on this center. Thus, if proton uptake is slow, it can control the rate of net reduction of Fe_{a3}. Again, similar behavior would be expected at Cu_B.

This model makes some predictions which are significantly different from earlier models where Fe_{a3} is always a good electron acceptor and where electron transfer was rate-limiting. If electron transfer from Fe_a to Fe_{a3} controls reduction of Fe_{a3}, the rate should be

$$\text{rate} = [Fe_a(II)]k_{\text{electron transfer}} \quad (1)$$

predicting a linear relationship between the reduction level at Fe_a and the rate at which Fe_{a3} becomes reduced. In contrast, if the rate-limiting process is proton uptake by a fraction of enzyme in which Fe_{a3} is reduced, the rate of net

reduction of Fe_{a3} will be

$$\text{rate} = [Fe_{a3}(II)]_{\text{before protonation}} k_{\text{protonation}} \quad (2)$$

where $[Fe_{a3}(II)]_{\text{before protonation}}$ is determined by (i) the (low) E_m of the unprotonated Fe_{a3} and (ii) the redox potential (E_h) of the species in fast equilibrium (Fe_c, Cu_A, Fe_a, and the unprotonated Fe_{a3}).

The energetics of this system are illustrated in Figure 1. (Note that low midpoint redox potential values will appear toward the top of the plot because the *y*-axis is "energy.") The solid curve shows the midpoint redox potentials in the system when the experiment begins, with the enzyme still oxidized. In this state, Fe_a is the best electron acceptor in the system. Fe_{a3} and Cu_B both have relatively low midpoint potentials since no protons have been taken up. As the reaction begins, electrons flow from Fe_c through Cu_A into Fe_a, which becomes essentially completely reduced because of its high E_m. Fe_{a3} and Cu_B are in fast redox equilibrium with the rest of the enzyme, but because of their low E_m's, only a very small subpopulation of these centers becomes reduced. Although small, these populations of reduced Fe_{a3} and Cu_B play a very important role in the reaction, because it is here that proton uptake occurs. This uptake of protons stabilizes the reduced Fe_{a3} and Cu_B, raising their E_m's, and reduced Fe_{a3} and Cu_B begin to accumulate.

Reduction of Fe_{a3} and Cu_B and proton uptake lead to a new energetic situation, which is illustrated by the dashed curve in Figure 1. Now, Fe_{a3} and Cu_B are the best electron acceptors in the system, whereas the midpoint redox potentials of Fe_a and Cu_A have dropped, due to the anticooperative redox interactions. Fe_a is now the lowest potential acceptor in the system, and any electron holes will migrate here. For this reason, Fe_a will begin to be re-oxidized as other metal centers and reduced, unless the driving E_h is extremely low.

The re-reduction of Fe_{a3} in these experiments is always biphasic. This presumably reflects the fact that electrons are flowing to Cu_B as well as to Fe_{a3}, and that there is a relatively strong anticooperative redox interaction between these two centers. This also indicates that the midpoint potentials of Fe_{a3} and Cu_B cannot be very far apart under these conditions. The same biphasicity is also seen, as expected, in the time course of cytochrome *c* oxidation, since that is the source of the electrons entering the binuclear center.

According to the model, the rate-limiting step in the reduction of Fe_{a3} and Cu_B is the uptake of protons which stabilize these metal centers in their reduced forms (eq 2). We thus set out to look for evidence of this, both by studying the pH dependence of the rate of re-reduction of Fe_{a3} and by trying to observe the proton uptake itself.

Figure 5 shows the time course of re-reduction of Fe_{a3} at three pH values in experiments with ruthenium hexaammine as reductant. In these experiments, dithionite was used as the ultimate reductant, and so the added oxygen could be in excess over enzyme (see Materials and Methods). The re-reduction process is biphasic at all pH values studied, but the rates of both phases show the same pH dependence (Figure 6): a 3-fold change per pH unit. A 10-fold change might be expected on theoretical grounds, but our result is consistent with other experimental findings on membrane proteins (Shinkarev et al., 1993). This apparent discrepancy has been explained in terms of acid/base groups (surface

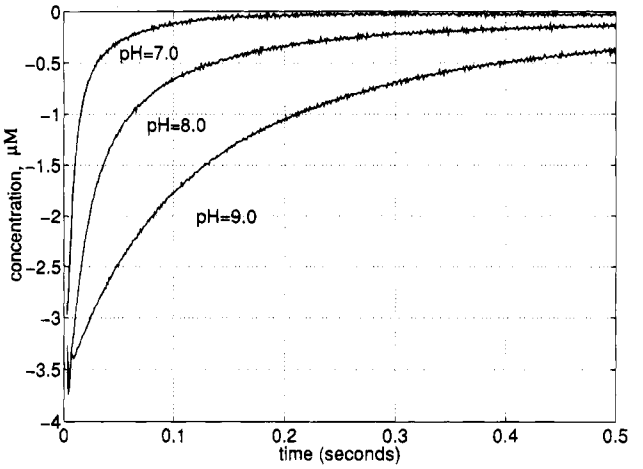

FIGURE 5: Re-reduction of cytochrome *c* oxidase by ruthenium hexaammine. Time courses for re-reduction of Fe_{a3} at different pH values. (The extended tail in the initial re-oxidation of Fe_{a3}, noted in the earlier experiments, is not present here. This is because ascorbate has been replaced by dithionite as reductant, and as a consequence, a much larger amount of oxygen could be used here to re−oxidize the enzyme, since the excess will be consumed by the dithionite; see Materials and Methods.) Conditions: buffers (200 mM), pH 7.0 MOPS, pH 8.0 Tricine, pH 9.0 CHES; *n*-dodecyl β-D-maltoside, 0.1%; ruthenium hexaammine, 5 mM; sodium dithionite, 30 mM; cytochrome *c* oxidase, 5 μM; oxygen, 130 μM.

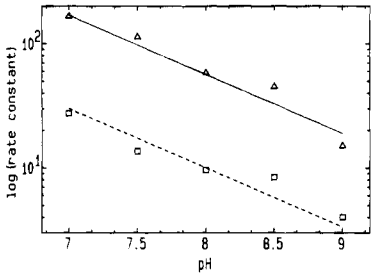

FIGURE 6: Re-reduction of cytochrome *c* oxidase by ruthenium hexaammine. pH dependence of the rate constants for re-reduction of Fe_{a3} (conditions as for Figure 5). In all cases, a two-exponential fit was used to arrive at the two rate constants shown.

charges) in the membrane/detergent environment of the enzyme, which can provide local buffering capacity and thus make the local pH differ from the pH of the bulk solution (Shinkarev et al., 1993).

In order to look for evidence of proton uptake (or release) directly, we carried out a similar experiment but included a pH-sensitive dye in the sample. Since the dye, phenol red, has a different spectrum from any of the redox carriers in the system, the time course of changes in the dye absorbance could be obtained from the kinetic data surface by including the dye spectrum in the basis set of spectra used for the decomposition (see Materials and Methods). In this way, (relative) changes in pH could be tracked at the same time as reduction of Fe_a and Fe_{a3}. As shown in Figure 7, when the enzyme is mixed with oxygen, there is an initial fast proton uptake, presumably due to net proton consumption when the enzyme reacts with oxygen, but following that, proton uptake proceeds with the same time course as the reduction of Fe_{a3}. Fitting a three-exponential function to the dye time course yields rate constants of 224, 61.7, and 10.3 s^{-1} while a similar fit to the Fe_{a3} time course gives 372, 85.0, and 10.4 s^{-1}. Only the two slowest phases are related to re-reduction of the enzyme, and in both cases the redox and protonation events have similar rate constants. The

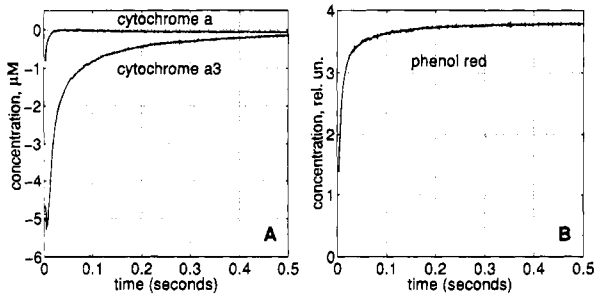

FIGURE 7: Re-reduction of cytochrome *c* oxidase by ruthenium hexaammine. Time courses for re-reduction of Fe_a and Fe_{a3} (left panel) and for phenol red absorbance (right panel). Conditions as in Figure 4 except that essentially no buffer was present, having been removed chromatographically, and that ruthenium hexaammine was prereduced by a substoichiometric amount of NADH; [phenol red], 10 μM.

fastest phase in each case is connected to oxidation by oxygen. Neither fast phase will be well-fit since both take place largely within the measurement dead-time.

Not only do we observe electron redistribution and proton uptake, we can also see a clear interaction between these two. Thus, the redox driving force and the availability of protons both influence the kinetics of reduction of Fe_{a3} in ways that would not be expected if the rate was controlled by heme−heme electron tunnelling, but as would be expected from eq 2.

Structural Considerations. Current structural ideas about the enzyme are consistent with the fastest rates which have been measured for Fe_a to Fe_{a3} electron transfer. Site-directed mutagenesis studies have led to a model in which Fe_a and Fe_{a3} are ligated by two histidines separated by only one intervening residue in the sequence (Figure 1). In the structure, this means that Fe_a and Fe_{a3} lie on opposite sides of the same transmembrane helix and that there is a direct pathway, through bonds, from one iron to the other. The metal-to-metal distance is about 16 Å, which is what would be expected for an electron transfer time constant of 3 μs (Verkhovsky et al., 1992).

One alternative explanation for slow reduction of Fe_{a3} which has recently been put forward is that under some circumstances an axial ligand exchange at Fe_{a3} breaks the iron−histidine bond. Such a disruption of a through-bond pathway might be expected to slow electron transfer considerably (Woodruff, 1993; Brunori et al., 1994). While our results do not address this issue directly, they show clearly that slow electron tunnelling is not responsible for the kinetics of reduction of Fe_{a3}.

Reduction of Fe_{a3} in "Resting" Cytochrome c Oxidase. As described earlier, the starting point for all of the present experiments was "pulsed" cytochrome *c* oxidase, an active oxidized form of the enzyme. The "pulsed" enzyme is obtained by complete reduction and re-oxidation of the enzyme. It is not stable but reverts to a slower "resting" form in a matter of minutes, unless there is continued enzyme turnover (reduction and re-oxidation). Prior to the discovery of the "pulsed" enzyme (Antonini et al., 1977), many experiments were done with the "resting" enzyme to study the kinetics of its reduction by cytochrome *c*. The arrival of electrons at Fe_{a3} in the "resting" enzyme is even slower [see, for example, Petersen and Cox (1980)], but here too it seems likely that the slow reduction is not due to slow electron transfer rates per se, but to an initially low midpoint redox potential of Fe_{a3} which slowly shifts to a higher potential. Some support for this can be found in the literature. Beinert and co-workers (Beinert et al., 1976) studied the reduction of the "resting" enzyme by ferrocytochrome *c*, using rapid-freezing and EPR spectroscopy, and found evidence that some electrons reached the binuclear site of the enzyme within the first few milliseconds of the reaction. They report that a small but significant population of Cu_B was rapidly reduced, as evidenced by the appearance of an EPR signal at $g = 6$, a resonance assigned to Fe_{a3}(III). In the fully-oxidized enzyme, Fe_{a3} and Cu_B interact antiferromagnetically with the result that no EPR signal is produced. Reduction of Cu_B breaks this coupling, allowing the $g = 6$ signal from Fe_{a3} to be seen (Wikström et al., 1981). Thus, electrons from cytochrome *c* are able to reach the oxygen reduction site rapidly even in the "resting" enzyme, but the redox potentials of Fe_{a3} and Cu_B are unfavorable for reduction. The slow net reduction of the site in the "resting" enzyme presumably reflects a shift in redox equilibrium, as it does in the "pulsed" enzyme. It has often been suggested that in the "resting" enzyme, there is an inert ligand at the distal site of Fe_{a3} [see Wikström et al. (1981)]. Such a ligand might cause reduction to be slow, either by lowering the redox potential of the site or by slowing protonation or both.

With the "pulsed" enzyme, reduction of Fe_{a3} is faster. In the present experiments, when cytochrome *c* was used as the donor, the fast phase of reduction of Fe_{a3} had a rate of 85 (electrons) s^{-1} (at pH 7.0). This is still too slow to account for turnover numbers in the range of 150 (electrons) s^{-1}, reported under similar conditions in the spectrophotometric assay (pH 7.0, 20 °C, with lauryl maltoside; Robinson et al., 1985). Our results and model (above) go some ways toward explaining this discrepancy. In the model, the rate of net reduction of Fe_{a3} depends on the extent to which the low-potential form of Fe_{a3} becomes reduced prior to protonation, and is thus a sensitive function of E_h. When catalytic turnover is measured, either in the spectrophotometric or in the polarographic assay, it is possible to maintain a large, and essentially constant, driving force (a low E_h). In the spectrophotometric assay, only a catalytic amount of cytochrome *c* oxidase is needed, and ferrocytochrome *c* is typically present in more than 10^4-fold excess (Robinson, 1982). At the time when the initial rate of the reaction is measured, the cytochrome *c* is essentially completely reduced. In the polarographic assay, not only is there a similarly large excess of ferrocytochrome *c* but also ascorbate and a mediator (TMPD) can be added to maintain the cytochrome *c* in the reduced state (Ferguson-Miller et al., 1976).

In contrast, the present ("electron counting") experiments call for both cytochrome *c* and cytochrome *c* oxidase to be monitored spectroscopically, which means that no large excess of the donor can be used. In the experiment in Figure 3, about one-third of the cytochrome *c* becomes oxidized. E_h rises very rapidly at the beginning of the reaction as the cytochrome *c* begins to become oxidized. When ruthenium hexaammine is the electron donor, a higher driving force is maintained, and the fast phase of reduction of Fe_{a3} has a rate of 165 (electrons) s^{-1} (at pH 7.0), presumably because with the lower applied E_h a larger subpopulation of low-potential Fe_{a3} is reduced prior to protonation. This is close to the turnover rate measured in the spectrophotometric assay at this pH (above).

Mechanistic Implications of the Model. We can now consider the catalytic cycle of the enzyme in light of our model for the reductive process. In the oxidized enzyme, the metals of the oxygen reduction site have relatively low midpoint redox potentials. Electron tunnelling rates to all points in the enzyme are high and remain high throughout the cycle. The cycle begins when electrons enter from cytochrome *c*. Electrons equilibrate rapidly throughout the enzyme, but the electron occupancies at Fe_{a3} and Cu_B are relatively small, because these centers have low midpoint redox potentials. This begins to change because the enzyme population in which Fe_{a3} or Cu_B are reduced begins to take up protons, raising the E_m at these sites, causing the populations of reduced Fe_{a3} and Cu_B to increase. Since electron transfer is fast, proton uptake is rate-limiting for this process.

Oxygen can bind and begin to react only when both Fe_{a3} and Cu_B are reduced, but the probability of such two-electron reduction is very small until at least one proton has been taken up. (The anticooperative redox interaction between Fe_{a3} and Cu_B decreases this probability even below the simple cross section.) This ensures that the oxygen reaction will not begin without the uptake of at least one proton. In effect, the enzyme has created a thermodynamic barrier which must be crossed before the start of the oxygen reaction (see Figure 1). Electrons cannot cross this barrier, except at a large energetic cost, unless they are "accompanied" by protons. However, once the oxygen reaction begins, the barrier is no longer there, and fast electron transfer rates ensure that electrons will be available to complete the reaction, even though the donor redox potential may not be very low.

This mechanism allows the oxidative part of the cycle to proceed quickly and smoothly even when net enzyme turnover is slow. The chemistry by which oxygen is reduced to water can potentially pass through several intermediates which could be dangerous to the cell. When the enzyme turns over at speed, the mechanism can pass rapidly through the steps where hazardous intermediates occur, minimizing the probability that they will be released. This would not necessarily be the case when turnover is slow. Cytochrome *c* is a one-electron donor, and oxygen is a four-electron acceptor. When the supply of electrons is limited, there is the possibility that the oxygen reaction could begin without enough electrons being available to complete it, and the reaction could become trapped with a species such as a ferryl or peroxide at the oxygen reduction site.

The low midpoint redox potentials of unprotonated Fe_{a3} and Cu_B allow control of enzyme turnover to be maintained. If enzyme turnover begins to deplete the electron donor pool, the E_h will rise, Fe_{a3} and Cu_B will be more oxidized, and enzyme turnover will slow down. Thus, enzyme turnover is never allowed to proceed to the point where the donor pool is seriously depleted of electrons. Because of this, when oxygen does react with a given enzyme molecule, there are always electrons available in the donor pool and the fast electron transfer rates ensure that reduction of the oxgen to water can be completed quickly. Thus, even when net turnover is slow, the steps between oxygen binding and the formation of water will proceed fast enough to prevent the release of hazardous intermediates.

In fact, this may allow the enzyme to move through the oxidative part of the cycle at approximately the same pace over a very wide range of net turnover rates. Since the steps in the cycle from peroxide to water are responsible for most of the energy conservation process in the enzyme (Wikström, 1989), the ability to maintain consistent timing during this part of the reaction might be important for coupling electron transfer to proton translocation.

The low midpoint redox potentials of unprotonated Fe_{a3} and Cu_B maintain the redox potential in the electron input pool by preventing excessive turnover. This explains the finding that k_{cat} of cytochrome oxidase increases continuously as the pH is lowered (Thörnström et al., 1984). Control of electron input into this pool is also important, not only for cytochrome *c* oxidase but also for the dynamics of the respiratory chain as a whole. The Rieske Fe-S center appears to be the most important element in this control. This electron carrier is situated in the output side of the cytochrome bc_1 complex and controls the oxidation of quinol in the Q-cycle [see Mitchell (1976)]. In the Q-cycle, quinol can become oxidized only when the Rieske Fe-S center is able to take the first of the two electrons (the second is transferred to the low-potential cytochrome *b* center). Rieske Fe-S has a midpoint potential higher than that of the donor pool for cytochrome *c* oxidase (cytochrome c_1 and cytochrome *c*), and will thus remain reduced until the E_h in this pool rises, due to electron demand from cytochrome *c* oxidase. Upon oxidation, the Rieske Fe-S center will immediately be re-reduced by the Q-cycle. Thus, the Rieske center behaves as a redox potential-sensitive gate, maintaining a low E_h upstream of this point and a high E_h downstream. Importantly, even though the downstream E_h is high, electrons can be provided quickly as needed for the cytochrome *c* oxidase reaction.

The Rieske center thus partitions the redox potential drop between the Q-cycle in cytochrome bc_1 and the oxygen reaction in cytochrome *c* oxidase. This partitioning is important for a number of reasons: If the redox potential at cytochrome *c* were allowed to become too high, cytochrome *c* oxidase would not be able to function, or would operate only at some risk to the cell. On the other hand, if the redox potential at cytochrome *c* oxidase were to become too low, there would not be a sufficient energy drop across cytochrome bc_1 for the Q-cycle to operate. In addition, an excessively low redox potential at cytochrome *c* could cause uncoupling in the cytochrome *c* oxidase reaction. On the basic of the model we have presented, if the electron donor redox potential is sufficiently low, both Fe_{a3} and Cu_B might become reduced and the oxygen reaction begin before protons could be taken up.

The fact that the electron population does not move before protonation is an effective "gating" (Malmström, 1985; Blair et al., 1986b) of electron transfer by proton transfer, even though it does not involve changes in the actual rate of electron tunnelling. The uptake of protons here is almost certainly a part of the energy transduction mechanism of the enzyme. Although we do not know the exact mechanistic role of protons taken up at this stage, we can speculate that one or both of them will be used in the proton pump. Efficient proton pumping is likely to require the protons to be preloaded into a pump site before the driving redox event takes place. In a model of proton pumping recently published by our group (Wikström et al., 1994; Morgan et al., 1994), the uptake of scalar protons (the ones which become part of the water) provides energy directly to displace the pumped protons from a carrier site in the enzyme. The

protons taken up in the reduction process studied here represent only a fraction of the total number required for one turnover, but their behavior may provide a window into a more general energetic coupling mechanism.

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