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COUPLED PROTON AND ELECTRON TRANSFER REACTIONS IN CYTOCHROME OXIDASE

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1. ABSTRACT

Cytochrome oxidase catalyzes the four-electron reduction of O_2 to water and conserves the substantial free energy of the reaction in the form of a protonmotive force. For each electron, two full charges are translocated across the membrane, resulting in a voltage. One of the mechanisms to generate the charge separation in cytochrome oxidase is *via* a proton pump. A single reaction cycle can be monitored over the course of about 1 msec using absorption spectroscopy, revealing distinct intermediates. Thus, the reaction cycle can be studied as a series of steps. Each of the reaction steps in the catalytic cycle involves a sequence of coupled electron and proton transfer reaction, where protons are either consumed in the chemistry of water formation or pumped across the membrane. The pumping mechanism requires consideration of both the thermodynamics of the various species but also the favored kinetic pathways that assure proton pumping is unidirectional. Hence, a knowledge of transition states and transiently, poorly populated intermediates is likely to be important to understand the mechanism of the pump.

2. INTRODUCTION

Cytochrome oxidase catalyzes the conversion of O_2 to 2 H_2O by aerobic organisms as the terminal reaction of the respiratory chain (1). The reaction is highly exergonic and free energy is conserved and tranformed to a biologically useful form by generating a proton electrochemical gradient across the membrane in which the enzyme is located. The eukaryotic oxidases are located in the mitochondrial inner membrane. There are many prokaryotic oxidases that are homologues of the mitochondrial enzyme (2), and these prokaryotic oxidases span the bacterial cytoplasmic membrane. A schematic of the enzyme is shown in Figure 1. The enzyme active site is a bimetallic center consisting of heme a_3 and Cu_B, buried within the protein; hence, these enzymes are called heme/copper oxidases. The chemical reaction requires 4 electrons and 4 protons plus O_2. Electron transfer to the heme a_3 /Cu_B center from the cytochrome c substrate is facilitated by Cu_A and heme a (see Figure 1). Heme a is a six-coordinated heme with two histidine axial ligands. This heme is either in the reduced (Fe^{2+}) or oxidized (Fe^{3+}) form. The electrons and protons used in the chemical reaction originate from opposite sides of the membrane. Protons are delivered to the active site *via* proton-conducting channels. By utilizing electrons and protons from opposite sides of the membrane (Figure 1), the enzyme moves the equivalent of one positive charge across the membrane per electron from the inside (electrically negative, bacterial cytoplasm or mitochondrial matrix) to the outside (electrically positive). This generates a voltage across the membrane (3).

In addition to this charge separation, the oxidase is also a proton pump. For each electron, one additional proton is translocated across the membrane from the inside to the outside (3). Hence, for each electron, two full charges are moved across the membrane (Figure 1), conserving the free energy from the chemical reaction in the form of a chemiosmotic gradient. The net reaction is the following.

$$4\ \text{cyt}\ c_{red} + 8\ H^+_{in} + O_2 \rightarrow 4\ \text{cyt}\ c_{ox} + 2\ H_2O + 4\ H^+_{out}$$

Four of the protons taken up by the enzyme from the inside are "chemical" protons, consumed to generate water, and another four protons are "pumped" protons, delivered across the membrane. Controlling the timing and pathways of proton transfers is critical to the proper function of the enzyme.

2.1. The active site

X-ray structures are available for cytochrome oxidase from several sources(4-10) (11). The heme a_3/Cu_B center from the *R. sphaeroides* oxidase is shown in Figure

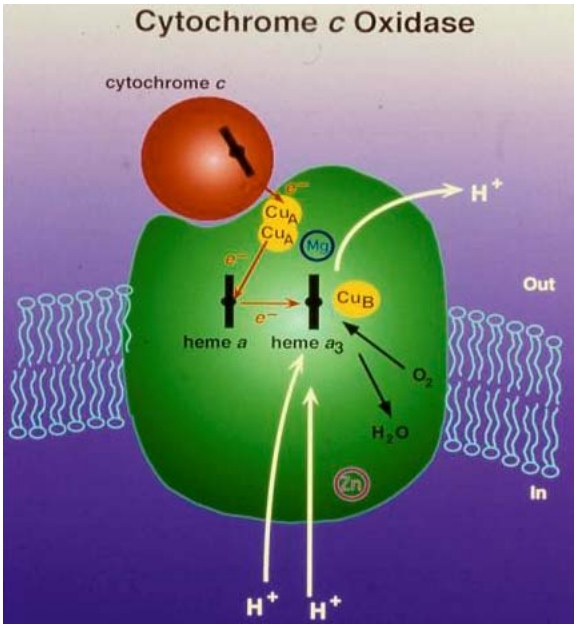

Figure 1. A schematic of cytochrome oxidase. The membrane separates the electrically negative aqueous phase (bacterial cytoplasm or mitochondrial matrix) from the "outside" electrically positive phase. Cytochrome c donates electrons from the "outside" via Cu_A and heme a to the heme a_3/Cu_B center. Protons from the opposite side of the membrane are destined to be consumed in generating water (chemical protons) or are pumped across the membrane. (Figure kindly provided by Dr. Shelagh Ferguson-Miller.)

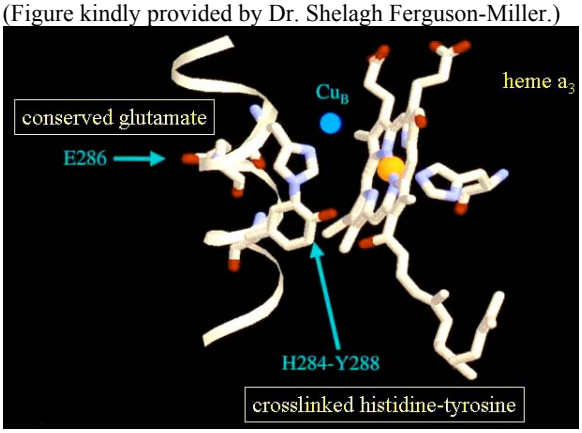

Figure 2. The heme a_3/Cu_B active site of the *R. sphaeroides* cytochrome oxidase. The cross-linked H284/Y288 pair is shown, along with E286. The tyrosine and glutamate are terminal residues of the K-channel and D-channel, respectively, and are internal proton donors during the catalytic cycle.

2. The copper is located about 4.5 Å from the heme iron and is ligated by three histidines, only one of which is shown in Figure 2. Of particular interest is the post-translational modification in which a one of the histidines ligating Cu_B is crosslinked to a tyrosine (H284/Y288 in the *R. sphaeroides* oxidase). The tyrosine is in position to donate either a proton or hydrogen atom to facilitate O-O bond cleavage and is an essential residue for catalysis (12-15).

2.2. Two channels facilitate proton transfer from solution to the active site

The arrows in Figure 2 point to the two residues that are thought to be proton donors during the reaction cycle. One is the tyrosine (Y288), mentioned above, and the second is glutamate E286. These residues are each terminal amino acid residues of channels that facilitate proton transfer from the "inside" aqueous phase. These two channels are named the D-channel and the K-channel, pictured in Figure 3. The entrance of the K-channel is a glutamate at the surface of subunit II ($E101^{II}$) (16, 17), and the entrance of the D-channel is aspartate D132 in subunit I. The two channels are functional during different steps in the catalytic mechanism (next Section), as shown by the examination of mutants. Proton transfer occurs by a Grotthuss mechanism, essentially by a series of steps, each of which involves a proton shift from a hydrogen bond donor to an acceptor (18, 19). Internal water molecules can act as the hydrogen bond donors and acceptors for this process. The D-channel contains a string of water molecules, observed in the X-ray structures, linking D132 to E286 (Figure 3). Other internal water molecules are inferred to be present in cavities (20-22), and are either disordered (and therefore not observable in the crystal structures) or are transient, or are not present in all states of the enzyme. For example, whereas it is certain that E286 is a source of protons transferred to the active site (about 12 Å away), there is no hydrogen bond pathway observable in the X-ray structures for this proton transfer. Molecular dynamics, however, suggests that water molecules will move into this region and form a hydrogen bonded pathway connecting E286 to the heme a_3 /Cu_B center, at least transiently (22). Another point worth noting is that the movement of E286 side chain may also be part of the dynamics of proton transfer, picking up a protons from a donor and then shifting position to deliver the proton to an acceptor (20, 21).

2.3. The Reaction Cycle

Oxygen is reduced to water in a series of steps (1), illustrated in **Figure 4A.** The states of the enzyme are shown in bold type (**O, E, R_2, P_m** and **F)** and, by convention, indicate the status of the heme a_3 /Cu_B center. The oxidized state, **O**, for example, contains Cu_B^{2+} and heme a Fe^{3+}. Reduction of the heme a_3 /Cu_B center by one or two electrons yields states **E** and **R_2**, respectively. Experimentally, electron transfer into the heme a_3/Cu_B site is always accompanied by the net uptake of one proton by the enzyme for charge compensation(23). Depending on the step in the mechanism, this proton is taken up *via* either the K-channel or the D-channel.

One can also have the enzyme in the fully oxidized and fully reduced (**R_4**) states, meaning that all four of the redox metals are either oxidized or reduced, respectively. In Figure 4B, the sequence of reactions observed when O_2 reacts with the fully reduced enzyme is shown. Note that the reaction steps shown in Figure 4A presume that heme a is oxidized (Fe^{3+}) when O_2 reactions

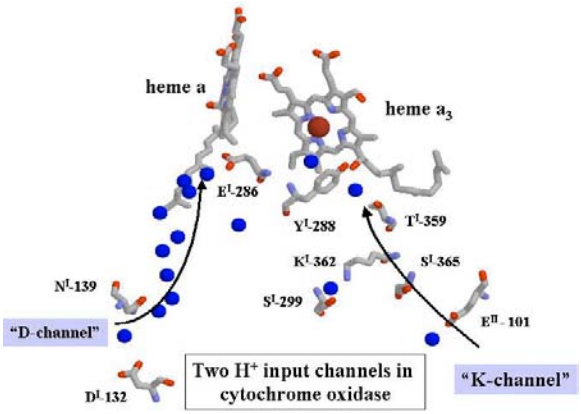

Figure 3. The key residues in the K-channel and D-channel within subunit I of the *R. sphaeroides* oxidase. Internal water molecules resolved in the X-ray structure of the *R. sphaeroides* oxidase are also shown. The large atom is Cu_B. Note the proximity of heme a, on the left, to heme a_3, on the right.

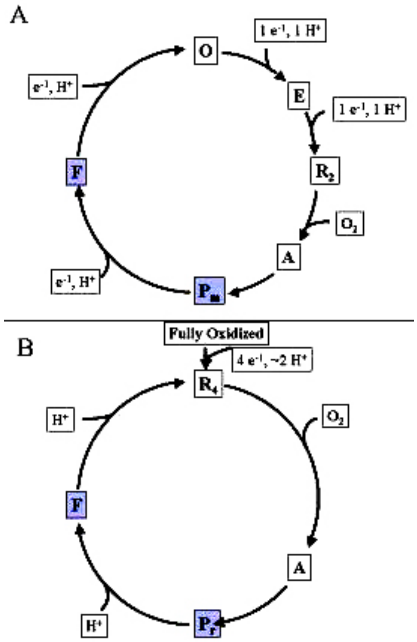

Figure 4. Schematic catalytic cycles of cytochrome oxidase. Part A depicts the cycle starting from the fully oxidized enzyme, in which electrons are added one at a time. Only the status of the heme a_3 /Cu_B center is indicated. Part B depicts the reaction of the fully reduced enzyme with O_2. Approximately 2 (depending on pH) protons are taken up by the enzyme upon full reduction.

with the heme a_3 /Cu_B center, whereas in Figure 4B, heme a is reduced (Fe^{2+}). A summary of the reaction steps shown in Figure 4A follows.

O→E

The one-electron reduced enzyme is referred to as the "E" state. The first electron transferred to the heme a_3 /Cu_B center is accompanied by a proton delivered via the K-channel (16, 24-28). Mutants disrupting the K-channel dramatically slow or block reduction of the heme a_3 /Cu_B center (e.g., K362M). The proton delivered almost certainly converts a hydroxide bound at the binuclear center to water.

E→R_2

The second electron delivered to the binuclear center also requires a proton to be taken up from solution for charge compensation. The experimental evidence is not definitive, but it appears that this may also be *via* the K-channel (29). It is not known where this proton resides within the protein.

R_2→A→P

Once the enzyme is reduced by two electrons, O_2 can bind and react. After forming an initial complex with reduced heme a_3 (called state **A**), the O-O bond is split, forming an intermediate (state **P**). Four electrons must be delivered to O_2 to split the O-O bond. Two electrons come from the heme a_3 iron (Fe^{2+}→Fe^{4+}), one from Cu_B (Cu^{+}→Cu^{2+}) and the fourth electron can come from one of two sources, depending on whether heme a is oxidized (Figure 4A) or reduced (Figure 4B). If heme a is reduced at the time O_2 reacts at the heme a_3 /Cu_B center, as in Figure 4B, the fourth electron is provided by heme a, which becomes oxidized. In this case the **P** intermediate is designated $\mathbf{P_r}$ (30). This state of the enzyme is observable only transiently since it has excess negative charge at the binuclear center. A proton is transferred from E286 within about 120 μsec, forming the next intermediate ($\mathbf{P_r}$→**F** transition in Figure 4B).

If heme a is oxidized and the "fourth" electron is not available from this source, as in the sequence in Figure 4A, then the reaction proceeds, several-fold slower, by taking an electron from the active site tyrosine (Y288; see Figure 2), along with a proton, forming a tyrosyl radical (12-14). Whether $\mathbf{P_r}$ or $\mathbf{P_m}$ is formed, Y288 provides a proton to the distal oxygen atom of O_2 (i.e., the oxygen atom not bonded to the heme a_3 Fe^{2+}), forming hydroxide. The proximal oxygen atom remains associated with the heme a_3 iron as an oxoferryl species (Fe^{4+}=O^{2-}) in both $\mathbf{P_r}$ and $\mathbf{P_m}$ (14, 30). Note that species $\mathbf{P_m}$ results from a chemical rearrangement at the enzyme active site and is a relatively stable species, whereas species $\mathbf{P_r}$ results from electron transfer from heme a into the heme a_3 /Cu_B center and exists only transiently until a charge-compensating proton from E286 is delivered.

P→F

If the intermediate formed is $\mathbf{P_r}$ (Figure 4B**)** the next intermediate, **F**, is formed by proton transfer from E286 (31), presumably protonating either the hydroxide or tyrosinate at the active site. If state $\mathbf{P_m}$ is formed (Figure 4A), then the generation of state F requires delivery of both a proton (from E286) and electron (from heme a, after it is reduced) to the binuclear center, reducing the tyrosyl radical to tyrosinate and protonating either this or the hydroxide at the active site.

F→O

The final step in the reaction is the transfer of another electron from heme a to the heme a_3 /Cu_B center, accompanied by a proton from E286 (31). This reaction reduces the heme a_3 $Fe^{4+}=O^{2-}$ to Fe^{3+} + ^-OH.

2.4. Principal Experimental Approaches to Examine Proton Transfer Reactions in Oxidase

There are several approaches to study the nature of the proton transfer reactions during catalysis. These mostly involve methods for examining either one turnover cycle or individual steps within the cycle.

2.4.1. Reaction of O_2 with theFully Reduced Enzyme (R_4)- the Flow-Flash Method

The protein is prepared in the fully reduced (i.e., 4-electron reduced) $\mathbf{R_4}$ state which is bound to CO. After rapid mixing of O_2, the reaction is initiated by the photolysis of CO. Optical monitoring of the hemes reveals steps: $\mathbf{R_4 \rightarrow A \rightarrow P_r \rightarrow F \rightarrow O}$ with approximate time constants of 8 μsec, 30 μsec, 120 μsec and 1 msec (*R. sphaeroides* enzyme)(32). This sequence is shown in Figure 4B.

2.4.2. Photoreduction of O, E, P_m and F

Individual steps can be examined in isolation by using a light-generated reductant, usually a derivative of Ruthenium (e.g., Ruthenium *bis* bipyridine, RuBpy), which is non-covalently bound to the enzyme (24, 33, 34). Methods have been devised to trap most of the enzyme in states **E**, $\mathbf{P_m}$ or **F**. The isolated one-electron steps correspond to those shown in Figure 4A: $\mathbf{E \rightarrow R_2}$, $\mathbf{P_m \rightarrow F}$ and $\mathbf{F \rightarrow O}$.

Generation of P_m

The fully oxidized enzyme is reduced by CO, a 2-electron reductant, in the presence of O_2. After the reaction of CO, two electrons are present in the enzyme (see **Section 2.4.4**), so the subsequent reaction with O_2 forms the product $\mathbf{P_m}$.(see Figure 4A). Photoreduction results in the $\mathbf{P_m \rightarrow F}$ transition.

Generation of F

Exposure of the oxidized enzyme to H_2O_2 at alkaline pH results in the generation of state **F**. This allows one to examine the $\mathbf{F \rightarrow O}$ transition by photoreduction. The peroxide reaction is actually a steady state cycle (35, 36).

Generation of E

Once state **F** is obtained by reacting with peroxide, the peroxide can be eliminated using catalase and the enzyme reduced by CO (29, 37). This 2-electron reduction brings the enzyme through **O** and to **E** directly. Photoreduction allows one to examine the $\mathbf{E \rightarrow R_2}$ transition (29). The CO that is present binds to and traps the product in which both electrons are at the heme a_3 /Cu_B center, so the actual transition studied is $\mathbf{E \rightarrow R_2(CO)}$.

2.4.3. $O \rightarrow R_4$, reduction of the fully oxidized enzyme

The anaerobic addition of a chemical reductant such as Ruthenium hexammine, dithionite or ascorbate/TMPD to the fully oxidized enzyme using a stopped flow rapid mixer allows one to time-resolve the rates of reduction of heme a and heme a_3 (38). Freshly oxidized enzyme (i.e., pulsed oxidase) is used in this experiment to avoid hysteric artifacts (39). Proton uptake (about 2.4 protons, depending on the pH) accompanies full reduction of the oxidase (40).

2.4.4. Backflow reaction (reverse electron transfer)

The two-electron reduced form of the enzyme ($\mathbf{R_2}$) is formed by the anaerobic reduction of the fully oxidized enzyme by CO, a two-electron reductant. CO binds to the reduced enzyme (to ferrous heme a_3), stabilizing the state where the two electrons are at the heme a_3 /Cu_B center. This is the "CO-mixed valence" form of the enzyme, $\mathbf{R_2(CO)}$. Photolysis results in dissociation of CO, which lowers the midpoint potential of heme a_3, causing a redistribution of the electron on heme a_3 to heme a and Cu_A (41-43). This "backwards" electron transfer is formally the reverse of the $\mathbf{E \rightarrow R_2}$ transition (Figure 4A). The backflow reaction is accompanied by release of a proton *via* the K-channel (28, 44, 45).

2.5. Monitoring proton transfer reactions in the oxidase

All the proton transfer reactions in the oxidase are coupled to electron transfer. The electron transfer steps are monitored by UV-vis spectroscopy and then correlated with proton transfer reactions. The methods used include the following.

1. pH-dependence of electron transfer reactions.
2. Solvent kinetic isotope effects on individual electron transfer reactions. The k_H/k_D ratio is determined by running the reaction in H_2O or D_2O. This can be used to identify electron transfer reactions that are rate-limited by coupled proton transfers (46, 47).
3. Proton release from oxidase reconstituted in phospholipid vesicles (48).
4. Net proton uptake/release from oxidase in solution (detergent-solubilized) (49).
5. Electrometric measurement of charge separation across the membrane by oxidase reconstituted in vesicles (24, 29, 34, 50, 51). This time-resolved voltage measurement monitors electrical work done by moving charges across the membrane.
6. FTIR difference spectroscopy. This method can identify individual residues, particularly glutamates and aspartates, that undergo changes in hydrogen bonding or protonation (52-57).

3. PROTON-COUPLED ELECTRON TRANSFER

Each electron transfer from heme a to the heme a_3 /Cu_B center is coupled to proton transfer. It is useful to consider some general principles. Figure 5 is a thermodynamic cycle showing the free energy linkage for the transfer of a single electron from A^- to B coupled to the binding of single proton. We start with an oxidized/deprotonated state of B ($A^-B + H^+$) and end up with the reduced/protonated species (AB^-H^+). Each of the electron transfers from heme a to the heme a_3 /Cu_B center can be considered using this formalism. There are two kinetic pathways, either through the reduced/deprotonated intermediate ($AB^- + H^+$) or *via* the oxidized/protonated species (A^-BH^+). Note that these intermediates can be

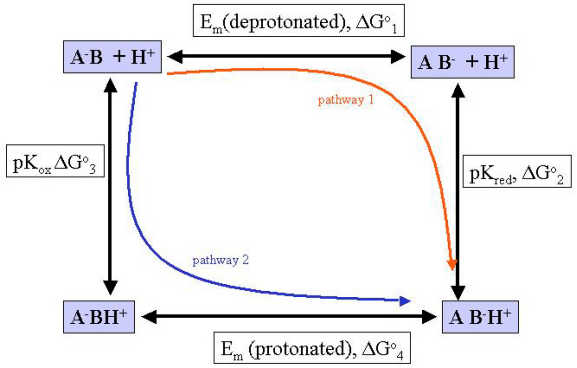

Figure 5. A thermodynamic cycle for a proton-coupled electron transfer reaction. Electron transfer from A^- to B is coupled to a proton transfer. Either or both pathways may be utilized depending on the rate constants for the individual steps. The intermediates may not be present in sufficient amount to directly observe.

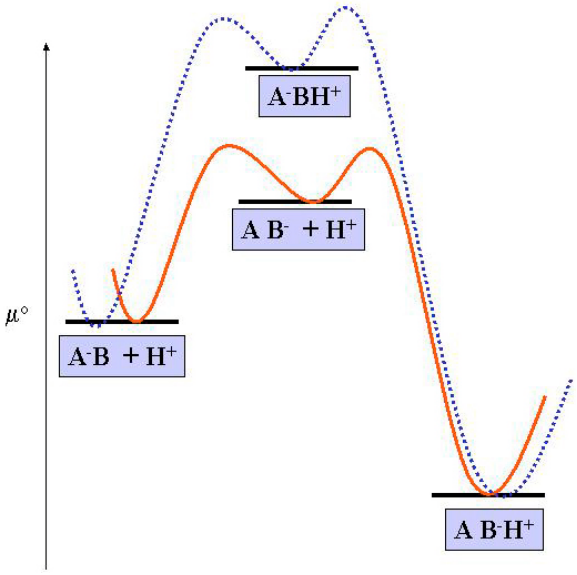

Figure 6. A free energy diagram showing the standard state chemical potentials of species in Figure 5. In this example, the lowest lying intermediate is the reduced product (B^-) , which is then "trapped" by a proton transfer. The alternative pathway is very slow because of the very unfavorable rate constant, in this case due to the high chemical potential of the intermediate.

unstable with respect to both the starting and final states of the system and, therefore, never highly populated. Two distinct cases are when 1) the intrinsic rate of electron transfer is much faster than the rate of proton transfer; and 2) the rate of proton transfer is much faster than the electron transfer rate.

A generic free energy diagram is shown in Figure 6 in which it is postulated that the reduced/deprotonated form of B has a lower chemical potential than the oxidized protonated form. In this example, the favored kinetic pathway is that in which the electron is transferred first (B^-), but to a small fraction of the population, and then the proton is transferred, essentially trapping the electron (B^- H^+). An example of this is the internal electron transfer between the semiquinones in the photosynthetic reaction center (58):

$$Q_A^{-\bullet} Q_B^{-\bullet} + H^+ \leftrightarrows Q_A^{-\bullet}(Q_BH)^\bullet \rightarrow Q_A(Q_BH)^-.$$
$$\text{rapid} \qquad \text{slow}$$

In this example, the protonation reaction of $Q_B^{-\bullet}$ is in rapid equilibrium and the slower electron transfer yields the $(Q_BH)^\bullet$ species. The observed rate of electron transfer is the intrinsic electron transfer rate constant for the reaction $Q_A^{-\bullet}(Q_BH)^\bullet \rightarrow Q_A(Q_BH)^-$ multiplied by the fraction of the population in the form $Q_A^{-\bullet}(Q_BH)^\bullet$:

$$k_{obs} = k_{ET}\ [f(Q_A^{-\bullet}(Q_BH)^\bullet)].$$

An unambiguous kinetic analysis requires measuring the rates as a function of the driving force for protonation (pH), the driving force for electron transfer (ΔE_m), and direct observation of the intermediate. This has not been possible with the proton-coupled electron transfer reaction in the oxidase, but pH-dependence and solvent kinetic isotope effects have generally been consistent with models in which it a proton transfer is rate-limiting and traps the electron in the heme a_3 /Cu_B center.

The dependence of the rate of reduction of the heme a_3 /Cu_B center by chemical reductants was examined as a function of both pH and the redox driving potential(38). The electrons are in rapid equilibrium, but the deprotonated intermediate is energetically disfavored and never present at a significant concentration. The rapid electronic equilibrium is followed by a slower protonation (Figure 7).

$$\mathbf{O \rightarrow E} \qquad a^-(a_3Cu_B)_{OX} + H^+ \leftrightarrows$$
$$a(a_3Cu_B)^- + H^+ \rightarrow a(a_3Cu_BH)$$
$$\text{rapid} \qquad \text{slow}$$

Electron transfer from heme a to the heme a_3 /Cu_B center is slow or is completely blocked by mutations in the K-channel(27). Reduction of the binuclear center is not observed unless the proton is delivered to trap the final product because the intermediate species $a(a_3Cu_B)^-$ is energetically disfavored

$$a^-(a_3Cu_B)_{OX} + H^+ \leftrightarrows a(a_3Cu_B)^- + H^+ \xrightarrow{\mathbf{x}} a(a_3Cu_BH)$$
$$\text{K-channel mutants}$$

The $\mathbf{P_r \rightarrow F}$ and $\mathbf{F \rightarrow O}$ transitions are also rate-limited by proton transfer (31). These reactions have substantial H/D kinetic isotope effects.

$$\mathbf{P_r \rightarrow F} \qquad a^-(P_r) + H^+ \leftrightarrows a(P_r)^- + H^+ \rightarrow a(P_rH) \quad k_H/k_D \approx 2.5$$
$$\mathbf{F \rightarrow O} \qquad a^-(F) + H^+ \leftrightarrows a(F)^- + H^+ \rightarrow a(FH) \quad k_H/k_D \approx 7$$

Proton inventory analysis indicates that the rate-limiting proton transfer involves just a single proton(59),

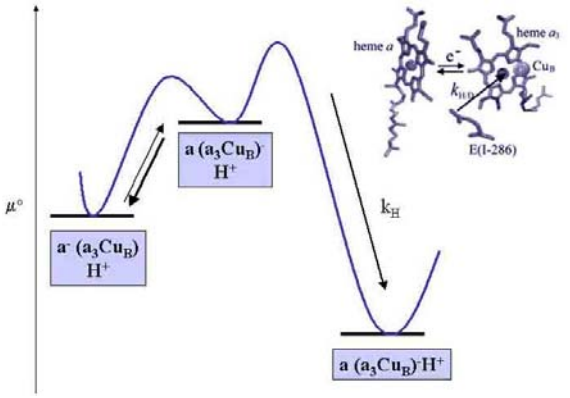

Figure 7. A schematic free energy diagram for the electron transfer from heme a to the oxidized heme a_3 /Cu_B center. The electron equilibrates between the starting state and the intermediate prior to slower proton transfer, which is via the K-channel. Blocking proton transfer by a mutation in the K-channel prevents significant electron transfer because the reduced/deprotonated state is very unstable (high chemical potential). The same situation is thought to apply to the electron transfer of the heme a_3 /Cu_B center in states $\mathbf{P_r}$ and **F**, but the rate-limiting proton transfer is from E286. Whether this proton transfer is related to the chemical or pumped proton is not known.

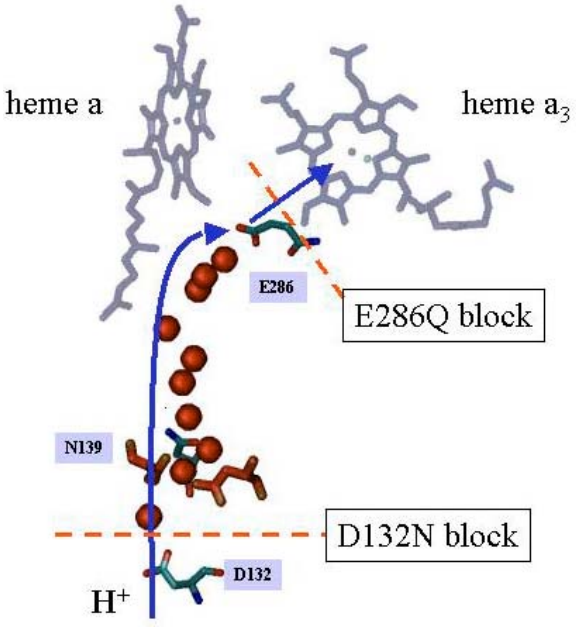

Figure 8. The D-channel, showing the two-step pathway of protons from D132 to E286 and from E286 to the heme a_3 /Cu_B center. Residues E286, N139 and D132 are indicated. There is a water bridging D132 and N139. The N139D mutant has 2- to 3-fold oxidase activity but is devoid of any proton pumping.

and the examination of mutants shows that the proton comes from E286(31). Replacing E286 with glutamine (E286Q) eliminates enzyme turnover and blocks the $\mathbf{P_r}\rightarrow\mathbf{F}$ and $\mathbf{F}\rightarrow\mathbf{O}$ electron transfer reactions, so the reaction of O_2 with the fully reduced enzyme stops at state $\mathbf{P_r}$. The E286D mutant has about half the turnover of the wild type oxidase and pumps protons normally. The $\mathbf{P_r}\rightarrow\mathbf{F}$ and $\mathbf{F}\rightarrow\mathbf{O}$ reactions are also slowed in the E286D mutant oxidase, and the H/D solvent kinetic isotope effects are altered.

4. INTERNAL PROTON CAPACITY AND RATE-LIMITING INTERNAL PROTON TRANSFERS

Two reaction steps are partially rate-limited by internal proton transfers and proceed without proton uptake from the bulk aqueous phase: $\mathbf{A}\rightarrow\mathbf{P_r}$ and $\mathbf{A}\rightarrow\mathbf{P_m}$. A third reaction, $\mathbf{P_r}\rightarrow\mathbf{F}$ is also rate-limited by internal proton transfer and, in the D132N mutant, can proceed without uptake of a proton from solution. The formation of states $\mathbf{P_r}$ and $\mathbf{P_m}$ in the reactions of O_2 with the fully reduced and two-electron reduced forms of the enzyme, respectively, both have deuterium solvent kinetic isotope effects (k_H/k_D = 1.4-1.9) (60). This suggests partial rate-limitation by a proton transfer, probably from the active-site Y288 to the distal oxygen atom.

The E286Q mutant does not proceed beyond $\mathbf{P_r}$ when when O_2 reacts with the fully reduced enzyme. In contrast, the D132N mutant proceeds to state **F**. However, whereas the $\mathbf{P_r}\rightarrow\mathbf{F}$ step is associated with a net proton uptake in the wild type oxidase, no proton uptake is associated with this step with the D132N mutant (61). As indicated in Figure 8, the D132N mutation blocks access of the D-channel from the bulk aqueous phase, but the proton needed for the $\mathbf{P_r}\rightarrow\mathbf{F}$ transition is provided by E286. The reaction of O_2 with the D132N mutant can go no further than state **F**, however, since the proton required for the $\mathbf{F}\rightarrow\mathbf{O}$ transition is not available. The sequential $\mathbf{P_r}\rightarrow\mathbf{F}$ and $\mathbf{F}\rightarrow\mathbf{O}$ transitions both require protons delivered *via* E286, with the requirement that E286 be reprotonated from D132 in between these steps. The D-channel has an internal proton capacity (at least the1 proton on E286) allowing the $\mathbf{P_r}\rightarrow\mathbf{F}$ transition to proceed even when the channel entrance is blocked.

Blocking the entrance of the K-channel ($E101^{II}$ mutants), in contrast, dramatically inhibits electron transfer from heme a to the oxidized heme a_3 /Cu_B center (16). There is no internal proton present within the K-channel to convert the active site hydroxide to water, as required for the reduction of the active-site metal centers.

5. REACTION STEPS LINKED TO PROTON PUMPING

Several electron transfer steps have been shown to pump protons. There are two independent assays used to assess proton pumping using the isolated oxidase reconstituted in a membrane bilayer.

1. Proton release from oxidase reconstituted in phospholipid vesicles (48, 62).

2. Time-resolved electrometric measurement of voltage generation across the membrane (34, 50, 51, 63). It is presumed that all charge movements across the membrane accompanying the electron transfer from heme a to the heme a_3 /Cu_B center are due to proton movements across low dielectric portions of the protein. These electrogenic charge movements can be due to the substrate protons or pumped protons.

The best characterized partial reaction is the **F→O** transition. Other steps which have been interpreted as being coupled to proton pumping are the **P_r→F**, **P_m→F**, **E→R_2(CO)** and **O˜→R_2** (see below). About half of the proton pumping occurs during the reduction of the heme a_3 /Cu_B center prior to the reaction with O_2 and about half occurs during the reduction of the oxygenated **P** and **F** states of the enzyme (64). Notably, steps that do not result in generating a transmembrane voltage are the formation of **P_m** or **P_r** from the reaction of O_2 with the mixed-valence (**R_2**) or fully reduced (**R_4**) forms of the oxidase, respectively(64).

F→O

Photoreduction of the peroxide-generated **F** state (**F→O** transtion) generates a transmembrane voltage that occurs in 3 time-resolved phases (24, 34, 50). The fastest phase (*R. sphaeroides* oxidase), $\tau \approx 15$ μsec, corresponds to the electron transfer from Cu_A to heme a. The second and third phases are linked to electron transfer from heme a to the heme a_3 /Cu_B center (intermediate phase, $\tau \approx 0.5$ msec; slow phase, $\tau \approx 1.5$ msec). Interpreting the data in terms of the number of charges moved across the membrane depends on the estimate of how much of the membrane is crossed by an electron transfer between Cu_A and heme a (between 0.3 and 0.5). The lowest estimate, however, indicates that the amount of charge moved across the membrane concomitant with the electron transfer from heme a to the heme a_3 /Cu_B center in state **F** is substantially more than can be accounted for by moving one proton to the active site to complete the chemistry (27, 34, 50, 65). Most of the voltage generated must be due to internal transfers of the pumped proton.

The voltage generated during the **F→O** transition has also been measured during the reaction of O_2 with the fully reduced enzyme, and the data are compatible with the photoreduction experiments (51). Between 1 and 2 protons are released to the outside of vesicles containing oxidase during the **F→O** transition. Without the membrane, one observes about 1 proton taken up from solution during the **F→O** transition. Presumably, this represents the net proton change resulting from the simultaneous release of 1 proton and the uptake of 2 protons by the enzyme (48).

P_r→F

The voltage generated during the **P_r→F** transtion has also been quantified during the reaction of O_2 with the fully reduced enzyme (51). This phase of the chemical reaction is equally electrogenic as the **F→O** transition, which has been interpreted as proton pumping. However, no proton is released from vesicles reconstituted with oxidase during the **P_r→F** phase of the reaction. Without the membrane, the **P_r→F** transition is associated with the uptake of one proton from solution (48). Possibly, the pumped proton is transferred to some site near the external surface, but its release is delayed, or the interpretation of the electrometric analysis is incorrect.

P_m→F

The **P_m** state of the enzyme has been generated in three different ways to examine the voltage generated during the **P_m→F** transition. All the experiments indicate that the voltage generated during the **P_m→F** transition is equal in kinetic pattern and magnitude to that generated during the **F→O** transition (51, 66). There is no measurement of proton release during the **P_m→F** transition.

E→R_2(CO)

The **E** state has been produced by reacting CO (2-electron reductant) with the peroxide-generated **F** state of the enzyme. Photoreduction of this state results in a transmembrane potential similar to that observed during the **F→O** transition, suggesting that this transition is also coupled to proton pumping (29). No proton release has been measured.

O˜→R

Chemical reduction of the enzyme immediately after its oxidation results in the generation of a transmembrane voltage and the release of about 2 protons from reconstituted vesicles (67). If the same experiment is performed with enzyme that has not recently been through a cycle of reduction and re-oxidation, no proton release is observed. It is proposed that there is a transient, high energy form of the oxidized enzyme (state **O˜**) that is formed after turnover of the enzyme with O_2 and that electron transfer from heme a to this state of the binuclear center is coupled to proton pumping (67).

6. ALL PUMPED PROTONS ARE TRANSFERRED THROUGH THE D-CHANNEL: THE CENTRAL ROLE OF E286

Several mutations in the D-channel result in decoupling the proton pump from oxidase activity. The D132N and D132A mutants exhibit less than 5% of the turnover of the wild type oxidase, and this residual activity is not coupled to proton pumping (68). Possibly, all proton flux to the D-channel is blocked and the turnover is due to the leakage of protons backwards through the exit pathway from the wrong side of the membrane. In the *E. coli* oxidase, the E286C mutant has significant oxidase activity but does not pump protons (69). These results demonstrate that there are alternate pumping and non-pumping kinetic pathways for oxidase turnover. The normal pathway is coupled to the proton pump, but mutants that inhibit proton flux through the D-channel can alter the favored pathway to one which is decoupled. Mutants in the K-channel, by contrast, retain full proton pumping despite low activity. Electrometric studies of the **E→R_2(CO)** transition in the *P. denitrificans* oxidase show that proton flux through the D-channel is essential for the proton pumping associated with

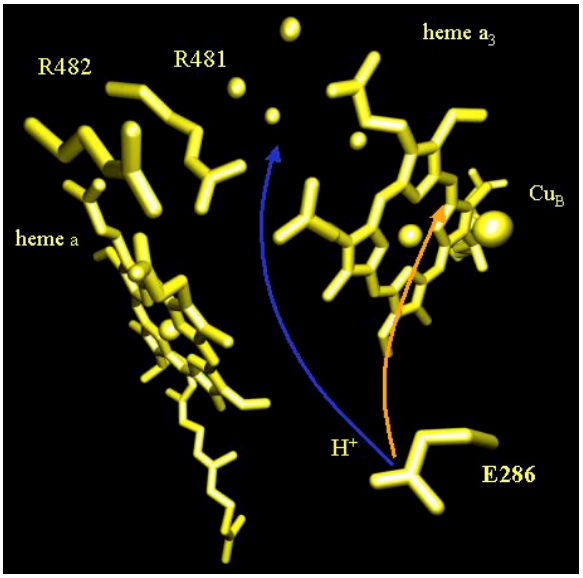

Figure 9. Postulated pathways from E286 to the postulated propionate/arginine proton accepting cluster for all pumped protons, and to the heme a_3 /Cu_B center, for the **P→F** and **F→O** transitions. (Figure kindly provided by Dr. Peter Brzezinski).

this step (29). Pumped protons must, therefore, be transferred from E286.

The best candidate for the proton acceptor from E286 is the complex formed by Arg481 (*R. sphaeroides* oxidase) and the Δ-propionate of heme a_3 (Figure 9) (3, 52, 69, 70). This is hydrogen bonded to the ring nitrogen of W172, which might assist in proton delivery to the propionate (see Figure 8). The double mutant R481Q/R482Q in the *E. coli* oxidase also decouples the proton pump, consistent with these residues being part of a proton accepting cluster (69).

The most interesting mutant influencing proton pumping is N139D (*R. sphaeroides* numbering)(71) (72). This introduces an additional aspartate residue within the D-channel just above the entrance residue D132 (see Figure 8). Remarkably, this mutation has an increased turnover (2- to 3-fold higher) in the steady state, but is totally decoupled from proton pumping (72). The **F→O** transition in the reaction of fully reduced enzyme with O_2 is similarly accelerated about 2-fold. The result indicates that coupling to the pumping apparatus must be very finely tuned. The subtle perturbation of the N139D mutation essentially changes the kinetic preference completely to the non-coupled pathway. The most likely location of perturbation is E286, connected to N139D by a string of water molecules.

All of the pumped protons must be transferred *via* E286, along with the chemical protons during the **P→F** and **F→O** transitions. There must be two different pathways to facilitate proton transfer from E286 to either the heme a_3 /Cu_B center or to the proton acceptor (propionate/arginine) in the exit channel. One can imagine a toggle switch, directing protons one way or the other, and the N139D mutant acts to hold the switch in the "chemistry" position (72). An important role for internal water molecules, not observed in the X-ray structures, has been indicated by molecular dynamics studies (20, 22). Water movements are known to play a crucial role in the dynamics of the light-driven proton pump, bacteriorhodopsin, influencing the energetic stability of protonated residues in intermediate states and providing pathways for proton transfers during the photocycle(73) (74). A critical role for the dynamics of internal water molecules is likely for the oxidase as well.

One can speculate that there is a favored kinetic pathway in which a proton is transferred to the exit pathway acceptor (arginine/propionate) rapidly as the electron is transferred to the heme a_3 /Cu_B center, and that there is a slower proton transfer which follows, in which the chemical proton is transferred to the heme a_3 /Cu_B center, displacing the proton in the exit channel. There must also be a kinetic pathway preference for the proton to be delivered to the exit pathway from the inside (E286) but dissociate on the opposite side of the membrane. This unidirectionality is an essential component of the pump.

7. PERSPECTIVE

Proton transfers are obviously at the heart of the cytochrome oxidase mechanism. Each of the steps coupled to the proton pump initiates a sequence of steps through transient intermediates, resulting in the efficient conversion of the free energy from the reaction at the active site to a proton motive force across the membrane. The internal plumbing has been partially mapped out, defining the K-channel and D-channel with an indication of the proton accepting propionate/arginine cluster "above" the hemes along the exit pathway for pumped protons. The reason for having two separate input channels is not evident. The main role of the K-channel is limited to proton transfers required for charge compensation at the heme a_3 /Cu_B center prior to cleavage of the O-O bond. It appears that all proton transfers after the O-O bond cleavage are through the D-channel.

The role of internal water molecules, whether observed in the X-ray structures or deduced from molecular dynamics or analysis of internal cavities, is critical for making and breaking proton-conducting pathways that are essential for the oxidase to function.

An unusual feature of the proton transfers within the oxidase is the utilization of one pathway leading to E286 for protons destined to two different destinations: the heme a_3 /Cu_B center or the pump exit channel. Since the electron-coupled proton transfer reactions provide a very strong driving force to pull protons into the heme a_3 /Cu_B center, there must be a safeguard mechanism to prevent all the protons coming through the D-channel from being consumed by the chemistry. In the N139D mutant, this safeguard is bypassed and the pump is decoupled. It is likely that the alteration caused by the mutation is transmitted to E286 through the water chain in the D-channel.

Finally, another feature of the coupled proton-electron transfers in the oxidase is that the kinetic pathway appears to involve unstable, but required, intermediates. This is similar to the example of the bacterial photosynthetic reaction (Section 2). Such intermediates may be essential for the proton-coupled pathway. Identifying the chemical nature of these intermediates will provide a substantial experimental challenge.

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