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Protons, Pumps, and Potentials: Control of Cytochrome Oxidase

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Cytochrome *c* oxidase oxidizes cytochrome *c* and reduces molecular oxygen to water. When the enzyme is embedded across a membrane, this process generates electrical and pH gradients, and these gradients inhibit enzyme turnover. This respiratory control process is seen both in intact mitochondria and in reconstituted proteoliposomes. Generation of pH gradients and their role in respiratory control are described. Both electron and proton movement seem to be implicated. A topochemical arrangement of redox centers, like that in the photosynthetic reaction center and the cytochrome bc_1 complex, ensures charge separation as a result of electron movement. Proton translocation does not require such a topology, although it does require alternating access to the two sides of the membrane by proton-donating and accepting groups. The sites of respiratory control within the enzyme are discussed and a model presented for electron transfer and proton pumping by the oxidase in the light of current knowledge of the trans-membranous location of the redox centers involved.

KEY WORDS: Cytochrome oxidase; proteoliposome; respiratory control; membrane potential; pH gradient; cytochrome *c*; oxygen; proton pump.

INTRODUCTION

Cytochrome *c* oxidase transfers electrons from cytochrome *c* to molecular oxygen, and simultaneously generates a membrane potential and a pH gradient by translocating charges across the membrane in which it is embedded. As the resulting gradients inhibit the enzyme turnover, membranous oxidase is subject to a biophysical "feedback" control, classically known as "respiratory control". The chemical nature of the charge translocation—either electron or proton movement—remains problematic. Both, but more especially proton movement, can result directly from the operation of the catalytic machinery of oxygen reduction or from an "indirect" process linked to the energy-providing steps by conformational changes in the protein complex.

Respiratory control can be exerted in one of three ways:

(i) thermodynamically upon all or a large part of the overall process;
(ii) kinetically upon one or more rate-determining steps of the mechanism also involved in the charge-translocating process; and
(iii) allosterically by a rate reduction in a noncharge-translocating step, induced by the gradient of potential or pH.

In each case the control may involve either bulk ΔpH and $\Delta\Psi$ gradients or local gradients of different magnitude from the bulk gradients and equilibrating more or less slowly with the latter. In this short review we intend to argue that the charge movement must be of protons rather than electrons, and that there is no clear analogy with processes involved in the operation of either bacterial and plant photosynthetic centers or

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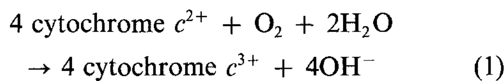
the cytochrome bc_1 complex. Both direct and indirect mechanisms of proton pumping may be involved; current evidence is inadequate to rule out one or the other. We shall also suggest that the control exerted by electrochemical gradients is not thermodynamic in origin but either kinetic or allosteric, and that effects of ΔpH and $\Delta\Psi$ are not equivalent.

The phenomenon of respiratory control was discovered and the concept developed by Chance and Williams (1955) as a consequence of their work on ATP synthesis by mitochondria. It was interpreted in terms of control of electron transfer in the respiratory chain by the formation of ligated species of the carriers less competent in electron transfer than their unligated counterparts. This earliest model of respiratory control was thus a kinetic one—the controlled site was necessarily a part of the energy-conserving process but the control did not require equilibrium to be established across the site in question. An alternative model—involving "thermodynamic" control—was developed by Wilson *et al.* (1979) as well as by Slater *et al.* (1973) in Amsterdam. Most of this theoretical and experimental work predated the acceptance of the chemiosmotic theory (Mitchell, 1968) as well as the discovery of proton pumping by the oxidase itself (Wikström, 1977; Krab and Wikström, 1978).

It is clear that it is either impossible or at least very difficult to bring the whole cytochrome oxidase reaction into equilibrium:



$$4 \text{ cytochrome } c^{2+} + O_2 + 2H_2O \rightarrow 4 \text{ cytochrome } c^{3+} + 4OH^- \quad (1)$$

In this respect it differs from the other two energy-conserving reactions of the mammalian respiratory chain, the NADH-Q reductase and the QH_2-cytochrome c reductase, both of which can be reversed at high ratios of [ATP]/[ADP][P_i]. Although overall equilibrium is not achieved in the mitochondrion, a form of thermodynamic control may nevertheless be possible for this enzyme if some rate-determining partial reactions can be brought close to equilibrium. Both "classical" mechanisms of control thus still remain possibilities. A third form of control was not considered by either group of schools when the phenomenon was first discovered. This, the "allosteric" type involves an inhibitory feedback by a product of energy conservation at a rate-determining step that may not be one of the steps involved in energy conservation. Such feedback could either involve a chemical product of energy conservation, such as ATP, or a physical product such as the membrane potential ($\Delta\Psi$) or pH gradient (ΔpH) known to act as intermediates in ATP synthesis.

The discovery of proton (Wikström, 1977) as well as charge (Hinkle *et al.*, 1972) translocation by the enzyme also raised questions concerning details of mechanism and control. Are the charge-translocating and H^+-translocating steps the same or different? Do these involve common or dual pathways? Azzi *et al.* (1984) found that the two could be experimentally distinguished using the inhibitor DCCD, and Nicholls *et al.* (1987) later claimed that there were two membrane potential generating processes, one sensitive and the other insensitive to DCCD. Does H^+ translocation occur with constant or with variable stoichiometry? If coupling between proton movement and electron transfer is close and obligatory, the stoichiometry involved should be constant. If, on the other hand, the coupling is more distant and voluntary, variable ratios of H^+ to e^- could occur, as a result of evolutionarily built-in "gearing" or of biophysically unavoidable "slippage" as postulated by Azzone's group (Petronilli *et al.*, 1991).

At least one of the evolutionarily conserved subunits of the enzyme, subunit III, has been regarded as having a functional role in controlling H^+ movement. The evidence for its direct role as a part of a proton channel has, however, been disproved by the observations (Gregory and Ferguson-Miller, 1988; Haltia *et al.*, 1992) that either subunit III-depleted enzyme or enzyme with subunit III whose evolutionarily conserved and DCCD-sensitive membrane-embedded glutamate residue is removed by site-directed mutagenesis can still pump protons efficiently. A controlling function of an indirect kind for this subunit remains a possibility and Prochaska's group remain strong protagonists of its functional importance (Prochaska and Fink, 1987).

METHODS AND MATERIALS

Beef heart cytochrome c oxidase was isolated essentially according to Kuboyama *et al.* (1972). Phosphatidylcholine (type IV-S, "Asolectin"), cytochrome c (type VI, horse heart), sodium ascorbate, TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine), and valinomycin were from Sigma Chemical Co. Nigericin was from Calbiochem, and pyranine (8-hydroxypyrene-1,3,6-trisulfonate) and 4-heptadecyl-7-hydroxycoumarin (HC) from Molecular Probes, Inc.

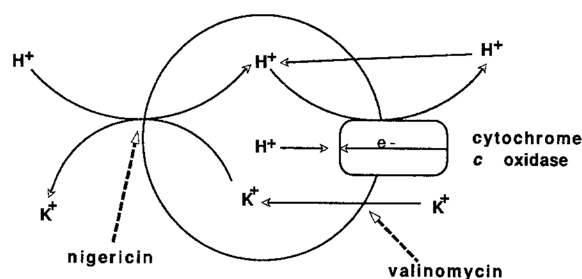


Fig. 1. The proteoliposomal steady state. The proton and potassium fluxes are required to establish the observed steady states. The intrinsic electrophoretic potassium permeability and electroneutral K^+/H^+ exchange can be catalyzed by valinomycin and nigericin, respectively (cf. Wrigglesworth *et al.*, 1990).

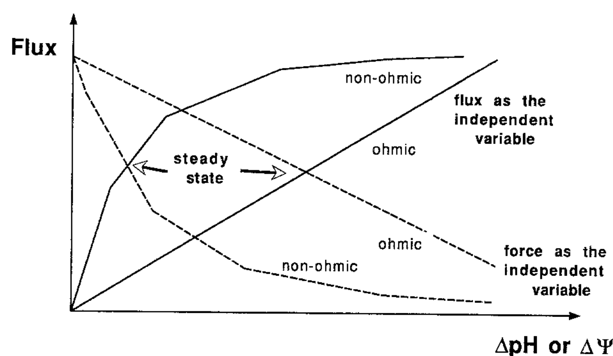


Fig. 2. Theoretical relationships between flux and membrane gradients (ΔpH or ΔΨ). The relationship between flux and gradient is linear in the "ohmic" and nonlinear in the "nonohmic" cases. If a steady state is established in any system, the respiration rate and controlling gradient will be determined by the intersection points shown where the generated and return fluxes are equal (cf. Nicholls *et al.*, 1990).

Vesicle Preparation

Asolectin was dissolved in chloroform; 3 mole % 4-heptadecyl-7-hydroxycoumarin in tetrahydrofuran was added (for HC-containing COV), and the solvent evaporated under N_2, leaving a lipid film. Dried lipid was dispersed in phosphate buffer, and cytochrome *c* oxidase then added (± 5 mM pyranine). The suspension was sonicated under N_2 (10 min, pulsed mode, 5°C), centrifuged to remove titanium and lipid aggregates, and external probe (if present) removed by Sephadex G-25 filtration.

Absorption and Fluorescence Spectroscopy

Reduction of cytochromes *c*, *a*, and $a + a_3$ was determined at 550–540 nm, 605–630 nm, and 445–470 nm, respectively. Outside-facing enzyme was reduced by ascorbate plus cytochrome *c* and inside-facing enzyme by TMPD. Fluorescences of pyranine and HC were measured either with a Perkin-Elmer LS-50 spectrofluorometer or in the multipurpose cuvette system described previously (Singh and Nicholls, 1985). Oxygen concentration in this cuvette was monitored by a Clark-type electrode.

DISCUSSION AND ILLUSTRATIVE RESULTS

When proteoliposomes respire in the presence of cytochrome *c* and ascorbate and in the absence of ionophores, both ΔpH and ΔΨ are generated in the steady state. Figure 1 summarises the processes involved. Cytochrome *c* oxidase pumps protons out of the vesicle. The protons can return either by electrophoretic or electroneutral pathways. Electroneutral return involves cation exchange, typically with potassium from the vesicle interior. The potassium is restored in the steady state because the electrogenic ΔΨ drives permeant cations as well as protons into the vesicle. An electrophoretic/electroneutral cycle of K^+/H^+ exchange is thus an intrinsic part of the proteoliposomal steady state. In the mitochondrion appropriate specific carriers are involved, but in the proteoliposome the intrinsic bilayer permeabilities control the final state, in which K^+ efflux equals K^+ influx. The former is determined by potassium ($\Delta[K^+]$) and proton (ΔpH) gradients, and the latter by K^+ gradient and membrane potential (ΔΨ). The permeability "constants" involved may themselves be functions of the gradients, especially ΔΨ.

What are the relationships between steady-state ΔpH or ΔΨ values and respiratory flux? Figure 2 illustrates the situation diagrammatically. If flux is varied by increasing the substrate level or decreasing an inhibitor concentration, the gradients created are functions of the increasing flux (Nicholls *et al.*, 1987). The increase may be linear ("ohmic"), or more characteristically nonlinear ("nonohmic"), especially in the case of ΔΨ. Conversely, the controlling gradient can be varied by adding an appropriate ionophore (small amounts of valinomycin in the presence of nigericin to abolish ΔΨ, or small amounts of nigericin in the presence of valinomycin to abolish ΔpH). If this is done, the flux increases to a maximal value at zero gradient. These profiles may also show an "ohmic" or "nonohmic" character. The steady-state flux and gradient will be obtained where the two characteristic curves cross, usually, but not necessarily, at a unique point.

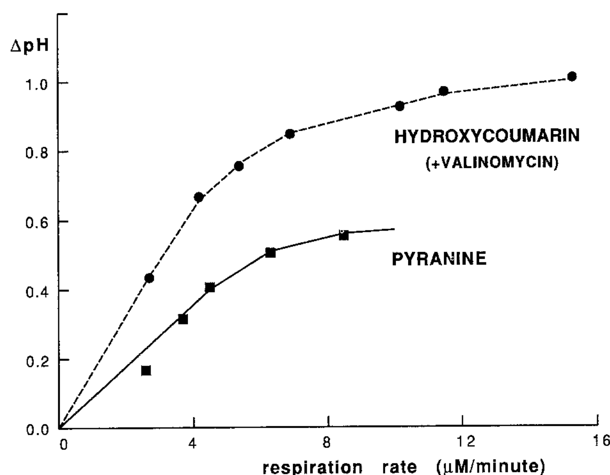


Fig. 3. Gradients of ΔpH in bulk phase and at the inner COV membrane surface as a function of respiration rate. The experiments shown were carried out in 50 mM potassium phosphate and 0.35 mM EDTA buffer at pH 7.9 and 25°C, with 0.1 mM TMPD, 1.8 mM ascorbate, and variable amounts of cytochrome *c* to modulate the measured flux. The vesicles contained ~1.2 mM phospholipid with 80 nM inlaid oxidase and 3 mole % HC (Butko and Nicholls, unpublished work).

Experimental examples of such relationships are presented in Nicholls (1990), as well as by Krishnamoorthy and Hinkle (1984). In general, the enzyme responds more "ohmically" to ΔpH than to ΔΨ. In Fig. 3 we see an example of the relationship between ΔpH and flux in pyranine-containing and in heptadecylhydroxycoumarin-containing vesicles. At low respiration rates the pH gradient produced is proportional to flux, whereas at higher rates ΔpH becomes flux-independent. In these vesicles ΔpH_{max} was ~0.55 pH units with trapped pyranine and up to 1.0 pH unit with a membrane-embedded probe. Is the apparent pH gradient a function of the intravesicular location of the probe molecules? If the steady-state pH at the inner membrane surface is higher than that in the bulk interior phase, then this must be a kinetic rather than an equilibrium difference—or it would be seen equally well in "passive" experiments. The possibility of kinetic differences between surface and bulk pH has been critically analyzed by Gutman and Nachliel (1990), but a recent study of bacteriorhodopsin (Heberle and Dencher, 1992) suggests that local pH gradients differing from bulk ones may also be observed in that pump.

The nonlinear plots (Fig. 3) also show that ΔpH does not develop proportionately to enzyme turnover. If two parallel paths for electron flow are associated with two cytochrome *c* affinity regions, only one, operating at low turnover, may be capable of proton pumping. This would give changes in $\Delta H^+/e^-$ ratio with turnover, as some have proposed (Murphy and Brand, 1988a, b; Papa *et al.*, 1991), and reflect differences between the two putative cytochrome *c* binding sites (for a review see Cooper, 1990), such that only one ("high affinity") is "coupled." Alternatively, one path may couple electron transport to proton pumping, but at high turnover rates the pump slips, and less than 1.0 H^+ is moved per electron transferred (Pietrobon *et al.*, 1982).

In a third mechanism, H^+ backflow varies (cf. Wrigglesworth *et al.*, 1990). As turnover increases, so does ΔΨ, and it becomes more difficult to pump protons out. If the lipid bilayer behave nonohmically (Krishnamoorthy and Hinkle, 1984; Singh and Nicholls, 1986), its conductivity will increase with the transmembrane potential. Such a mechanism implies that even during high turnover, when the relationship between ΔpH and flux is nonlinear, cytochrome oxidase may operate with unchanged kinetics and H^+/e^- ratio. It cannot account for nonlinearity in the presence of valinomycin (Fig. 3), unless local potentials are not dissipated by the ionophore.

Calculations of ΔpH_{max} indicate that the vesicles cannot generate a similar ΔpH at all values of flux. If ΔpH/[*c*] intercepts from Eadie–Hofstee plots are multiplied by the K_m values in the rate measurements, ΔpH_{max} values are obtained which *would* be achieved if the system remained ohmic at high flux rates. "ΔpH_{max}" values calculated in this way are very large (⩾8.0 pH units in the presence or absence of valinomycin). Such values are thermodynamically unrealizable; the enzyme cannot maintain the expected ΔpH values at high flux proportionate to those achieved at low flux.

An adequate kinetic model for the active system of Fig. 1 remains to be developed (cf. Nicholls, 1990; Wrigglesworth *et al.*, 1990). In the simplest case we expect that in the steady state, the rate *J* of charge efflux will be given by

$$J = v_{resp} \times n \tag{2a}$$

where *n* is the number of charges moved per turnover (between 0 and 2 if v_{resp} is in $e^-/aa_3/s$). This flux should be equal to the rate of proton or cation efflux, $k \times \Delta pH \times B$ or $k' \times \Delta[OH^-] \times B$, according to

$$n \times v_{resp} = k \times \Delta pH \times B \quad \text{or(?)} \quad k' \times \Delta[OH^-] \times B \tag{2b}$$

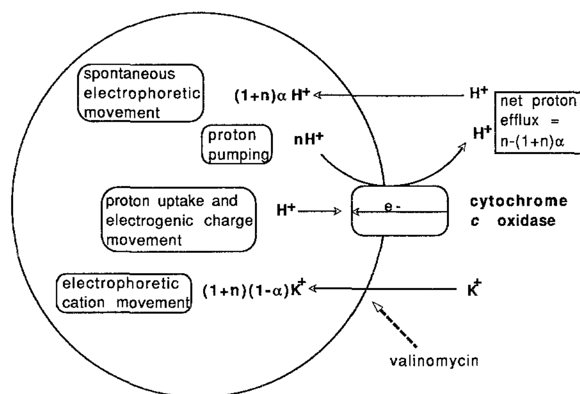


Fig. 4. Charge movements occurring in a proteoliposome during a proton "pulse" experiment. A cytochrome *c* oxidase vesicle is shown with the electron, proton, and cation movements during and following a proton pulse experiment involving the transfer of a single electron to oxygen (see text). n = intrinsic proton pumping activity (H^+ ions translocated per electron). α = proportion of electrophoretic flux carried by protons. $(1 + n)(1 - \alpha)$ = proportion of electrophoretic flux carried by cations.

where B is the buffering power of the system. If we know B and obtain k from the rate of ΔpH collapse upon anaerobiosis, we can calculate n from data such as that in Fig. 3 according to

$$n = \Delta pH_{ss} \times k = B/v_{resp} \tag{2c}$$

When reasonable values of B are inserted into Eq. (2), the value of n obtained is rather low (Nicholls *et al.*, 1990). There are, however, substantial variations in estimated buffering power (cf. Grzesiek and Dencher, 1986). It is therefore difficult to decide directly whether H^+ pumping is continuing at its stoichiometric rate when the pH gradient is present. Other methods are needed to resolve this question (cf. Murphy and Brand, 1988a, b).

Steverding and Kadenbach (1990a, b) have concluded that proton translocation cannot take place at all in the absence of a compensating charge transfer, for example, of K^+ by valinomycin. They report apparent "reverse" H^+ transfer in proteoliposomes pulsed with oxygen or cytochrome *c* in the absence of ionophores, involving a fast alkalinization, followed by a slow alkalinization to the characteristic overall scalar 1.0 OH^-/e^- [Eq. (1)]. But for every H^+ pumped out by the enzyme, two charges are moved (Fig. 4). In the steady state (+ valinomycin), or after an O_2 pulse, up to two K^+ ions enter for every emerging H^+ (Sigel and Carafoli, 1978; Proteau *et al.*, 1983). The resulting alkalinization is compensated by electroneutral cation–proton exchange (Fig. 1). If no single ionophore

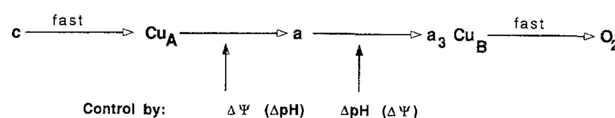


Fig. 5. The electron transfer pathway: cytochrome *c* oxidase. A linear pathway (Hill, 1991) is assumed and the probable sites of ΔpH and $\Delta\Psi$ control (Gregory and Ferguson-Miller, 1989; Capitanio *et al.*, 1990) indicated.

activity dominates ion movement, the extent and direction of external pH change will depend upon the permeabilities of the compensating ions. Some K^+ ions enter the vesicles even without valinomycin (Singh and Nicholls, 1984). Both protons and cations must move fairly rapidly during turnover (Wrigglesworth *et al.*, 1990).

If $n/H^+/e^-$ are pumped out ($n \leqslant 1.0$), then $1 + n$ charges, including both H^+ and K^+, must return (Nicholls *et al.*, 1987). The α ratio, $[H^+]/([H^+] + [K^+])$ will depend upon H^+ and K^+ permeabilities, differing in different COV preparations and experimental conditions. When $(1 + n)\alpha < n$ (i.e., if $\alpha < 0.5$ when $n = 1$), then $n - (1 + n)\alpha$, the "immediate" external acidity after a pulse, will exceed 0, and rapid external acidification will occur. If $(1 + n)\alpha > n$, then $n - (1 + n)\alpha < 0$, and external alkalinization will be observed (see Fig. 4). This is what is found (Nicholls and Wrigglesworth, 1982). No special significance should be read into H^+ transfer stoichiometries determined in the absence of ionophores. Positive, zero (Hinkle, 1981), and negative (Steverding and Kadenbach, 1990a) acidifications can all be seen, and do not reflect intrinsic properties of the pump.

At what level is respiratory control exerted? Figure 5 summarizes the electron transfer pathway within the oxidase as determined by a number of workers, including most recently Hill (1991) and Han *et al.* (1990). The reduction of Cu_A by cytochrome *c*, once the initial complex has formed, is rapid (Hill, 1991). The reaction between reduced enzyme and oxygen is also rapid (Hill *et al.*, 1986); a bimolecular rate of $1.5 \times 10^8\,M^{-1}\,sec^{-1}$ assures [oxygen] independence down to submicromolar levels (Petersen *et al.*, 1974), and the first electron transfer step within the oxygenated complex proceeds at $> 30{,}000\,sec^{-1}$. Internal reaction steps are slower; reduction of cytochrome *a* by Cu_A proceeds at $10^4\,sec^{-1}$, and is reversible. Oxidation of cytochrome *a* by the binuclear cytochrome a_3Cu_B center is also slow, and probably

contributes a major part to the rate control of the enzyme under deenergized conditions.

The equilibrium between cytochromes *c* and *a* can be determined either under inhibited conditions (Rich *et al.*, 1988) or in the proteoliposomal steady state (Nicholls, 1990). In the presence of cyanide the equilibrium is largely $\Delta\Psi$-dependent (Hinkle and Mitchell, 1970), at the Cu_A–cyt. *a* rather than the cyt. *c*–Cu_A level (Rich *et al.*, 1988). In the steady state, a ΔpH effect can be seen, as abolition of $\Delta\Psi$ by valinomycin addition does not raise the apparent redox potential by as much as does abolition of both $\Delta\Psi$ and ΔpH (Nicholls, 1990). The level of cytochrome *a* reduction during respiration depends upon the system used. In proteoliposomes appreciable reduction is seen (Gregory and Ferguson-Miller, 1989; Capitanio *et al.*, 1990; Nicholls, 1990). In mitochondria, cytochrome *a* appears to stay largely oxidized, even at relatively high flux levels (Morgan and Wikström, 1991). This may reflect the higher turnover of the mitochondrial enzyme and the difficulty of ensuring high levels of reduction of the endogenous cytochrome *c*. In the steady state proteoliposomal oxidase shows a special sensitivity to ΔpH. Both fully controlled and fully released systems have maximal reduction levels of up to 35%. In the absence of ΔpH (+ nigericin) but with full ($\sim$ 180 mV) $\Delta\Psi$, steady-state reduction decreases. On the other hand, in the absence of $\Delta\Psi$ (+ valinomycin) but with substantial ($\sim$0.6 pH units) ΔpH, steady-state reduction is markedly increased (to 50% or greater). Valinomycin addition to respiring proteoliposomes induces an initial rate increase (and oxidations of cytochromes *c* and *a*) followed by a progressive slowing down and an increase in cytochrome *a* reduction as ΔpH is established (Gregory and Ferguson-Miller, 1989; Capitanio *et al.*, 1990; Nicholls, 1990). This is consistent with the control point in Fig. 5.

Corresponding kinetic events are not easy to follow, but Brunori's group have made the appropriate attempt (Antonini *et al.*, 1991; Sarti *et al.*, 1992). Stopped-flow experiments involving re-reduction following oxidation of reduced enzyme show that valinomycin accelerates approximately tenfold the second phase of the biphasic process monitored both by oxidation of cytochrome *c* and reduction of the binuclear center (trapping the reduced form with CO). It may be noted that the overall reaction being observed is only a partial one; steps involving the oxygen reaction with the binuclear center and associated proton pumping (Wikström, 1989) are not involved. It may therefore

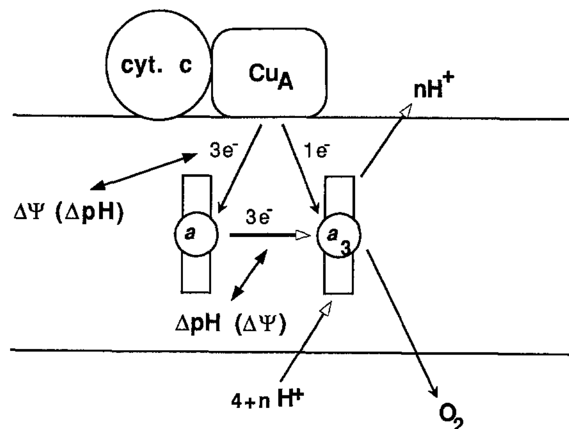


Fig. 6. Membrane arrangement and control: cytochrome *c* oxidase. The diagram summarizes current information [cf. Babcock and Wikström (1992) for a recent thorough review] concerning the topochemical arrangement of the cytochrome oxidase metal centers within the membrane. Unlike the simple scheme of Fig. 6, we assume here that at least one of the four electrons involved in reduction of oxygen proceeds from Cu_A to the binuclear center bypassing cytochrome *a*. All the charge separation occurs either in the reduction of the cytochrome *a* center or in proton flux to and from the binuclear center. The electrons passing between the two heme groups do so almost at the same membrane depth.

be suggested that the two rate-limiting steps are the first two in the sequence of Fig. 5. Addition of one electron from cytochrome *c* will be at the level of Cu_A and $\Delta\Psi$-insensitive; addition of a second electron at cytochrome *a* will be $\Delta\Psi$-sensitive. The succeeding electron transfers to the binuclear center may not be sensitive to either $\Delta\Psi$ or ΔpH, unless proton translocation is involved. This is because the probable arrangement of metal centers across the inner mitochondrial membrane is as sketched in Fig. 6 (cf. Babcock and Wikström, 1992). Electron transfer between cytochrome *a* and the binuclear center is parallel to, and not across, the membrane. This interpretation thus differs from that offered by Sarti *et al.* (1992) who believe that the $\Delta\Psi$-sensitive step is that between cytochrome *a* and the binuclear center. Further experiments will be required to settle the question.

In the overall reaction (Fig. 6) both electron and proton movements take place and are constrained by both types of transmembrane gradient. During the reduction of one dioxygen molecule, four protons are pumped across the membrane, probably during the reduction of the initial intermediate compound(s) to the ferric state. Control is exerted at two levels: (i) electron transfer to cytochrome *a* and (ii) the proton-pumping cytochrome *a* to binuclear center

step. Whether the latter process is "direct" or "indirect" cannot be determined from the data presently available. The fact that two protons seem to be moved at each of two one-electron steps (Wikström, 1989) *may* support a mechanism of a more indirect type.

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