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The Histidine Cycle: A New Model for Proton Translocation in the Respiratory Heme-Copper Oxidases

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A model of redox-linked proton translocation is presented for the terminal heme-copper oxidases. The new model, which is distinct both in principle and in detail from previously suggested mechanisms, is introduced in a historical perspective and outlined first as a set of general principles, and then as a more detailed chemical mechanism, adapted to what is known about the chemistry of dioxygen reduction in this family of enzymes. The model postulates a direct mechanistic role in proton-pumping of the oxygenous ligand on the iron in the binuclear heme-copper site through an electrostatic nonbonding interaction between this ligand and the doubly protonated imidazolium group of a conserved histidine residue nearby. In the model this histidine residue cycles between imidazolium and imidazolate states translocating two protons per event, the imidazolate state stabilized by bonding to the copper in the site. The model also suggests a key role in proton translocation for those protons that are taken up in reduction of O_2 to water, in that their uptake to the oxygenous ligand unlatches the electrostatically stabilized imidazolium residue and promotes proton release.

KEY WORDS: Proton-pumping; cytochrome oxidase; oxygen reduction.

PRINCIPLES OF PROTON TRANSLOCATION

Peter Mitchell's chemiosmotic theory holds, in general terms, that energy is transmitted from the redox processes of respiration and photosynthesis to the systems which produce ATP through the intermediary of an electrochemical proton gradient across the mitochondrial, chloroplast, or bacterial membrane. This amounts to the storage of charge in a biological capacitor. This paper discusses the mechanism by which the flow of electrons is coupled to the generation of this protonmotive force, in one specific type of respiratory chain component, the heme-copper terminal oxidases.

In Mitchell's model, the coupling between electron flow and the protonmotive force was achieved entirely by what he termed "vectorial chemistry." The respiratory chain was arranged in such a way that it zigzagged between the inside and the outside of the coupling membrane (or more precisely, the osmotic barrier) a number of times. On each round trip (a *redox loop*), redox equivalents traveled from the outer side of the barrier to the inside as electrons, but returned by hydrogen transfer borne on a redox carrier. For the electron to return to the outer side of the barrier, the redox carrier thus had to pick up a proton and carry the two together as hydrogen. The result was that every time an electron crossed the barrier and returned, there was net translocation of one proton across the membrane.

This absolutely direct, covalent coupling between proton translocation and electron transfer is a distinguishing feature of the redox loop mechanism, uniquely achieved by hydrogen-transferring redox carriers, such as quinones, flavins, and the dioxygen/water couple. An example of such an arrangement is the ubiquinone (Q) cycle mechanism for the cytochrome bc_1 and b_6f

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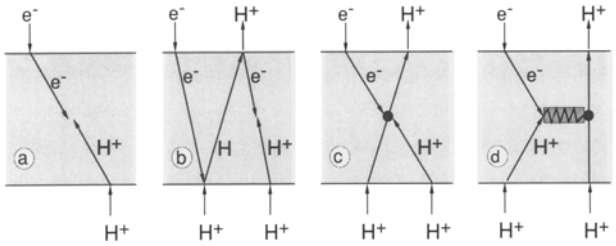

Fig. 1. Various schemes for generation of protonmotive force by cytochrome *c* oxidase: (a) Mitchell's original proposal; (b) mechanism with an extra redox loop; (c) a directly coupled proton pump; (d) an indirectly coupled (conformational) proton pump.

complexes (Mitchell, 1976), for which there is now strong support (Crofts and Wraight, 1983; Gennis *et al.*, 1993). Mitchell believed that the whole respiratory chain could be described as nothing more than a series of *redox loops*, each of which transports electrons across the membrane and back, and in the process carries one proton per electron across the membrane.

The mechanism of terminal oxidases also involves vectorial chemistry. In the cytochrome *c* oxidase reaction, four electrons from cytochrome *c* on the outside of the membrane are electrically annihilated in the reduction of O_2 to 2 H_2O at the active site by an equal number of protons originating from the inside of the membrane (Mitchell, 1966, 1976, 1981; Wikström, 1988). Thus, the reaction makes up the electron-carrying arm and proton-input site of a redox loop (Fig. 1a). In Mitchell's original view, this was the full extent of energy conservation by cytochrome *c* oxidase, leading to a predicted thermodynamic $1H^+/e^-$ stoichiometry. It was later shown, however, that in addition, the oxidase actually pumps protons across the membrane so that the overall charge stoichiometry is doubled (Wikström, 1977; Wikström and Saari, 1977; Krab and Wikström, 1978, 1979; Wikström and Krab, 1979).

The redox loop mechanism has more difficulty explaining this additional translocation of protons in the enzyme. There is, of course, the possibility that cytochrome oxidase might contain an additional redox loop (Fig. 1b), although in the late 1970's this possibility was thought to be excluded simply because no hydrogen-carrying redox couple could be found in the enzyme (Wikström *et al.*, 1981). (In retrospect, we note that there was a failure to appreciate that (Mitchell, 1988) certain redox couples in the dioxygen reduction chemistry could play such a role, e.g., the O_2/H_2O_2 couple; but see below.)

To account for proton translocation in the absence of a redox loop, two types of models were proposed: a directly coupled proton pump (DCP) mechanism (Fig. 1c; Wikström and Krab, 1978, 1979), and an indirect or "conformational" mechanism involving a *membrane* or *redox Bohr* effect, as suggested by Chance *et al.* (1977) and by Papa (1976), which calls for long-range protein-mediated coupling between the redox centers and the proton-carrying residues (Fig. 1d). (Indirect coupling has the same mechanistic requirements as a DCP mechanism but, in addition, requires that energy be transmitted through the protein. An Occam's razor argument might suggest that indirect mechanisms need not be considered unless the DCP mechanism can be disproved, but see below.)

In the directly coupled pump (DCP) model, redox-linked ligand exchange at one of the enzyme's metal centers controls both the binding of protons at a pumping site and the directionality of their uptake and release. Proton binding properties are controlled by locating the proton-carrying sites on metal center ligands, while the geometrical rearrangement upon

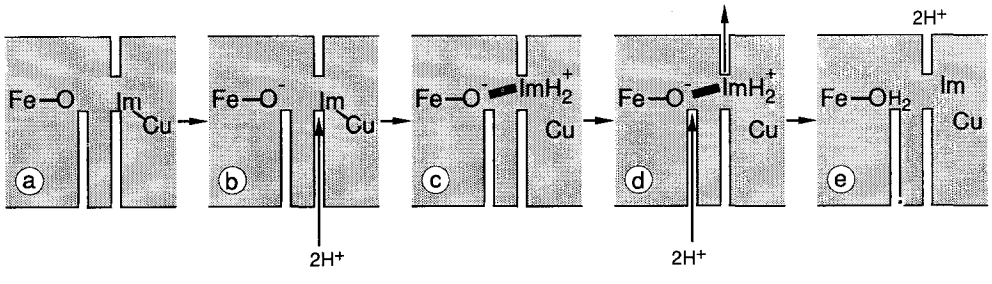

Fig. 2. The histidine cycle—a generalized scheme: Fe, iron of the oxygen-binding heme; Cu, Cu_B; O, generalized oxygen-reduction intermediate; Im, imidazole side chain of histidine 284; the dark rectangle represents the electrostatic interaction between the negative charge on the oxygen intermediate and the protonated imidazole; channels for scalar and vectorial proton input are shown on the lower left and right, respectively.

ligand exchange could provide alternate access to input and output proton channels (Wikström and Krab, 1978, 1979). Note that if the enzyme were to use a redox loop mechanism to translocate these protons, the redox equivalents would have to cross the osmotic barrier two more times (Fig. 2b), but in the case of the DCP model (Fig. 2c), *the redox chemistry involved in proton translocation need not be vectorially orientated.* Furthermore, no formal hydrogen redox carrier is required. In spite of this, the proton/electron linkage in such a model should be considered "direct" since the chemical entities that carry the protons across the osmotic barrier would be ligands of the redox center itself (Wikström and Krab, 1978, 1979). A detailed chemical model for a proton pump based on the DCP principle was developed by Chan and co-workers (Blair *et al.*, 1986; Gelles *et al.*, 1986). This model hypothesized Cu_A as the redox center but, more recently, other proposals have been published following the same motif (Larsen *et al.*, 1992; Rousseau *et al.*, 1993; Woodruff, 1993).

The fact that Mitchell's chemiosmotic theory was so intimately based on the notion of vectorial chemistry and redox loops was presumably one basis for the persistent opposition against the finding that cytochrome *c* oxidase functions as a true proton translocator (see, e.g., Moyle and Mitchell, 1978; Mitchell, 1981). This controversy ended in 1985 (Wikström and Casey, 1985; Mitchell *et al.*, 1985). Mitchell then suggested that the oxidase reaction may be arranged as a redox loop utilizing the H_2O_2/O_2 couple as the hydrogen carrier ("O-loop" or "O-cycle"). He soon abandoned this, however, in favor of a model based on redox-linked OH^-/H_2O ligand exchange and conformational switching at either the Cu_A or the Cu_B center, of which the latter was preferred subsequently (Mitchell, 1987, 1988). Although it was argued otherwise (Mitchell, 1988), this was a fundamental departure from the original redox loop concept, and represented, in fact, a version of the DCP principle proposed ten years earlier (Wikström and Krab, 1978, 1979).

However, some recent models (Vygodina and Konstantinov, 1987; see also Papa *et al.*, this issue) propose that the protons which are pumped are carried covalently on oxygenous intermediates of the redox reaction, as H_2O_2 for example. This is reminiscent of Mitchell's "O-cycle" or "O-loop" and can be regarded as a version of vectorial chemistry whereby the heme-copper oxidases contain an additional redox loop.

CONSTRAINTS ON THE MECHANISM OF PROTON PUMPING IN THE HEME-COPPER OXIDASES

Structural Considerations

The primary structures of heme-copper respiratory oxidases from over 80 species are known at present. The common features are a binuclear heme-copper dioxygen reaction center, and a low-spin heme, which functions as electron donor to this center.[3] The main structural relationships between these centers are presently being revealed from mutagenesis data (Hosler *et al.*, 1993). Three enzyme subcategories can be discerned: the cytochrome *c* oxidases of aa_3 type, the quinol oxidases exemplified by cytochrome bo_3 of *E. coli*, and the recently discovered cytochrome cbb_3-type oxidases, which accept electrons from cytochrome *c*, but lack the Cu_A center, which is found in all previously described cytochrome *c* oxidases as the site of electron entry. It is now well established that both the aa_3-type cytochrome *c* oxidases and the quinol oxidases are able to translocate protons (Puustinen *et al.*, 1989; Lauraeus and Wikström, 1993; Wikström *et al.*, 1994). Recently, it has also been shown that the cbb_3-type oxidase of *P. denitrificans* pumps protons (Raitio and Wikström, 1994).

This most recent finding puts important structural constraints on possible mechanisms of proton translocation. The cbb_3 enzymes are much more distantly related to the rest of the family (see van der Oost *et al.*, 1994) in that, with few exceptions, only the six histidines in subunit I which have been implicated in the binding of the hemes and of Cu_B are conserved (see Hosler *et al.*, 1993). This suggests that the metal centers and their ligands play a central role in the proton translocation mechanism and argues against long-range conformational coupling mechanisms. Such indirect mechanisms (e.g., the *redox Bohr* type) depend on the protein structure to transmit energy from the redox centers to proton carriers. It is difficult to see how this could be accomplished without a much larger number of functionally important amino acid residues than are conserved in the cbb_3 enzymes.

[3] In this paper, we will use the cytochrome *c* oxidase nomenclature in which these centers are known as Fe_{a3}, Cu_B, and Fe_a, respectively.

Mechanistic Considerations

In considering proton translocation mechanisms it is essential to define which partial reaction steps in the catalytic cycle are associated with proton-pumping. Wikström (1989) addressed this issue directly from an experimental standpoint, and found that the main energy-requiring steps of proton translocation are linked to those catalytic steps that are associated with the oxygen chemistry: more specifically, to the conversion of the "peroxy" (P) intermediate of the binuclear center to the "ferryl" (F) state, and of the latter to the oxidized (O) state.

This restriction excludes several models proposed in the past. It not only suggests that the proton translocation mechanism is closely associated, structurally and functionally, with the binuclear center, but also indicates that oxygen may play a mechanistic role rather than only being a thermodynamically effective oxidant. Furthermore, it suggests that the chemical mechanism of proton translocation may be one where two protons are translocated at a time.

The "O-loop" class of mechanisms (above) is appealing insofar as they offer solutions to the problems of the proton/electron stoichiometry and the role of the oxygen, but they pose one basic design challenge: In such a model, the translocated protons must be chemically bonded to the oxygen at some point in the cycle (as H_2O_2, for example). In order to complete the cycle, these protons must leave the oxygen and be ejected on the outside of the membrane, while additional protons must be taken up from the inside of the membrane to bind oxygen and make water. However, it is not clear why the protons which are to be pumped do not simply remain bound to the oxygen, but instead are expelled, with significant expenditure of energy, only to be replaced by chemically identical protons taken up, with additional expenditure of energy, from the inside of the membrane. Therefore, in the new model to be proposed here (cf. Wikström *et al.*, 1994) we have avoided the covalent bonding between vectorial protons[4] and oxygen as in the "O-cycle" or "O-loop" type of mechanisms, but yet implemented the appealing feature of direct coupling of proton translocation mechanism to the chemistry of oxygen reduction, and retained basic features of the earlier DCP principle.

[4] Reduction of O_2 to $2\,H_2O$ by four electrons requires uptake of four protons as part of the redox chemistry, and is observed experimentally irrespective of membranous barriers for proton diffusion. For this reason we call these *scalar protons* even though they do exhibit the important property of sidedness in the membranous enzyme; they are taken up from the negatively charged side (inside) of the membrane. Coupled to the redox reaction, the respiratory heme-copper oxidases additionally translocate protons across the mitochondrial or bacterial membrane. These are distinguished here as *vectorial protons*.

THE HISTIDINE CYCLE: BASIC POSTULATES

This model was designed to satisfy the constraints described above, and in the present chemically more reasonable form it is a refined version of our original proposal (Wikström *et al.*, 1994) arrived at after discussions with several colleagues (see Acknowledgments). The present version is also better adjusted to conform with the net proton uptake observed in the isolated enzyme upon reduction of the binuclear site, and formation of the "peroxy," "ferryl," and "oxidized" states (Mitchell and Rich, 1994). We also consider some alternative possibilities for important transition states in the pump cycle (below).

We present the model as a fairly detailed structural scheme in which some functions are assigned to specific conserved amino acids. On a more conceptual level, however, the model has been designed to meet certain requirements of a proton pump mechanism, and in this sense, its solutions may be valid even if the precise details are not. This is especially true of the proposed interaction between the iron-bound oxygen intermediate and the uptake and release of pumped protons. For this reason, we will first present several general postulates upon which the design is based, before turning to the details.

1. Two-proton-per-electron stoichiometry is achieved by using histidine as the proton carrier. Proton translocation takes place in accordance with the DCP concept (Wikström and Krab, 1978, 1979), where redox-coupled proton binding occurs to a ligand of a metal center, and where movement of this ligand between input and output proton channels provides the necessary gating of the process. *A conserved histidine residue (H-284) is suggested to be this ligand, and to cycle between formal imidazolium (ImH_2^+) and imidazolate (Im^-) states* with translocation of $2\,H^+$ per cycle. *The Im^- residue is stabilized by forming a bond to Cu_B^{2+}, and by hydrogen bonding.*

2. The oxygen intermediate is directly involved in the proton pumping mechanism but does not bind the pumped protons covalently. In this model, the oxygen

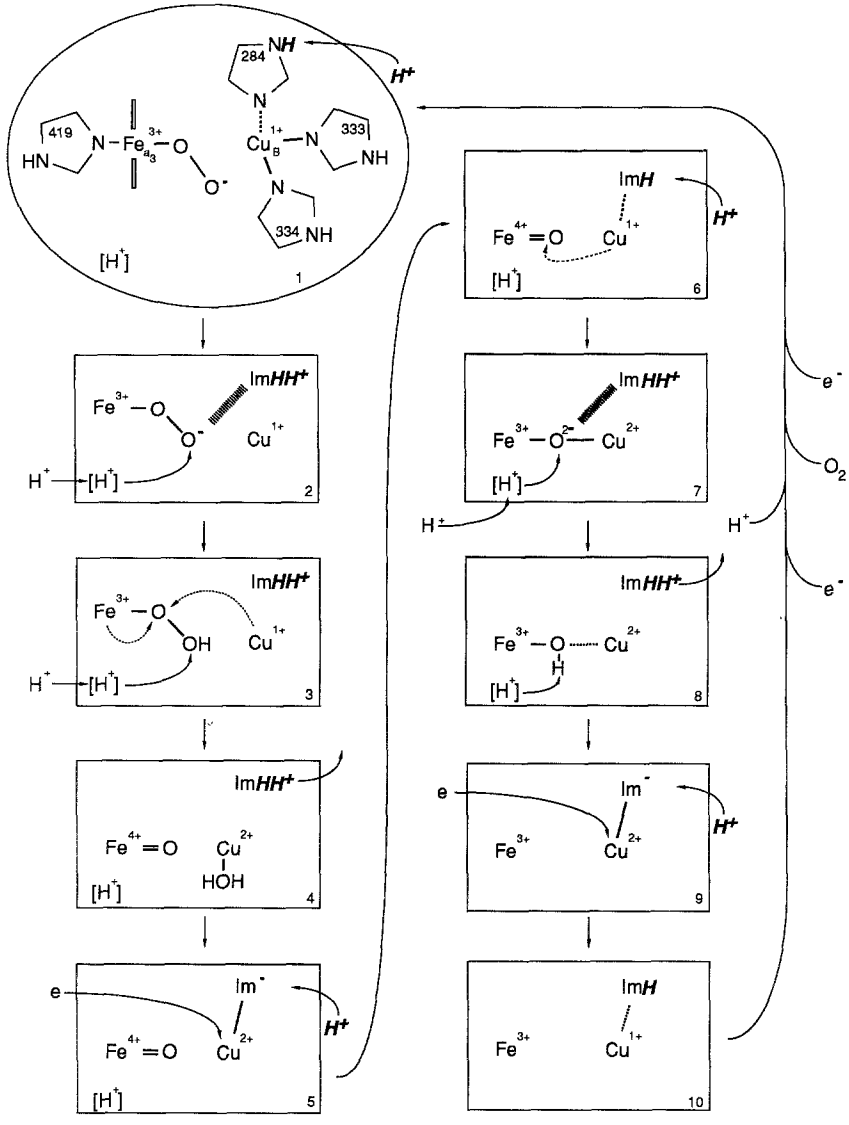

Fig. 3. The histidine cycle in a complete turnover of the enzyme: Boxes numbered in the lower right corner describe reaction states. State 1 is depicted in more detail structurally; only the changes to this are shown in states 2 through 10. Translocated protons (both input and output) are indicated by boldface italics on the upper right-hand side of the boxes. The scalar uptake of H^+ is indicated by arrows which approach from the left. The crossbar grate between the oxygenous ligand and the imidazole residue of H284 (states 2 and 7) indicates an electrostatic interaction. The reaction events are described in the text.

in the binuclear site is not simply a high-potential electron acceptor (as it is in the DCP models), but plays a mechanistic role in the proton pumping process. However, *the protons which are to be translocated* ("vectorial protons") *are never allowed to bind covalently to the oxygen.* Instead, the essential transition states are stabilized by a *purely electrostatic nonbonding interaction between* ImH_2^+ *and the oxygenous ligand.*

3. The electrostatic interaction between oxygen and protons, in turn, facilitates electron transfer to the iron–oxygen intermediate. We propose that both the peroxy and ferryl intermediates have low redox potentials until protonated; without protons they

are not good electron acceptors (Williams, 1985). However, because of the electrostatic interaction described above, protonation of H284 acts in an analogous way to direct protonation of the oxygen, and stabilizes the intermediate, raising its redox potential and making electron transfer possible. Thus, in the usual terminology used for DCP pumps, the electron transfer is gated by the uptake of protons at the pump site.

4. Binding of scalar protons to oxygen drives the ejection of the vectorial protons from the pump. The electrostatically stabilized transition state leads to electron transfer to the oxygen-liganded Fe_{a3} center, which is followed by uptake of protons to complete a step in the oxygen chemistry. The arrival of these scalar protons results in a neutral oxygen species at the iron, canceling out the interaction that had stabilized the ImH_2^+ state. (Note that this occurs twice during the reduction of O_2 to water, i.e., on conversion of "peroxy" to "ferryl" and "ferryl" to "oxidized.") In effect, the binding of scalar protons to the oxygen displaces the vectorial[4] protons from H284, since the latter were held there by the negative charge on the oxygen intermediate. The displaced protons move to the output channel and the external medium. This amounts to a *transfer of energy from the scalar to the vectorial protons.*

5. Access by scalar and vectorial protons must be controlled separately. The mechanism calls for a clear mechanistic (or temporal) separation between the protons which will bind to the reduced oxygen to make water (scalar protons) and those which will be translocated. The vectorial protons must not be allowed to react with oxygen because they would presumably remain there and no protons would be pumped. Similarly, the scalar protons must not arrive in advance of the vectorial protons, since stabilization of the vectorial protons on H-284 depends on the charge of the unprotonated oxygen intermediate. Early arrival of the scalar protons would prevent uptake of the vectorial protons and thus short-circuit the proton pump. In the model this separation is achieved by having two separate pathways of proton uptake, one for vectorial, and one for scalar protons. We propose that the pathway which leads the scalar protons to the oxygen intermediate imposes a kinetic delay, relative to vectorial proton input and electron transfer. This allows time for the proton pump element H284 to be protonated and those protons to be stabilized by the negative charge on the oxygen species before the oxygen becomes protonated, eventually leading to destabilization and ejection of the pumped protons.[5]

A MODEL FOR THE MECHANISM OF PROTON PUMPING

The key element in this model is that the negative charge of the oxygen intermediate, bound to Fe_{a3}, directly stabilizes the protonation of the pumping element. This interaction holds the vectorial protons in place up to the point when the scalar protons arrive at the oxygen, when the negative charge on the oxygen intermediate is neutralized and the vectorial protons are destabilized, forcing them into the external medium. We propose that this mechanism operates in essentially the same way during the P → F and F → O transitions of the cycle. By switching between formal imidazolium and imidazolate states, the proton pumping element (H284) is able to carry the necessary two protons in each of these one-electron steps. Figure 2 shows a generalized scheme for one cycle of this pump, which could be either the P → F or the F → O transition. (A more detailed scheme which traces this mechanism through the entire catalytic cycle follows as Fig. 3.)

The scheme begins with the pump element (H284) completely deprotonated (imidazolate) and bound as a ligand to Cu_B^{2+} (Fig. 2a). (The unprotonated H284 may be further stabilized by hydrogen bonding with the conserved tryptophan, W280, located one helical turn above H284 in helix VI of subunit I). Subsequent steps lead to reduction of Cu_B, and the formation of a negatively charged oxygen intermediate bound to Fe_{a3} (Fig. 2b). These events promote the uptake of two protons at H284, together with the de-binding of this ligand from Cu_B (Figs. 2b and c). The electrostatic interaction of the negatively charged oxygen intermediate and the protonated H284 stabilizes both of these species. One result of this is to raise the redox potential of the oxygen intermediate, making electron transfer to it favorable from Cu_B (or Fe_a). At this point, but not before, the oxygen intermediate is allowed to take up

[5] The electroneutrality principle of electronation and protonation of the enzyme's centers proposed by Mitchell and Rich (1994) is satisfied in a specific sequential fashion that is essential for the mechanism proposed here: first by electrostatic stabilization of a transition state necessary for electron transfer-coupled proton translocation, and then by secondary, direct protonation of oxygen.

(scalar) protons to form water (Fig. 2d). These protons neutralize the negative charge on the intermediate directly, thus *destabilizing* the protons on H284, with the result that they are ejected on the outer side of the membrane (Fig. 2e). H284, now in the deprotonated, imidazolate form, is again free to return as a ligand to the newly reoxidized Cu_B, and the cycle can begin again.

ENERGY TRANSFER IN THE PROTON PUMP

As described above, a crucial aspect of this model is that the energy for proton pumping comes directly from the oxygen chemistry, and not just from the flow of electrons through the redox carriers in the enzyme. The uptake of the two vectorial protons by H284 is made energetically possible by the nearby negative charge on the oxygen intermediate. Subsequently, the ejection of these protons takes place when the negative charge is neutralized by scalar protons binding directly to the oxygen intermediate to produce water. Thus, the energy of binding the scalar protons to form water is directly used to destabilize the vectorial protons, or in other words to pump them. One way to view this is that the oxygen intermediate first takes up the two vectorial protons, but instead of binding them they are kept on H284. These protons are then, in effect, displaced by the scalar protons which bind to the oxygen intermediate to form water. In order to achieve the full stoichiometry of proton pumping, it is important that the charged oxygen intermediate is *protonically* isolated from H284. The protons which go to make water *must not* come from H284, since this would short-circuit the pump. This, as well as the strong electrostatic interaction between ImH_2^+ and the negatively charged oxygen ligand, may be achieved by a short distance between these residues, and an intervening medium of low polarizability.

CONTROL OF SIDEDNESS IN PROTON PUMPING (PROTON GATING)

It is not enough to impart energy to the vectorial protons in order to translocate them; a pump must also ensure that protons are taken up from the correct side of the osmotic barrier and released on the opposite side. We propose that this control may be achieved in two ways: (1) access of H284 to proton input and output channels is controlled kinetically by controlling the distances and electrostatic environment that protons have to pass through to get from H284 to the two channels. Since proton uptake and release takes place in states that differ with respect to the valence and coordination of Cu_B, the geometrical switching at the copper should be able to create the necessary changes in the kinetics of proton input and output. (This is what has been called kinetic control; Blair *et al.*, 1986). (2) The rates of input and output can also be controlled by placing a proton well (groups with high pK) directly to the input side of the pumping element and a "barrier" (groups with low pK) directly on the output side (cf. Warshel and Russell, 1984). The result of this would be to reverse the natural occupancies of protons, making them plentiful on the input side and rare on the output side. Thus protons will be much more readily available from the input side whereas "proton holes" will be found much more easily on the output side. (Although this would also manifest itself in the kinetics as a difference in the relative rates of proton uptake and release in the forward and backward directions, the effect is achieved by controlling the populations in various states. This has therefore been called thermodynamic control; Blair *et al.*, 1986).

CONTROL OF SCALAR PROTONATION

If protons are allowed to arrive at the oxygen intermediate before H284 is fully protonated, the pump will switch prematurely from its (proton) input to output configuration, and less than the full complement of protons will be translocated. In order to prevent this we propose that the enzyme limits the rate of this scalar protonation so that there is time for the protonation of H284 to occur first. This could be accomplished by having separate pathways for the entry of vectorial and scalar protons into the active site, the latter of which is slower. The existence of such dual pathways would be consistent with what is observed in the D135N mutant of cytochrome bo_3 (Thomas *et al.*, 1994), where substitution of an apparently peripheral acidic residue leads to the abolition of proton pumping without a corresponding loss of oxidase activity (about 50% remains). We propose that D135, which is in the helix II–III "loop" of subunit I, may be part of the opening of the channel which carries protons from the input side of the membrane to the proton pump element, H284.

CONTROL OF ELECTRON TRANSFER IN THE PUMP (ELECTRON GATING)

In addition to proton gating, a redox-driven proton pump requires control of redox events (often called electron gating). If redox processes take place prematurely, this will uncouple the pump. (This is exactly analogous to what will happen if the scalar protons reach the oxygen intermediate prematurely.) In the present mechanism, electron gating is achieved, for the most part, by controlling the redox potential of the Fe_{a3}-oxygen intermediate species. We propose that, in the absence of protons, the redox potentials of the peroxy and ferryl intermediates are quite low, so that they may be reduced only when protons are available. However, these protons do not have to be scalar protons binding directly to the oxygen; the electrostatic interaction by which the charge on the oxygen stabilizes vectorial protons on H284 has the reciprocal effect of stabilizing the oxygen intermediate, in a way analogous to direct protonation. In the absence of direct protonation, which is slow, electron transfer will occur only when the proton-translocating element, H284, is "loaded" with protons.

A second aspect of electron gating in this mechanism is control of the reduction of Cu_B. If Cu_B were to be re-reduced before the vectorial protons on H284 leave, this might dissipate enough energy to prevent their ejection and decouple the proton pump. Although it appears that reduction of Cu_B from Fe_a through Fe_{a3} should be very fast, other mechanisms might work to prevent such premature electron transfer. Because such mechanisms might be difficult for the $P \rightarrow F$ and $F \rightarrow O$ transitions, they will be described below in the context of the complete oxygen-reduction cycle.

PROTON PUMPING AND THE OXYGEN-REDUCTION CYCLE

Reduction of the Binuclear Site

We begin our discussion of the scheme (somewhat arbitrarily, since this is a cycle) with box 9, the fully oxidized enzyme: In this state H284 is completely deprotonated (imidazolate), bound as a ligand to Cu_B^{2+}, and further stabilized by hydrogen bonding (perhaps to W280). The first electron to enter the binuclear site goes primarily to Cu_B which has the highest potential. This causes weakening of the Cu_B–H284 bond and uptake of a proton on H284 (box 10). The next step shows two electrons, one proton and oxygen being taken up by the enzyme.

Reduction of the Ferric-Peroxy Intermediate (Scission of the O–O Bond)

The overall result of this (see Babcock and Wikström, 1992; Verkhovsky *et al.*, 1994) is the ferric-peroxy intermediate (box 1), where an extra proton (lower left) has been taken up in the course of reduction of the enzyme's hemes. This peroxy compound is characterized by a negative charge on the ligand. This triggers the uptake of another proton from the vectorial input channel into H284, formally producing the imidazolium cation (ImH_2^+), whereby the bond to Cu_B is broken. A transition state is reached where ImH_2^+ is stabilized by an electrostatic interaction with the negatively charged peroxy ligand on Fe_{a3} (box 2). No proton transfer is possible between these species, but the peroxy ligand is likewise stabilized, which makes electron transfer favorable from Cu_B.

The reaction now proceeds by protonation via the scalar input channel and scission of the O–O bond with formation of the oxyferryl structure (Varotisis and Babcock, 1990; see also Babcock and Wikström, 1992 and references therein; box 3), in which there is very little, if any, negative charge on the oxygen (Sawyer, 1991). The electrostatic stabilization of ImH_2^+ is thus lost, its pK_a values are drastically decreased, and the newly formed Cu_B^{2+} binds Im^-, favoring release of two protons into the protonic output channel. Im^- is further stabilized by hydrogen bonding, possibly to W280, as mentioned above. Once H284 is bonded to Cu_B, the next electron enters the site. We propose that Cu_B may be reduced initially in preference to the oxyferryl because scalar proton uptake is still restricted, and the E_m of the oxyferryl could well be much lower than that expected from aqueous solution data. Premature re-reduction of Cu_B from Fe_a before the ejection of the vectorial protons could interfere with proton pumping, as mentioned above. This could simply be prevented by either slow (kinetic control) or inefficient (thermodynamic control) electron transfer to Cu_B^{2+} when it is unliganded to H284. Another possibility is that the presence of the oxyferryl structure between Fe_a and Cu_B (see Hosler *et al.*, 1993) prevents fast electron transfer between these two centers. If, as we suggest above, the E_m of the oxyferryl is low at this point, then the occupancy of the transiently reduced

Fe_{a3} would be expected to be small, thus reducing the effective rate of electron transfer to Cu_B, even though the rate constant was large.

Reduction of the Ferryl Intermediate

As shown in boxes 5 to 6, the reduction of Cu_B is accompanied by fast uptake of a proton from the vectorial input channel to the Im^- ligand, analogously to the events during primary reduction of the oxidized binuclear site (above). A second proton is taken up from the vectorial input channel by the imidazole (boxes 6 to 7), which then dissociates from Cu_B. These events provide an electrostatically stabilized transition state where electron transfer from Cu_B to Fe_{a3} is favorable; the product may be drawn as a negatively charged μ-oxo bridge between Fe_{a3} and Cu_B (box 7; Lee and Holm, 1993; Nanthakumar *et al.*, 1993; Karlin *et al.*, 1994). This is followed by protonation of the ligand via the scalar input channel, whereby the electrostatic interaction is broken. ImH_2^+ loses its protons into the output channel, and is stabilized as Im^- by bonding to Cu_B^{2+}, and hydrogen bonding, with cleavage of the μ-oxo bridge. These steps (boxes 8–9) may include the ferric hydroxide intermediate that has been detected in Raman experiments (Han *et al.*, 1990).

Must the Fourth Electron Pass through Cu_B?

In this version of the model, the fourth electron (which converts F to O) takes up residence on Cu_B before moving to the oxygen intermediate. However, an alternative scenario is also possible where the fourth electron reduces the Fe^{4+}=O, directly. In the absence of scalar protons, electron transfer to the ferryl might nevertheless be thermodynamically possible in a transition state where the two vectorial protons have been taken up by the H284 residue. The resulting ImH_2^+ would dissociate from Cu_B, and electrostatic interactions between oxygen and ImH_2^+ and the free coordination site at Cu^{2+} could stabilize the reaction product, in the same way as the μ-oxo species Fe^{3+}–O^{2-}–Cu^{2+}. Although in this alternative scheme, the proton pumping is no longer associated with a redox change in Cu_B, the main principles of the mechanism remain the same.

EPILOGUE

The model presented here (and see Wikström *et al.*, 1994) only bears resemblance to a redox loop or O-cycle type of mechanism insofar as the translocated protons interact *directly* with the electronated oxygenous ligand. However, the purely electrostatic nonbonding interaction suggested here not only contrasts with the covalent H^+/e^- linkage in a redox loop, but is a key feature of the model that may help to explain coupling of the redox events to proton translocation in a way that is still direct, but avoids the serious problems that arise from "mixing" the mechanistic fates of scalar and vectorial protons. On the other hand, DCP models have in the past been drawn to pass electrons to an acceptor in a manner coupled to proton translocation, where the role of the acceptor was simply that of a thermodynamically efficient electron sink. In contrast, in the present model the oxygenous ligand to the Fe_{a3} center plays a direct mechanistic role in proton translocation. Nevertheless, the histidine cycle shows many of the essential features that distinguish the DCP principle from the redox loop and its variants: The redox chemistry does not determine the sidedness or directionality of proton movements, which is the essence of vectorial redox chemistry, nor does the oxygen move together with the proton on the pathway of proton translocation, as in "O-cycle" type of redox loop models. Finally, the histidine cycle does not depend on global conformational changes or long-range energy transfer events in the protein as in redox Bohr or other "conformational" models of proton translocation.

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