MINI-REVIEW

Cytochrome Oxidase as a Proton Pump

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Abstract

The general structure of cytochrome oxidase is reviewed and evidence that the enzyme acts as a redox-linked proton pump outlined. The overall H^+/e^- stoichiometry of the pump is discussed and results [Wikström (1989), *Nature* **338**, 293] which suggest that only the final two electrons which reduce the peroxide adduct to water are coupled to protein translocated are considered in terms of the restrictions they place on pump mechanisms. "Direct" and "indirect" mechanisms for proton translocation are discussed in the context of evidence for redox-linked conformational changes in the enzyme, the role of subunit III, and the nature of the Cu_A site.

Key Words: Cytochrome *c* oxidase; redox-linked pump; proton translocation; electron transfer.

Introduction

Over the last few years three journals have devoted entire volumes to papers concerned with the structure and function of cytochrome *c* oxidase (Brunori *et al.*, 1985a; Vänngård, 1988; Brunori and Chance, 1988). This fact, taken with the continuous stream of research articles, reviews, and books (e.g., Wikström *et al.*, 1981) on the subject of this enzyme, gives a clear idea of its importance. This importance stems from the central role it plays in cell respiration and the complex and exquisite way in which the different aspects of its chemistry are combined and integrated. Not only does cytochrome *c* oxidase couple oxidation of cytochrome *c*, a single-electron donor, to the

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reduction of molecular O_2 to water, a four-electron reaction, while stabilizing and retaining possible toxic intermediates within its interior, but in so doing it also establishes a proton gradient across the inner mitochondrial membrane (for eukaryotes) by uptake of substrate protons and by vectorial proton transport. It thus transduces redox free energy into a proton electrochemical gradient subsequently used for ATP synthesis in accord with the chemiosmotic theory of Mitchell (1966).

Composition and Structure of the Enzyme

The functional unit of the enzyme isolated from mammalian sources comprises a complex set of thirteen different subunits, three of mitochondrial origin and ten of nuclear origin, together with two copper atoms and two hemea groups (for recent reviews see Azzi and Müller, 1990; Malmström, 1990a, b; and Capaldi, 1990). In addition, zinc and magnesium atoms are known to be associated with this complex (Einarsdottir and Caughey, 1985), and a third copper atom has also been reported (Steffens et al., 1987). The functions of these latter, if any, are as yet unknown (but see below). The functional unit is so-called because it can carry out all the redox chemistry involved in the reduction of molecular oxygen to water. The assembly of the complex involving the coordinated synthesis of the subunits and insertion into the membrane poses a fascinating problem which has recently been reviewed by Capaldi (1990).

The prokaryotic enzyme, which contains identical metal centres to those of the eukaryotic enzyme, has in general only three subunits, analogous to the mitochondrially coded subunits I, II, and III of eukaryotes. Subunit III may play a role as a template for correct assembly (Haltia et al., 1989). However, it can be removed (from both the eukaryotic and prokaryotic enzyme) without loss of any redox metal centres, hence localizing these centres in subunits I and II (see Fig. 1).

The role of the nuclear-coded subunits remains unclear. Work with mutants deficient in a particular subunit suggests that these may have a role in assembling the total complex (although the prokaryotic enzyme assembles without their aid) (Poyton et al., 1988).

The smaller, nuclear-coded subunits are suggested to have a regulatory role in the enzyme's activity and to be tissue specific (Kadenbach et al., 1987; Poyton et al., 1988).

Despite the considerable effort expended, only two studies giving direct structural information have been reported, namely the very successful electron microscopy investigation (Fuller et al., 1979) and the recent first report of a
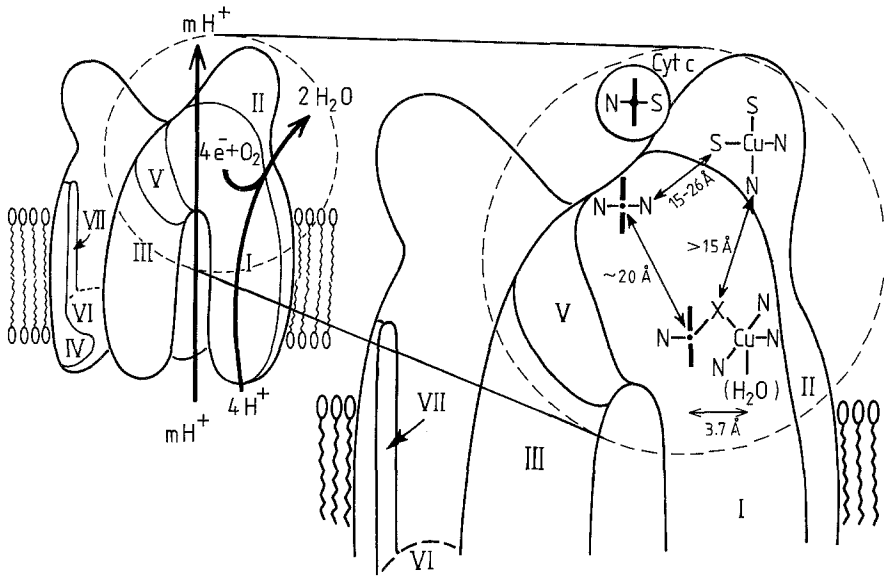
Fig. 1. The left-hand figure shows the overall disposition of the dimeric form of the enzyme spanning the inner mitochondrial membrane. The relative positions of the mitochondrially coded subunits (I, II, and III) are shown together with those of some of the subunits coded for in the nucleus. The figure also illustrates that the four protons used in the reduction of O_2 to H_2O are taken up from the matrix. In addition, protons are pumped from the matrix to the exterior of the mitochondrial inner membrane. The stoichiometry of the redox linked pump, m protons/ $4e^-$, is discussed in the text. To the right is shown an enlargement of a portion of the enzyme. The redox metal centres, Cu_A, cytochrome a, and the binuclear $Cu_B \cdot$ cytochrome a_3 centre, are shown together with their ligands and geometric relationship. Evidence suggests that Cu_A is in subunit II while the others are located in subunit I, although it is possible that cytochrome a may also be located in subunit II. The bridging ligand found between Cyt a_3 and Cu_B in the resting enzyme is probably a Cl^- anion. Cyt. c denotes electrons to cytochrome a and possibly Cu_A which are themselves in rapid redox equilibrium and in turn denote electrons to the oxygen-binding binuclear centre (a_3Cu_B). For clarity, the metal centres are somwhat displaced toward the aqueous phase. Cyta is probably just within the membrane.

crystal structure (Yoshikawa *et al.*, 1988). The E.M. method has given important information regarding the overall shape of the complex and its topology with respect to the membrane. However, as yet it does not have the capacity to yield the high-resolution pictures we really require if they are to be useful in understanding mechanism. Unfortunately, the crystal structures are as yet almost devoid of useful structural information.

Structural information has been obtained indirectly from consideration of the amino acid sequences of the individual subunits, e.g., hydropathy plots, and a knowledge of the positions of these evolutionary invariant amino acids which are considered, from spectroscopic measurements, to be metal ligands (e.g., Holm *et al.*, 1987). Comparison between the eukaryotic and the
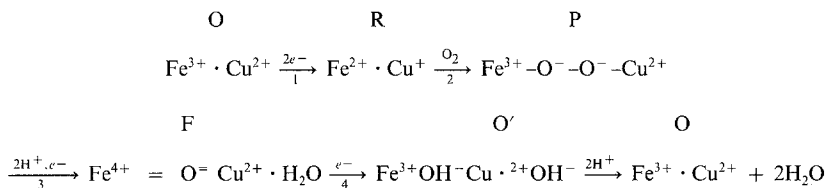
simpler prokaryotic systems has also proven extremely useful, as have crosslinking and antibody-binding studies. Spectroscopic techniques have yielded estimates of the distances between the metal centres (Brudvig *et al.*, 1984) as well as their ligands (see Brunori *et al.*, 1988 for review).

The picture which emerges, although lacking in resolution and indeed open to counterinterpretation in some respects, is summarized in Fig. 1.

The reaction mechanism for the reduction of dioxygen to water is reasonably well understood. For our purposes we need only indicate the major steps. The binuclear centre is reduced by electrons from the Cu_A and cyt. *a* centres, possibly in what is effectively a concerted two-electron process. Dioxygen binds to this ferrous/cuprous centre and is very rapidly converted to peroxide by electron transfer from the metals (Chance *et al.*, 1975). The peroxide remains bound to the now ferric/cupric centre (a peroxidic adduct P) which now receives an electron from Cu_A or cyt. *a* to form a three-electron reduced intermediate (in which the oxygen-to-oxygen bond is broken), here depicted as a ferryl species (F) (protonated) (Hansson *et al.*, 1982; Blair *et al.*, 1985; Wikström, 1988). Finally, a fourth electron (and two protons) completes the reduction to water and returns the binuclear centre to its original oxidized state (O).

This reaction sequence may be written as follows:

<div align="center">

Scheme 1

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$$\overset{O}{Fe^{3+} \cdot Cu^{2+}} \xrightarrow[1]{2e^-} \overset{R}{Fe^{2+} \cdot Cu^+} \xrightarrow[2]{O_2} \overset{P}{Fe^{3+}-O^--O^--Cu^{2+}}$$

$$\xrightarrow[3]{2H^+, e^-} \overset{F}{Fe^{4+}} = O^= Cu^{2+} \cdot H_2O \xrightarrow[4]{e^-} \overset{O'}{Fe^{3+}OH^-Cu \cdot {}^{2+}OH^-} \xrightarrow{2H^+} \overset{O}{Fe^{3+} \cdot Cu^{2+}} + 2H_2O$$

Here $Fe \cdot Cu$ refers to the binuclear centre comprising the heme group of cytochrome a_3 and Cu_B. O, R, P, and F refer to the oxidized, reduced, peroxy, and ferryl forms, respectively.

Evidence for Cytochrome c Oxidase as a Proton Pump

Figure 1 shows that in addition to protons taken up to form water, protons are also vectorially transported from the inside to the outside of the membrane which the enzyme spans.

In 1977, Wikström reported in the first of an important series of papers that cytochrome *c* oxidase (in rat liver mitochrondria), while catalyzing the oxidation of ferrocytochrome *c* by oxygen, also ejects protons into the "C-phase" outside the mitochondrial inner membrane (Wikström, 1977).

The complexities of studies using whole mitochondria, however, made the simpler system comprising cytochrome c oxidase reconstituted into liposomes an attractive alternative for investigating this proton pump. In this system, interference from other redox complexes is eliminated and both spectroscopic and pH measurements (either electrometrically or spectroscopically) are simplified. Using this system, Krab and Wikström (1978) demonstrated the redox-linked acidification of the external medium and provided evidence that these protons had been translocated from the interior of the liposome.

Experiments in which cation uptake (e.g., K^+) was shown to accompany acidification and to balance exactly the charge carried by protons across the membrane (Sigel and Carafoli, 1980), provided convincing evidence that the protons appearing in the outer phase had been transported from the interior of the liposome and were not due to scalar proton release, e.g., cytochrome c binding to the exterior portion of the enzyme (Fig. 1). Similar conclusions have been drawn from experiments using fluorescent lipid probes to monitor disappearance within the liposome (Thelen *et al.*, 1985).

Following the resolution of the complexities of using mitochondria (Wikström and Casey, 1985), it is now generally agreed that cytochrome c oxidase does act as a true pump, coupling the free energy of the redox reactions to the vectorial transport of protons from the inside to the outside of the inner mitochondrial membrane.

Stoichiometry of the Pump

The H^+/e^- ratio of the pump may in principle be measured rather simply by determining the number of protons ejected for each molecules of cytochrome c oxidized. One way to obtain the former is through a comparison of the magnitude of the initial pH change (corrected for scalar protons) seen on adding ferrocytochrome c to a solution of cytochrome oxidase liposomes with that seen on addition of a known quantity of acid to the solution. This procedure eliminates the need for a detailed knowledge of the buffering capacity of the system. The amount of cytochrome c oxidised (in e^- equivalents) is obtained directly from the absorbance changes in the α-band region of this protein.

Some problems arise with this method as there is a tendency for protons to leak back into the liposome, especially in experiments in which the enzyme turns over many times, requiring extrapolation to time zero. Nevertheless, this methodology has yielded a consensus, giving an H^+/e^- ratio of close to unity.

In order to avoid the problems of proton leak back into the liposome, Sarti *et al.* (1985) used a rapid kinetic method, stopped flow spectrophotometry,

to resolve proton ejection from the subsequent proton leak back. These experiments demonstrated that at the shortest times observed (millisecond time range) proton ejection and cytochrome c oxidation occurred synchronously and that the pump had a H^+/e^- stoichiometry of 0.93 ± 0.2.

The overall H^+/e^- stoichiometry of the pump therefore appears to be unity and thus in Fig. 1 the value of m is 4. The overall stoichiometry of the catalyzed reaction is therefore

$$4\text{cyt}.c^{2+} + O_2 + 8H^+_{\text{inside}} \longrightarrow 4\text{cyt}.c^{3+} + 2H_2O + 4H^+_{\text{outside}}$$
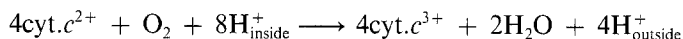
Although in each "turnover" of the enzyme four electrons are passed to dioxygen in order to reduce it to water and four protons are pumped out of the mitochondrion, it appears that these electron-transfer reactions are not equivalent in their proton tranlocating capacities. By varying the protonmotive force and redox potentials of mitochrondrial suspensions, Wilkström (1989) has stabilized the redox intermediates of dioxygen reduction O, P, and F (see Scheme 1). Using the ratio of [ATP] to [ADP] and [Pi] to set the value of the membrane protonmotive force and monitoring the relative concentrations of O, P, and F, Wikström (1989) determined the number of protons translocated by the individual electron transfer steps in the turnover cycle. In terms of Scheme 1, Wikström (1989) interpreted these results as follows: the initial two-electron electron transfer to the binuclear centre, step 1, is not coupled to proton translocation, nor is the initial reaction with dioxygen. However, the single-electron transfer steps, P → F and F → O (steps 3 and 4 in Scheme 1), each translocate two protons.

While the overall stoichiometry of this mechanism remains $4H^+/4e^-$, the four protons translocated are coupled with the transfer of only the final two electrons of the turnover cycle, i.e., those taking hydrogen peroxide to water. These results have several important consequences for describing the cytochrome oxidase proton pump. First, any mechanism for the pump must be able to justify two values, 0 and 2, for the ratio of protons translocated to electrons delivered to the binuclear centre. One way to do this is through an alternating electron transfer pathway. This, as we shall see, is a feature of some of the current proposals for the proton pump.

It should be noted, however, that the electron transfer in step 1 is to a site very different from the acceptor for steps 3 and 4. In these latter cases, the binuclear centre is complexed with intermediates of oxygen which greatly increases its redox potential, and hence the free energy available for proton translocation on reduction.

If, as Wikström (1989) reports, only the last two electrons used to reduce a molecule of oxygen to water are coupled to proton pumping, this will impose strict and valuable constraints on possible models for the pump. It is

most important therefore that these experiments be confirmed in other systems, e.g., vesicles, and if possible by other techniques.

General Mechanism of a Pump

The general theoretical framework for proton pumping, by which the passage of electrons down their electrochemical gradients is coupled to the "uphill" transport of protons, will be given elsewhere in this volume and have been reviewed extensively (Krab and Wikström, 1987; Blair *et al.*, 1985). Here we will briefly indicate some of the essential features which must constitute the mechanism of any such pump.

1. The pump must provide a way to couple redox reactions, i.e., the flow of electrons, to translocation of protons.

2. The pump must operate between two compartments, the boundaries of which are impermeable to the coupled ligands (electron and protons).

3. The pump must provide a "mobile element" coupled to the redox process which allows translocation of the protons between the compartments. The "mobile element" could range from a small configuration change around a specific metal centre to large conformational change of the whole protein complex, but in any case this element must provide a mechanism by which protons are moved from compartment to compartment and a gating or, better, an "alternating access" mechanism, providing a kind to "turnstile" to allow the undirectional passage of protons.

These considerations may conveniently be encapsulated into a general cubic model (Fig. 2) in which orthogonal edges correspond to electron-transfer steps, protonation steps, and transitions between enzyme states probably configurational in nature (see, for example, Krab and Wikström, 1987). The faces, on opposite sides of the cube, linked by edges depicting these transitions represent the electron and proton "input" and "output" states of the enzyme. The input state is accessible only to protons on the matrix side of the mitochrondrial membrane, while the "output" state is accessible only from the outside, c-side, of the membrane. Thermodynamic and kinetic constraints on the interconversion of some enzyme species, represented by the vertices of the cube, must ensure that only when the input state has accepted the requisite number of electrons and protons does it convert to the output state from which both electrons and protons and released prior to return to the "input" state. More complex schemes may, of course, be written (e.g., the "hypercube" with sixteen vertices) in which the output and input states of the electron and proton are distinct and systems such as that in Scheme 1 in which the P $\rightarrow$ F $\rightarrow$ O transition involve one electron and two protons require the additional complexity of nested cubes.
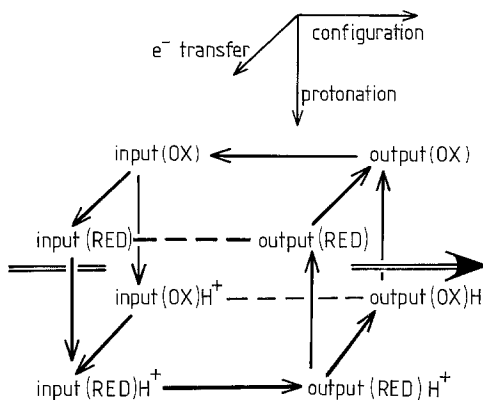
Fig. 2. A general cubic scheme for a redox-linked proton pump. The vertices of the cube represent enzyme species which are linked by edges representing electron transfer, protonation, and transitions from input to output states. Ox and Red represent the oxidized and reduced state of the metal site(s) to which pumping is coupled. The arrows indicate the direction of the redox free energy driven "flow" between species. The edges denoted by broken lines indicate transitions which are kinetically or thermodynamically unfavored and which, if operational, would uncouple proton translocation from the redox events.

Before examining details of possible pump mechanisms we first address some pertinent issues.

Redox-Linked Conformational Changes in Cytochrome c Oxidase

Several lines of evidence point to the fact that conformational/configurational changes, essential for any pump, accompany redox changes occurring during the turnover cycle of cytochrome c oxidase. Internal electron transfer from the cytochrome a and Cu_A centres to the binuclear oxygen binding site which is suggested to be rate limiting (Wilson et al., 1981; Brzezinski et al., 1986; Sarti et al., 1988) is sensitive to the redox and conformational state of the enzyme. For example, activation of the enzyme to a form termed "pulsed" by Antonini et al. (1977), is likely to involve changes in the spatial relationships between these sites and configurational changes around cytochrome a_3 and Cu_B.

Reduction of cytochrome a and/or Cu_A appear to trigger a number of significant conformational changes in the enzyme (Alleyne and Wilson, 1987; Kornblatt et al., 1988; Chan, 1988). These seem to be associated with opening access to the ligand-binding site (Jones et al., 1984; Jensen et al., 1984; Scholes and Malmström, 1986). Malmström has argued that it is the rate of these redox-linked conformational changes which limit the throughput of electrons within the enzyme and constitute the conformational change needed for the "alterating access" of a pump (see Malmström, 1990a, b).

Role of Subunit III in Proton Pumping

Subunit III, a large ($Mr \sim$ 29900 Da) hydrophobic protein coded for in the mitochondrion and containing six to seven regions considered to constitute membrane spanning helices (see Fig. 1), has been considered an important element of the pump. This conclusion was based on experiments in which dicyclohexycarbodiimide (DCCD) was found to inhibit (but not completely) the proton pumping activity of cytochrome c oxidase (Casey *et al.*, 1979). This reagent preferentially binds to subunit III at glutamate 90 a residue which lies within a hydrophobic region considered to be helical and which spans the membrane. This binding site is similar to a decapeptide which forms the DCCD binding site of the F_0 subunit of bovine mitochondrial ATPase.

However, experiments in which subunit III was removed by detergent or proteolysis (see Brunori *et al.*, 1987, for review) have revealed that subunit III-less cytochrome c oxidase incorporated into lipid vesicles retains its ability to pump protons. Fast reaction techniques, which enable proton ejection processes to be resolved from proton leak-back into the vesicle, have however, shown that the stoichiometry of the pump has dropped significantly, having an overall maximum value of $\sim 0.5 H^+/e^-$ (Sarti *et al.*, 1985). These results are in agreement with those of Solioz *et al.* (1982) who report that the two subunit enzyme from *Paracoccus denitrificans* exhibits a $0.5 H^+/e^-$ pump stoichiometry. We believe the simplest way to interpret these results is to suppose that subunit III acts as a conduit or part of a pathway which ensures that protons only reach the pump mechanism from the inside of the vesicle. If this pathway is impeded (e.g., by DCCD), then the pump is inhibited. If, however, the conduit is removed, then what is lost is the specificity in abstracting protons solely from the inside of the vesicle. The pump continues to eject protons with an unchanged stoichiometry of $\sim 1 H^+/e^-$, but protons are supplied to the pump from either side of the membrane. If proton access to the pump is random, with equal likelihood of protons reaching the pump mechanism from either side, then the expected apparent stoichiometry would be $0.5 H^+/e^-$, as found experimentally.

If this conclusion is correct, one might suppose that disruption of the proper spatial relationship between subunit III and the remainder of the complex, and hence the proton pathway to the pump, may yield an enzyme which exhibits a low H^+/e^- stoichiometry while yet retaining subunit III. There are derivatives of oxidase which seem to conform to this picture. For example, Sone and Nicholls (1984) observed that mild denaturation of the enzymes by heat treatment (43.5°C) reduced the pump stoichiometry to $0.5 H^+/e^-$.

Can Monomeric Oxidase Pump Protons?

Cytochrome c oxidase as isolated from beef is dimeric (see Fig. 1) but can be converted to the monomeric form by detergent treatment, exposure to elevated pH values, or by removal of subunit III. The enzyme from some other sources, most notably the Hammerhead shark, is isolated as a monomer while retaining all the major subunits (Wilson *et al.*, 1980; Georgevich *et al.*, 1983). When reconstituted into vesicles, both the shark and the subunit III-less bovine enzyme pump protons, indicating that the monomeric enzyme is competent to translocate protons (Bickar *et al.*, 1985; Sarti *et al.*, 1985). However, several lines of evidence suggest that reconstitution of the subunit III-less monomeric bovine enzyme promotes dimerization or at least some functional association, possibly of a transient nature (Antonini *et al.*, 1987; Finel and Wilkström, 1986). Recently experiments in which the rotational mobility of the enzyme reconstituted into the membrane of single-shelled liposomes have been used to monitor the aggregation state of the protein suggest that the shark and the subunit III-less bovine enzyme do under appropriate conditions remain largely as monomers within the membrane and retain their pumping capacity (Pride and Wilson, unpublished data). The balance of this evidence favors the view that monomers can pump protons, a conclusion also drawn by Moody and Rich (1989) on the basis of experiments in which only one monomer per dimer is active. If direct coupling (see below) were found to be the mechanism of the pump, the dimeric state would in any cases have little relevance to the pump as the full complement of metal centres and ligands is present within each monomer.

Mechanism for Coupling Redox-Free Energy to Proton Translocation

Models for the mechanism of proton pumping can be divided into two groups; direct-coupling models, in which proton translocation is carried out by protonation and deprotonation of the ligands of one or more of the metals during their oxidation and reduction cycle, and indirect-coupling models, in which oxidation and reduction reactions cause conformational changes in the enzyme structure which are transmitted to proton-translocating groups remote from the redox centre (see West, this volume). Carefully reasoned arguments for both model types, and even for hybrid models requiring both direct and indirect coupling, have been proposed, but no evidence for any of the models has been generally accepted as conclusive. To be consistent with the new information provded by Wikström (1989), both direct and indirect coupling will need to provide an H^+/e^- pump ratio of zero for the first two electrons delivered to the $Cu_B a_3$ site, while the subsequent two electrons are each coupled to two proton translocations. One way the H^+/e^- pump ratio might alternate between these values would be if there were two pathways for

internal electron transfer, only one of which was coupled to proton pumping. In fact, parallel pathways for internal electron transfer (i.e., from either cytochrome a or Cu_A to the bimetallic centre) have been shown to occur, both by rapid kinetic measurements (Hill and Greenwood, 1984) and by chemically modifying the Cu_A site to make it redox inactive (Gelles and Chan, 1985), and these features are manifested in several of the present models.

An example of a direct mechanism has been proposed by Mithcell *et al.* (1985), the essential feature of which is that oxygen acts as a hydrogen-transferring agent within the enzyme, thus effectively ferrying electrons and hydrogen ions from the cytochrome a and Cu_A centres to the cytochrome a_3 and Cu_B centres. The electrons effect the reduction of a second molecule of oxygen while the protons are ejected, providing the pump. The oxygen, which acts as the ligand (hydrogen) conductor, returns to the cytochrome a/Cu_A centres where it receives electrons from the metals and picks up protons, forming hydrogen peroxide. This O-loop mechanism thus couples the passage of electrons through the enzyme to proton transfer. The mechanism is clearly "direct" as the protons and electrons travel together as hydrogen atoms.

This model has been superceded by others (Mitchell, 1988) in which the site of coupling is at the a_3-Cu_B centre with the Cu_B site forming the osmotic barrier. Alternating access is accomplished by reorganization of the ligands around the Cu_B site as it passes through its redox cycle during the reduction of oxygen to water. Transitions of the Cu_B site from pyramidal through square planar to inverted pyramidal constitute the mobile element coupled to the redox reaction. The specific proposal by Mitchell (1988) has now been overtaken by the need to achieve a $2H^+/e^-$ stoichiometry for some steps. A modified version has now been proposed (Rich, 1990, personal communication) which maintains direct coupling at the $a_3 \cdot Cu_B$ centre and incorporates the stoichiometry of $2H^+/e^-$ for the $P \rightarrow F$ and $F \rightarrow O$ transitions as proposed by Wikström (1989).

At present, however, such direct mechanisms involving the a_3Cu_B site seem to require consecutive steps in which $2H^+$ are ejected from the a_3Cu_B site to the cystol side followed immediately by $2H^+$ uptake by the binuclear centre from the matrix. This puts heavy demands on the geometric and temporal requirements around the site if short circuits are to be avoided. These mechanisms have the advantage of being more amendable to direct experimental verification and, as Wikström (1989) has pointed out, the most parsimonious hypothesis is that the bimetallic centre is itself coupled to the proton pump, since it is the only site for which the four-electron transfers are necessarily nonequivalent. However, the mechanism through which this centre is coupled to proton translocation need not of course be direct.

Direct (or semi-direct) mechanisms involving both cytochrome a and copper A have been proposed. Babcock and Callahan (1983) have shown by

Raman spectroscopy that the strength of hydrogen bonding between the heme a formyl group of cytochrome a and some proton donor in the protein changes by some 110 mV on reduction of the iron. These authors have argued that this change in bonding strength may constitute part of the energy required for proton pumping and that changes in the hydrogen bonding configuration around the formyl group may act as a mobile element and gating mechanism transferring protons between proton channels with access to opposite sides of the membrane. Alternatively these configurational changes may be transmitted to remote sites for protonation and deprotonation as part of an indirect mechanism. Chan and his coworkers have presented evidence for the involvement of Cu_A in proton translocation (Gelles and Chan, 1985; Chan, 1988). They suggested that upon reduction a change in Cu_A liganding from Cys (see Fig. 1) to nearby Tyr allows a proton to be taken up from the matrix by the now free Cys anion. When Cu_A is reoxidised, the proton is transferred to the Tyr anion, to be released on the cytoplasmic side of the membrane during the next round of reduction and ligand exchange. However, this mechanism, as stated, yields a constant H^+/e^- pump ratio of 1 from each electron passed to the $Cu_A a_3$ site.

   An interesting and novel suggestion regarding the nature of the Cu_A site of oxidase may have a bearing on this problem. Recently it has been pointed out (Kroneck et al., 1988) that the copper centre in nitrous oxide reductase isolated from *Pseudomonas stutzeri* is in fact a mixed-valence [Cu(II), Cu(I)] binuclear centre. In addition to these authors, Kroneck et al. (1989) noted the similarity in the EPR properties between this site and Cu_A and indeed suggested that the Cu_A centre, although a single-electron acceptor, itself comprised both cupric and cuprous copper atoms. Although this view has been critisized (Li et al., 1989), other supporting evidence has been cited including the similarities of the MCD and EXAFS spectra and amino acid sequence homologies between the nitrous reductase and subunit II of cytochrome c oxidase (Scott et al., 1989). Were this sugestion to prove correct, one would at least have the possibility that transfer of a single electron into or out of this binuclear centre could induce coordination changes or ligand exchanges at both copper atoms and hence two protons transferred across the osmotic barrier by a mechanism similar to that proposed by Gelles and Chan (1985). Such speculations are perhaps unlikely in the light of the careful metal analyses which have recently been conducted in a number of laboratories (e.g., Yewey and Caughey, 1988) and which indicate that the copper-to-iron ratio (2.5 : 1) is too low to admit a binuclear Cu_A centre.

   Nevertheless, the comparison between one of the sites in the *P. stutzeri* protein and Cu_A of cytochrome c oxidase cannot be dismissed. Recent MCD data (Farra and Thomson, 1990) unequivocally indicate the identical nature

of these sites. Therefore it appears that either nitrous oxide reductase does not contain a binuclear mixed-valence copper centre, contrary to the suggestion of Kroneck *et al.* (1988), and Cu_A, as Fig. 1 indicates, is an isolated copper atom, or, if nitrous oxide reductase does contain a mixed-valence centre, so also must cytochrome *c* oxidase, i.e., "Cu_A" must be a mixed-valence dimer. Developments are awaited with interest.

In order to accommodate the $2H^+/e^-$ stoichiometry (Wikström, 1989) discussed above, Chan and Li (1990) in a review on proton pumping in cytochrome oxidases have now proposed a hybrid model. In this model, electron transfer to the binuclear centre occurs by an alternating pathway, with the first two electrons of an oxygen reduction cycle coming via cytochrome *a*, and the second two electrons passing through Cu_A (see Scheme 1). The electron transfer through cytochrome *a* are not coupled to proton translocation, while the electron transfers through Cu_A are each directly coupled to one proton translocation, and indirectly coupled to one proton translocation by subunit III. This model, including the role of Cu_A, the alternating internal electron transfer pathway, and the indirect coupling through subunit III, rests on (a) the earlier ligand exchange model involving Cu_A (Chan, 1988), (b) alternate pathways for electron transfer from either cytochrome *a* or Cu_A to the $a_3 \cdot Cu_B$ centre, and (c) the coupling of Cu_A to a second indirect pump, subunit III, in order to achieve the correct proton stoichiometry. Following this hypothesis, removal of subunit III, and hence a pumping element, lowers the pump stoichiometry to one H^+/e^- even for the steps involving Cu_A, and thus the overall stoichiometry becomes $0.5H^+/e^-$. However, the absence of discernible structural changes in subunit III on reduction of Cu_A (Wilson *et al.*, 1988) and the availability of simpler explanations for the lowered pumping stoichiometry on subunit III depletion (see above) may argue against the involvement of subunit III and hence this hybrid hypothesis.

Recently Malmström (1990a,b) has used the cubic scheme (Fig. 2) as a framework to argue eloquently the merits of the case for indirect coupling between e^- and H^+ transfer reactions. Starting with an input state (E_1) which can only equilibrate with protons from the marix side of the membrane and an output state (E_2) which can only equilibrate with protons from the cytoplasmic side of the membrane, he has suggested that the initial reduction of both cytochrome *a* and Cu_A causes a conformational change which, possibly through reorientation of transmembrane helices (Williams, 1987), allosterically affects the bimetallic centre. These allosteric interactions lower the potential of cytochrome *a* and raise the potential of cytochrome a_3 as well as decreasing the reorganization energy of the centres for oxidation and reduction. The combined effect of these changes is to increase dramatically the rate of internal electron transfer to the bimetal centre. However, in order to be consistent with an H^+/e^- ratio of zero for the first two electrons,
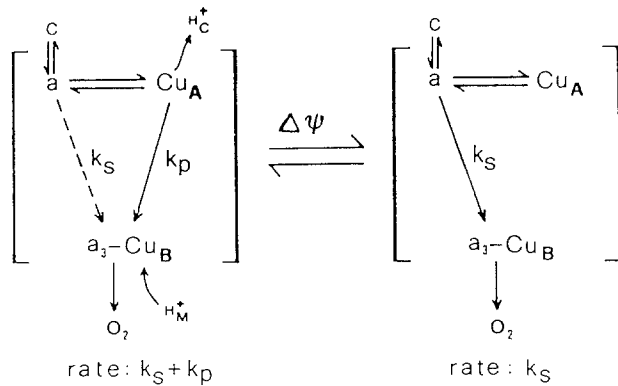
Fig. 3. Electron transfer pathways to the a_3-Cu_B site under control of membrane potential ψ. (From Malatesta *et al.*, 1988 with permission.)

he suggests that these first two internal electron transfers are uncoupled from proton pumping. The third and fourth internal electrons are transferred individually, probably through Cu_A. This transfer is slow in the input (E_1) state but 10^5 times faster in the output (E_2) state (Malmström, 1990b). If the transition from E_1 to E_2 is coupled to the binding of two protons, in this case at sites possibly remote from the metal centres, it would give an H^+/e^- ratio of 2 for these reactions, in keeping with the required stoichiometry.

The alternation of internal route by which electrons reach the a_3Cu_B site, with electrons arriving via cytochrome a being uncoupled from proton translocation and those via Cu_A coupled, is also central to a recent model proposed by Malatesta *et al.* (1988). In this model two conformations of the enzyme P (pumping) and S (slipping), characterized by their different internal electron transfer pathways, are in equilibrium. The position of this equilibrium is affected by the membrane ψ (Brunori *et al.*, 1985b), P and S forms (see Fig. 3) being equally populated at 80 mV (Sarti *et al.*, 1990). On increasing ψ to 160 mV (inside negative) the S state becomes fully populated. The potential thus acts in an analogous fashion to an allosteric effector or ligand to perturb conformational equilibrium and similarly displays saturation behaviour. These authors have provided evidence confirming that it is the internal electron transfer which limits the rate of enzyme turnover and that only reduction, and not oxidation of metal centres, is coupled to the pump.

The models proposed by Malmström, Brunori, and coworkers have similarities in that they share a branched pathway, a feature they also have in common with the model of Chan and Li (1990). However, in the former cases, the site of proton uptake is not made explicit.

A general concensus of most models for the pump would appear to be that reduction of the a_3Cu_B site is the essential feature which provides the

necessary free energy for proton translocation. Coupling is effected either (i) directly at the binuclear centre with proton ejection stoichiometric differences between the electron transfer steps due to the different chemical states that the a_3Cu_B site may take, or (ii) through the electron transfer pathway by changes at the electron donor sites, e.g., Cu_A, or by transmission to remote protonation/deprotonation sites, changes in stoichiometry throughout the cycle being achieved by different branches of the internal electron transfer pathway being used at different stages.

The near future will no doubt bring to light new features of the redox-linked proton pump of cytochrome c oxidase. We may confidently expect further elucidation of the internal electron transfer pathways and the effect of membrane potential on these. Site-directed mutagenesis will also allow specific changes in the coordination of the metal centres to be made, hence opening the way for an experimental investigation of the role of specific amino acid residues, whether they be metal ligands or remote from the metals, in proton pumping. However, all such approaches are at present hampered by the lack of high-resolution structural information. We must, therefore, hope that either X-ray diffraction or the exciting developments in electron microscopy (Henderson, 1990) will provide this fundamental information, without which a full understanding of the pump mechanism remains very unlikely.

Acknowledgments

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