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SHORT REVIEW

Molecular Mechanism of Proton Translocation by the Cytochrome System and the ATPase of Mitochondria. Role of Proteins[1]

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Introduction

It is agreed today that protonic currents represent a major device for energy transfer in coupling membranes. The hypothesis (Mitchell, 1966; 1979) that oxidative and photosynthetic phosphorylation are mediated by cyclic proton flow between redox enzymes and reversible ATPase complexes, each acting, separately, as proton translocators, has met with general acceptance (Boyer *et al.*, 1977). It is, however, disputed whether protonic coupling involves bulk-phase to bulk-phase thermodynamic potential difference of protons, without direct interaction of the redox of ATPase enzymes (Mitchell, 1966, 1979), or if it takes place within or at the surface of the membrane, thus involving some closer interaction of the two systems in membrane microenvironments (Williams, 1978; Van Dam *et al.*, 1978). Experimental support in favor of the latter view is thought to be provided by observations showing no direct relation between the rate of phosphorylation and the bulk-phase $\Delta\mu H^+$ (Sorgato *et al.*, 1980; Westerhoff *et al.*, 1981), as well as variability of the phosphate potential to the $\Delta\mu H^+$ ratio in mitochondria (Azzone *et al.*, 1978; Van Dam *et al.*, 1981) and chromatophores (Melandri *et al.*, 1981). It should, however, be pointed out that these observations, provided they do not derive from technical problems, can still be explained by bulk-phase chemios-

[1]Abbreviations: Adenylylimidophosphate, AMP-PNP; Sodium dodecylsulfate, SDS.

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mosis on the basis of variable H^+/e^- and H^+/ATP stoicheiometries (see Van Dam et al., 1981 and Papa, 1976).

According to the chemiosmotic rationale, coupling, in redox and ATPase enzymes, between chemical catalysis and vectorial proton translocation is an immediate consequence of diffusion of the same substrates and/or chemical groups along specifically oriented conduction pathways through the catalytic system anisotropically arranged in the membrane (Mitchell, 1966; 1979). On this basis Mitchell developed the protonmotive redox-loop model (Mitchell, 1966), the quinone cycle (Mitchell, 1976), as well as the protonmotive hydrodehydration loop for the ATPase complex (Mitchell, 1966; 1979). These hypothetical models should now be looked at in the light of the new progress made in the elucidation of the protein structure of redox and ATPase enzymes. The question arising is whether the new body of knowledge can still be accommodated within the framework of chemiosmotic models and, if so, what type of revision these models might need. Furthermore one might ask whether different models, essentially based on cooperativity principles (Boyer *et al.*, 1977 Papa, 1976; Wikström *et al.*, 1981), can better fit the molecular characteristics of the energy-transfer enzymes.

The Cytochrome System

In Fig. 1 a schematic view of the structure of the cytochrome system of mitochondria is presented. It can be seen that the apoproteins gain particular emphasis here with respect to the formalism of the chemiosmosis, which deals essentially with redox prosthetic groups. The b–c_1 (Bell *et al.*, 1979) and cytochrome c oxidase (Fuller *et al.*, 1979) complexes of the cytochrome chain have similar oblong shapes traversing the membrane. Both complexes have four well-characterized redox centers and eight (Marres and Slater, 1977) or nine (Leonard *et al.*, 1981) and seven (Capaldi *et al.*, 1977) or 12 (Steffens *et al.*, 1979; Merle and Kadenbach, 1980) polypeptides respectively, whose amino acid sequence is being actively resolved (see Von Jagow and Sebald, 1980, and Azzi, 1980, for a review).

The b cytochrome protein of the b–c_1 complex (Leonard *et al.*, 1981; Von Jagow and Sebald, 1980; Von Jagow and Engel, 1981) (subunit III) (Bell *et al.*, 1979) spans the membrane and has two distinct heme groups (Von Jagow and Sebald, 1980; Von Jagow and Engel, 1981): b_{566}, apparently displaced toward the P side, and b_{562}, toward the N side of the membrane (Mitchell, 1976; Papa *et al.*, 1981c). Cytochrome c_1 is identified with subunit IV (Bell *et al.*, 1979), located at the P side, where it interacts with cytochrome c shuttling between the two complexes. The fourth essential redox component is an Fe–S protein (Rieske *et al.*, 1964; Trumpower *et al.*, 1980), subunit V

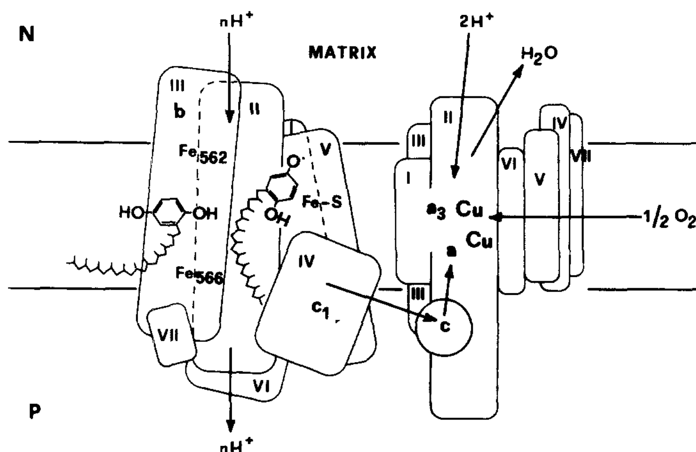

Fig. 1. Scheme showing the minimum polypeptide composition of the b–c_1 complex (Bell *et al.*, 1979; Marres and Slater, 1977) and cytochrome *c* oxidase (Capaldi *et al.*, 1977; see also Steffens *et al.*, 1979, and Merle and Kadenbach, 1980) of beef-heart mitochondria and their tentative membrane topology (Bell *et al.*, 1979; Fuller *et al.*, 1979; Azzi, 1980; Capaldi, 1981). The location of the redox components is drawn on the basis of data from Papa *et al.*, 1981c; Trumpower *et al.*, 1980; Ohnishi and Trumpower, 1980; Winter *et al.*, 1980; and Steffens and Buse, 1979. The matrix side of the membrane is indicated as negative (N) and the cytosolic side as positive (P) with reference to the sites of proton uptake and proton release by the cytochrome system.

(Bell *et al.*, 1979). Recently a special semiquinone of the b–c_1 complex has been discovered (Yu *et al.*, 1978). This has a midpoint potential of +150 mV at pH 7 and is probably associated to the Fe–S protein (Ohnishi and Trumpower, 1980).

In cytochrome *c* oxidase subunits I, II, and III traverse the membrane (Fuller *et al.*, 1979; Azzi, 1980; Capaldi, 1981). Copper is apparently associated to subunit II (Winter *et al.*, 1980; Steffens and Buse, 1979), and hemes a and a_3 to subunit II and perhaps I (Winter *et al.*, 1980). Cytochrome *c* binds to subunits II and III (Azzi, 1980; Capaldi, 1981).

Evidence has been produced showing that the protons for protonation of reduced oxygen to H_2O reach the reaction domain from the matrix or N phase (Papa, 1976; Mitchell and Moyle, 1970; Papa *et al.*, 1974a). The site where ubiquinol is oxidized by electron carriers and protons separate from electrons is, on the other hand, unknown.

In addition to these protolytic oxido-reductions the cytochrome system displays a different category of scalar proton-transfer reactions, these arising from cooperative linkage between redox transitions of the metals and protolytic events elsewhere in the redox enzymes (Papa, 1976; Papa *et al.*, 1973).

Linkage between protolytic events and oxido-reduction, called redox Bohr effect by analogy to the phenomena exhibited by hemoglobin (Perutz, 1976; Kilmartin, 1976), appears to be a general attribute of electron-carrying proteins (Papa *et al.,* 1977a; Dutton and Wilson, 1974). It is exhibited by *b* (Urban and Klingenberg, 1969) and *c* cytochromes (Papa *et al.,* 1977a), Fe–S proteins (Prince and Dutton, 1976) and cytochrome *c* oxidase (Dutton and Wilson, 1974; Van Gelder *et al.,* 1977) although this occurs, in some cases, outside of the physiological pH range (Papa *et al.,* 1977a; Dutton and Wilson, 1974).

Papa *et al.* (1973; 1976; 1977a) have proposed (see also Wikström *et al.,* 1981; Von Jagow and Sebald, 1980) that these linkage phenomena can be involved in proton pumping. It should be noted that the terms *proton pump* and *proton pumping* are used in this paper in the general sense for the process of active transmembrane proton translocation, i.e., metabolism-driven uphill proton transport, without reference to the specific molecular mechanism. To accomplish proton pumping (Fig. 2), a redox-linked protolytic group has to be exposed at one side of the membrane, the negative, when electrons arrive at the metal; the p*K* of the group is consequently enhanced, and protons are taken up at this side. Then the protonated group, or another which accepted protons from the first, attains exposure at the opposite side of the membrane when electrons leave the metal, the p*K* is lowered, and protons are released at this side (Papa, 1976; Wikström *et al.,* 1981).

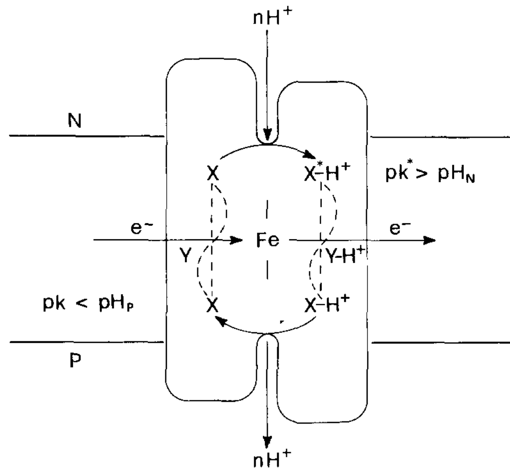

Fig. 2. Scheme describing the minimal possible steps of a redox proton pump (vectorial Bohr mechanism) (Papa, 1976) based on linkage between oxido-reduction of the metal and protolytic events in the apoprotein of a redox carrier (Redox Bohr effects, Papa, 1976; Papa *et al.,* 1979; Guerrieri *et al.,* 1981).

Bohr effects in redox enzymes have been directly determined by measurement of net pH changes associated with redox transitions of metal centers under conditions where vectorial processes are abolished (Papa *et al.*, 1979; Guerrieri *et al.*, 1981). In this way Bohr effects in isolated b–c_1 complex and cytochrome c oxidase were shown amounting, at pH 7, to 0.84 H^+ transferred per Fe–S protein of the b–c_1 complex undergoing oxidation-reduction and 0.8 H^+ transferred per hemes a plus a_3 (Papa *et al.*, 1979; Guerrieri *et al.*, 1981).

The H^+/e^- stoichiometry for actual transmembrane proton translocation associated to electron flow down the cytochrome system is a matter of controversy. A series of determinations produced a general stoichiometry of $2H^+/2e^-$ *per site* in mitochondria and bacteria (Papa, 1976; Mitchell, 1980c; Haddock and Jones, 1977). These stoichiometries were questioned by Lehninger (1978) who introduced the so-called *steady-state rate method* and reported that the H^+/O ratio could reach, under conditions defined as optimal, a value of 8 with succinate and 12 with NAD-linked substrates. Others found ratios of 6 and 9 respectively (Brand, 1977; Wickström and Saari, 1977).

It was, however, pointed out that the *steady-state rate method* was probably introducing more uncertainties that it eliminated (Mitchell, 1980c; Papa *et al.*, 1980a; 1980b).

Papa *et al.* (1980a, 1980b) found that at neutral pH the H^+/O quotient for succinate oxidation was, under the conditions used by Lehninger (1978), 8 when oxygen consumption was measured with a Clark electrode covered with a standard membrane, around 5–6 when the electrode was covered with a thin membrane (see also Wikström and Krab, 1979), but exactly 4 when computed on the basis of accurate spectrophotometric determination of respiration with hemoglobin. Thus the authors concluded that the H^+/O quotient higher than 4, found with polarographic measurement of oxygen consumption, may result from underestimation of the initial respiratory rate (see, however, Lehninger *et al.*, 1980). An H^+/O ratio of 4 was also obtained by replacing succinate with duroquinol, whose oxidation was measured spectrophotometrically (Papa *et al.*, 1980b).

Measurements of the $H^+/2e^-$ quotient for electron flow from the substrate terminus of the cytochrome system to ferricytochrome c (Mitchell, 1980c; Wikström and Saari, 1977; Papa *et al.*, 1980b) show that four H^+ are released at the P side of the mitochondrial membrane—two H^+ equivalents being effectively contributed by succinate or quinol, the other two, with their positive charges, being translocated from the matrix or N side to the P side—as $2e^-$ flow from quinol to cytochrome c.

Thus if the $H^+/2e^-$ ratio with succinate or quinols is the same, irrespective of whether electrons flow to oxygen or only to cytochrome c—and, in fact, no objections have so far been raised against the measure-

ments of Papa *et al.* (1980a; 1980b) (see also Mitchell, 1980b)—it would follow that proton pumping in the cytochrome system is confined in the b–c_1 complex and that electron flow from ferrocytochrome c to oxygen does not cause proton ejection from mitochondria.

These observations would substantiate the view, originally proposed by Mitchell (1966; 1979), that the oxidase, normally turning over in its natural environment in the membrane, functions as a pure electronic $\Delta\mu H^+$ generator (see also Papa *et al.*, 1978; Lorusso *et al.*, 1979). Thed shed, on the other hand, doubts on the physiological relevance of various observations by Wikström *et al.* (1977; 1981) and others (Casey *et al.*, 1979; Sigel and Carafoli, 1980) which apparently show that isolated mammalian cytochrome c oxidase functions as a proton pump when incorporated in artificial phospholipid membranes. This is obviously a highly disputed issue. In fact, a proton pump in the oxidase would mean that such a function could be performed by redox enzymes through linkage between electron transfer at the metal and protolytic events in apoproteins, without intervention of hydrogen-carrying organic prosthetic groups. It is, however, the opinion of this author (see also Papa, 1981) that the various results so far produced are open to criticism (cf. Mitchell, 1980a) and cannot be taken as a proof for the operation "*in situ*" of cytochrome c oxidase as a proton pump.

While the occurrence of transmembrane proton transport in cytochrome c oxidase remains controversial, the proton transport activity of the b–c_1 complex is a clearly established and generally accepted process. The finding that when a pair of electrons flow from quinol to cytochrome c four protons are released from mitochondria is still explained by Mitchell in terms of direct ligand conduction by the protonmotive quinone cycle (Mitchell, 1976; 1980b). This model implies (see Fig. 3) that there should be four separate redox sites, specific for each of the couples $QH_2/\mathring{Q}H$ and $Q\mathring{H}/Q$, and that the connection of the conduction pathways of e^-, H, H^+ and their carriers should be as shown by the arrows, but not otherwise (Mitchell, 1980b). The midpoint redox potential by the couples $QH_2/\mathring{Q}H$ and $\mathring{Q}H/Q$ should be brought by complexation to different values at the N and P side to be kinetically competent and inhibit uncontrolled dismutation of quinones (Mitchell, 1980b).

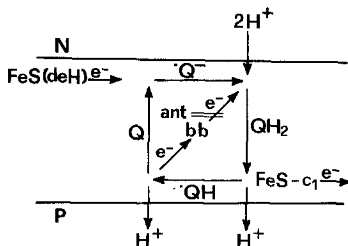

Fig. 3. The ubiquinone cycle system of the b–c_1 complex (after Mitchell 1976; 1980b).

There seem to be difficulties here, arising from the tendency of the redox processes to reversibility and the physical properties of long-chain natural quinones, making these molecules quite rigid and not so apt to flip flop across the insulating layer of the membrane. Proteins appear essential not only for specific stabilization of semiquinones but also for transmembrane proton conduction through amino acid residues specifically adapted to eschange protons (cf. Mitchell, 1980c).

A pure vectorial Bohr mechanism, based exclusively on proton conduction by ionizable groups in apoproteins, could lead to variable stoichiometries, depending upon the actual value of the pK's of the groups involved with respect to the H^+ activity in the N and P domains. Papa *et al.* (1973) originally proposed cytochrome b as a candidate for the proton pump, but later preferred another component of the complex located between b and c cytochromes (Papa, 1976; Papa *et al.*, 1975; 1978). Two lines of observations led them to this: (i) the characteristics of crossover effects exerted on oxido-reduction of b and c cytochromes by the $\Delta\psi$ and ΔpH component of the protonmotive force (Papa *et al.*, 1975; 1981c) and (ii) the peculiar pH dependence of the H^+/e^- stoichiometry in the span from succinate or quinone to cytochrome c (Papa *et al.*, 1977; 1981a; 1981b).

Collapse of $\Delta\psi$, induced by valinomycin addition to steady-state respiring mitochondria, caused preferential oxidation of cytochrome b_{566} with respect to cytochrome b_{562} (Papa *et al.*, 1981c). The oxidation of cytochrome b_{566} decreased as the pH of the medium was raised from neutrality to alkalinity (Papa *et al.*, 1981c). These and other observations (Papa *et al.*, 1981a; 1981c) show that $\Delta\psi$ controls forward electron flow from b_{566} to b_{562}, thus substantiating the proposal that the two hemes of cytochrome b are arranged in a sequence with b_{566} located at the P side of the membrane and toward the substrate and b_{562} at the N side toward oxygen (Papa, 1976; Mitchell, 1972; Wikström, 1973). Since of redox Bohr effects as the pH is brought to alkalinity, transfer of electrons by cytochrome b is replaced by effective hydrogen and the control by $\Delta\psi$ disappears (Papa *et al.*, 1981c).

Nigericin addition to respiring submitochondrial particles caused, on the other hand, oxidation of cytochrome b_{562} and reduction of c cytochromes (Papa *et al.*, 1981a; 1981c). Thus a crossover effect exerted by transmembrane ΔpH between cytochrome b_{562} and c cytochromes can be shown, which would locate the H^+ pumping step between these two carriers (Papa, 1976; Papa *et al.*, 1974b; 1977; Hinkle and Leung, 1974).

Vectorial H^+ translocation by b cytochromes would thus go in the direction opposite to that of the respiratory H^+ pump. Further evidence of this comes from the observations on the effect of pH on the H^+/e^- stoichiometry for H^+ release from mitochondria, associated to electron flow from quinols or succinate to cytochrome c (Papa *et al.*, 1977; 1981a; 1981b).

Interesting enough, the H^+/e^- ratio was found to exhibit the same values irrespective of whether electrons were transferred to oxygen or only to cytochrome *c*. The ratio decreased from 2 to around 1.5 as the pH was raised from 7 to 8.5. In both cases, however, further increase of the pH to 9 raised the H^+/e^- ratio above 2 (Papa *et al.*, 1977; 1981a; 1981b). The H^+/e^- ratio for electron flow from succinate to ferricyanide exhibited a pH-dependence similar to that observed with quinols as substrates (Papa *et al.*, 1981a). The H^+/e^- ratio was 2 at pH 7 and fell down to 1.5 at p. 8.5. Then it rose again at pH 9 but, in contrast to that observed with quinols, it did not exceed the value of 2 (Papa *et al.*, 1981a).

These observations cannot be explained in terms of the ubiquinone cycle, nor by the two-quinone redox-loop in series (Mitchell, 1980b; Crofts *et al.*, 1975). These models, at least as presently formulated, predict a fixed H^+/e^- quotient of 2 (Mitchell, 1976; 1980b; Crofts *et al.*, 1975).

In Fig. 4 a mechanism for H^+ pumping by the b–c_1 complex is presented which considers, as an essential element, the special quinone/semiquinone system of the complex (Yu *et al.*, 1978; Ohnishi and Trumpower, 1980). It is proposed that substrate quinol is oxidized to semiquinone by the Fe–S protein of the complex in an antimycin-insensitive reaction (Mitchell, 1972; 1976; Wikström, 1973; Erecinska *et al.*, 1972). The semiquinone is supposed to be oxidized at the *P* side of the membrane by b_{566} which then transfers electrons to b_{562} at the *N* side and from this to the Fe–S protein, through an antimycin-sensitive reaction. The two electrons reunite at the level of the Fe–S protein quinone center of the complex and from this pass to cytochrome c_1. The cytochrome *b* shunt explains the well-known phenomenon of the antimycin-promoted oxidant-induced reduction of *b* cytochromes (Mitchell, 1972; 1976; Wikström, 1973; Erecinska *et al.*, 1972) as well as the decrease of the H^+/e^- ratio from 2 to 1.5 from pH 7 to 8.5 (Papa *et al.*, 1977a; 1981a; 1981b), when *b* cytochromes change from electron to effective hydrogen carriers (Papa, 1976; Urban and Klingenberg, 1969).

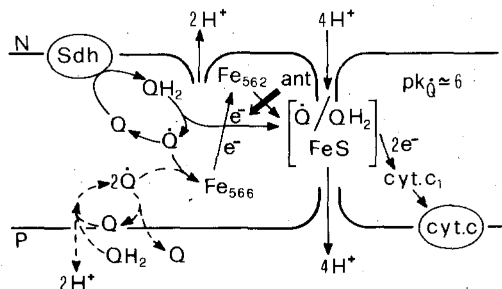

Fig. 4. Possible model for proton pumping in the b–c_1 complex (Papa *et al.*, 1981a; 1981b).

Protonation of the $\mathring{U}Q/UQH_2$ couple from the N side of the membrane upon reduction and proton release and upon oxidation at the P side can result in transmembrane translocation of two to four H^+ per $2e^-$ traversing the system. The actual stoichiometry will depend on the pK of the semiquinone and the actual pH at the N and P domains. Since direct measurements assign to the bound semiquinone a pK around 6 (Ohnishi and Trumpower, 1980), this transfers two H^+ per e^- over the pH range 7 to 9 (Ohnishi and Trumpower, 1980).

Another problem concerns the site where substrate quinol is oxidized by the b–c_1 complex. Recent experiments (Papa *et al.*, 1981a; Degli Esposti *et al.*, 1981) on the kinetics of reduction of endogenous c cytochromes by exogenous quinols in mitochondria and submitochondrial particles show that this process is much faster in "inside-out" particles than in intact mitochondria. The difference is higher with the more hydrophylic ubiquinol-1 than with ubiquinol-3 (Papa *et al.*, 1981a; Degli Esposti *et al.*, 1981). These and other observations (Lenaz *et al.*, 1981) indicate that substrate quinols reach the oxidation site in the b–c_1 complex from the N side of the membrane. The resulting scalar proton release might occur at the same side. The Fe–S protein–quinone center can therefore be conceived as translocating four H^+ per $2e^-$ over the entire pH range. Two of the H^+ removed from the N space are replaced by two H^+ from substrate oxidation, and two positive charges are compensated by net transfer of $2e^-$ from the dehydrogenase at the N side to cytochrome c at the P side. Dismutation at the P side of exogenous quinol with oxidized quinone, promoted by alkaline pH, can account for the extra H^+ release from mitochondria, observed at alkaline pH when exogenous quinols are used as substrates.

Do Bohr effects play any role in this model? Bohr effects of b cytochromes play a regulatory role in adjusting the rate of electron flow. pK shifts of ionizable groups (amino-acid residues) linked to oxido-reduction of the Fe–S protein–quinone center (Guerrieri *et al.*, 1981) can favor H^+ transfer in the N half of a proton channel to the ionized reduced quinone, and/or transfer of the H^+, produced in the oxidation of quinol to semiquinones, to ionizable groups in the P half of the channel. A general feature of this model is that Bohr effects in redox proteins, probably arising from electrostatic pairing of protolytic positive (arginine, lysine, or α-NH_2 residues) and negative groups (glutamic or aspartic residues) (see Perutz, 1976; Kilmartin, 1976), cooperate with protonmotive covalent bond exchange so as to result in coupling between metabolism and transport (see Fig. 6).

The possibility that weak, salt-bridge interactions between ionizable residues in apoproteins can contribute to anisotropic protonmotive covalent group exchange at the catalytic center seems in fact to gain general support, and evidence is emerging for its applicability to proton pumping in bacterio-

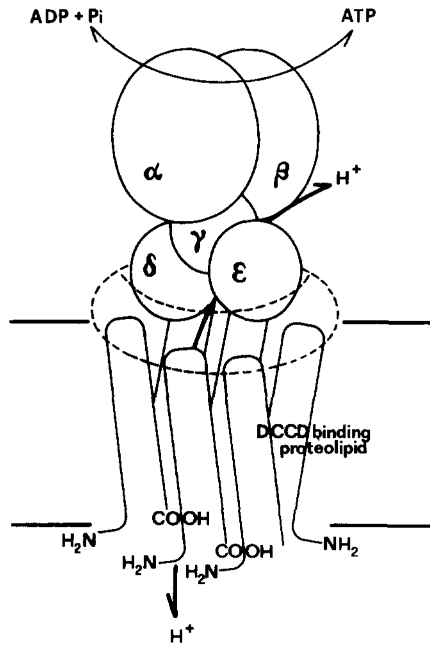

Fig. 5. Schematic membrane topology of polypeptides of F_1 and DCCD-binding proteolipid of F_0. Other membrane subunits are indicated by the dotted pad (Papa and Guerrieri, 1981). It should be noted that the subunit stoichiometry and arrangement of F_1 can be of the type $\alpha_3\ \beta_3\ \gamma\ \partial\ \epsilon$ or $\alpha_2\ \beta_2\ \gamma_2\ \partial_2\ \epsilon_2$ (Senior, 1979; Amzel *et al.*, 1981). The DCCD-binding proteolipid is considered to be present in the membrane in the hexameric state, each monomer being bent on itself so as to form two rods traversing the membrane. Thus the proteolipid oligomer could form a bundle of 12 rods traversing the membrane. The monomers could be oriented parallel, i.e., all the NH_2-terminus could be exposed at one side of the membrane (the outer, as, for example, shown in the figure), or antiparallel.

rhodopsin (Engelman *et al.*, 1980; Packer *et al.*, 1979) and ATPase complex (Pansini *et al.*, 1979; Guerrieri and Papa, 1981).

The H^+-ATPase

In Fig. 5 a schematic view of the polypeptide composition and membrane topography of the F_0–F_1 complex is presented.

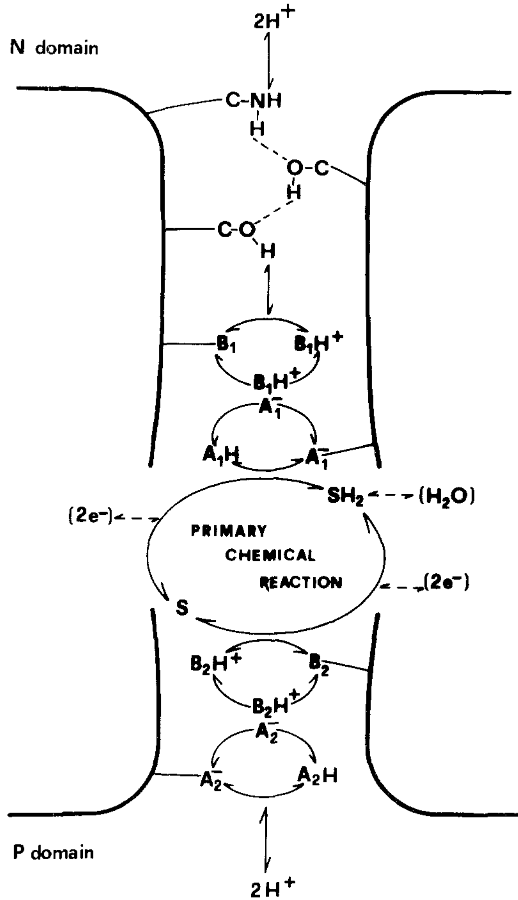

Fig. 6. Scheme showing a possible general model for proton pumps based on the combination, in series, of anisotropic arrangement of the protonmotive primary chemical reaction (energy source) and vectorial proton translocation by polar amino acid residues arranged in hydrogen-bonded network and salt-bridge relay. The model shown here for oxido-reduction or hydrodehydration systems could also apply to light-driven proton translocation by photoredox systems and bacteriorhodopsin.

Of the 7–11 polypeptides which constitute the F_1–F_0 complexes of coupling membranes so far characterized, the DCCD-binding proteolipid of F_0 and the five polypeptides of F_1 have been strictly preserved during evolution (Senior, 1979; Sebald *et al.*, 1979a). They should, thus, be essential to the basic function of the complex. The α and β subunits of F_1 are responsible for nucleotide binding and chemical catalysis (Senior, 1973; De

Pierre and Ernster, 1977). The γ, ∂, and ϵ subunits seem to provide the connection of F_1 to F_0 (De Pierre and Ernster, 1977; Kagawa, 1978). These and additional proteins present in the membrane sector, indicated altogether by the dotted pad, probably represent the gate of the pump (Kagawa, 1978; Papa *et al.*, 1977b).

The DCCD-binding proteolipid of F_0 seems to constitute by itself a transmembrane proton channel (Nelson *et al.*, 1977, Sigrist-Nelson and Azzi, 1980). Of the 70–80 residues of the proteolipid, around 80% are apolar (Sebald *et al.*, 1979a; Sigrist-Nelson and Azzi, 1980). Most of them are clustered in two segments of 20–25 residues, separated by a polar segment of 10–20 residues (Sebald *et al.*, 1979a; Altendorf *et al.*, 1979). The proteolipid could be bent on itself (see Fig. 5) with the two hydrophobic segments embedded in the membrane and the hydrophilic loop exposed at the surface (Altendorf *et al.*, 1979). The proteolipid exists in the membrane probably as a hexamer (Sebald *et al.*, 1979a, 1979b). It is possible, in analogy to the arrangement of bacteriorhodopsin in the purple membrane (Engelman *et al.*, 1980), that the double hydrophobic segments of the six monomers—probably coiled as α-helices (Sebald *et al.*, 1980)—extend side by side across the membrane perpendicularly to its plane so as to form a bundle of 12 rods with a central channel where H^+ translocation would take place.

Chemical modification with DCCD, as well as genetic amino acid substitution, have identified the essential role for proton conduction of the glutamic acid residue in position 65 (*Neurospora Crassa* sequence numbering) of the DCCD-binding proteolipid (Sebald *et al.*, 1979a; Altendorf *et al.*, 1979).

Evidence that other polar residues are involved in proton conduction by F_0 is provided by studies with chemical modifiers of amino acid residues. Sone *et al.* (1979; 1981) reported that tyrosine nitration with tetranitromethane or arginine modification with phenylglyoxal in F_0 of thermophilic bacterium PS3 inhibits H^+ conduction in liposomes.

Pansini *et al.* (1979) and Guerrieri and Papa (1981) have tested the effect of amino acid modifiers on proton conduction by F_0 still attached to the mitochondrial membrane. Anaerobic decay of the $\Delta\mu H^+$ generated by succinate respiration in submitochondrial particles deprived of F_1 takes place practically through the proton conduction pathway in F_0, as judged from the titer of inhibition of the decay by oligomycin of DCCD (Guerrieri and Papa 1981; Pansini *et al.*, 1978). Treatment of the particles with tetranitromethane or phenylglyoxal resulted in a marked depression of H^+ diffusion through F_0 (Pansini *et al.*, 1979; Guerrieri and Papa, 1981).

The kinetics of $\Delta\mu H^+$ relaxation in "inside-out" submitochondrial particles with F_1 still attached to F_0 is more complex (Pansini *et al.*, 1978). The process exhibits two apparent first-order phases. The fast phase ceases when approximately one-third of the $\Delta\mu H^+$ is relaxed. The biphasicity of H^+

release depends on ΔpH. It is, in fact, observed in the presence of $\Delta\psi$-collapsing valinomycin. Both phases are expression of H^+ conduction by H^+-ATPase, as judged from the inhibition by DCCD and oligomycin (Pansini *et al.*, 1978).

Thus transmembrane ΔpH that is more acidic inside the vesicles enhances H^+ conductivity of F_0. As part of $\Delta\mu H^+$ decays, the system reverts to low conductivity. In the mitochondrial membrane F_0 spans the membrane (Ludwig *et al.*, 1980). In "inside-out" particles F_0 can directly sense intravesicular pH changes. Acidification of this space can induce high H^+ conductivity by causing protonation of critical residue(s) of F_0.

AMP-PNP and alkyl cations, which inhibit ATP hydrolysis in particles or in isolated F_1 (Pansini *et al.*, 1979), also slow down the decay of the aerobic $\Delta\mu H^+$ (Pansini *et al.*, 1978). This inhibition presents a sigmoidal titration curve and is synergistic with inhibition by oligomycin (Pansini *et al.*, 1978). Furthermore it disappears when F_1 is removed from the particles (Pansini *et al.*, 1978). Thus binding of inhibitory ligands to F_1 induces a structural change in the F_0–F_1 complex, resulting in decreased H^+ conductivity.

The inhibition by lipophilic cations of H^+ translocation by H^+-ATPase indicates that negatively charged groups, located in hydrophobic environment of F_1, play an essential role (Pansini *et al.*, 1978; 1979). Inhibition of ATP hydrolysis and H^+ conduction in submitochondrial particles could also be produced by an anionic amphiphile, such as SDS (Pansini *et al.*, 1979). Furthermore the inhibition caused by one of the two amphiphiles was reversed by stoichiometric amounts of the other (Pansini *et al.*, 1979). These observations suggest that interactions of the type of salt bridges between acidic and basic residues in hydrophobic regions of F_1 and F_0 are involved in H^+ translocation.

The F_1 and the membrane integral proteins, which joined to contribute a gating region in the complex, are apparently engaged in the $\Delta\mu H^+$-dependent interaction of F_1 with F_0. This interaction, which probably involves salt bridges between acidic residues in F_1 and basic residues in F_0, might play a specific role in the coupling of H^+ translocation to chemical catalysis (Pansini *et al.*, 1979; Papa and Guerrieri, 1981; cf. Olivier and Jagendorf, 1976).

Knowledge of the primary structure of the DCCD-binding proteolipid of F_0 allows one to outline more specific proposals on the molecular mechanism of proton conduction by this component. The DCCD-reactive residue lies, in all the proteolipids so far characterized, in the middle of a hydrophobic sequence (Sebald *et al.*, 1979a; Altendorf *et al.*, 1979), which could raise the p*K* of the carboxylic group so as to correspond to the p*K* of 6.8 observed for H^+ conduction by isolated F_0 in liposomes (Okamoto *et al.*, 1977).

Being the acidic residues-65 located in a hydrophobic segment, it is difficult to visualize how it could, by its own, pick up H^+ from a water phase and release them to the opposite one. H^+ could, however, be exchanged at the

membrane surface by other residues and then be transferred to residue-65. Such a role could be played by arginine and tyrosine residues, whose modification also causes inhibition of H^+ conduction. The position of these residues remain to be identified. It seems, however, significant that only one arginine is present in the DCCD-binding proteolipid of mitochondria at position 45 and this is also occupied by arginine in the other DCCD-binding proteolipid so far analyzed (Sebald *et al.*, 1979a; Altendorf *et al.*, 1979). Beef-heart and *Neurospora* mitochondria have only one tyrosine residue at position 56 (Sebald *et al.*, 1979a). In the DCCD-binding protein of PS3 and chloroplasts, tyrosine-56 is replaced by an isofunctional threonine residue. In *E. Coli* and *S. Cerevisiae,* hydroxyl residues are missing at position 56 but a threonine is present at position 55 in the first, and at position 52 in the second case (Sebald *et al.*, 1979a). In the two hydrophobic segments of a monomer there are three to seven hydroxyl residues in the various species examined (Sebald *et al.*, 1979a), and 18–42 hydroxyl residues in all the hexamers. The monomers could be slightly displaced with respect to one another. It follows that these residues are numerous enough to be distributed along all the hypothetical channels of the hexamer. It is conceivable that hydroxyl residues from adjoining chains, joined by hydrogen bonds, constitute a continuous network (Dunker and Marvin, 1978; Nagle and Morowitz, 1978) with the acidic residues-65 in the middle, along which protons can rapidly move across the membrane from the entry to the exit mouth of the channel. Motion of residues can cover possible gaps. It is, however, possible that, intercalated with hydroxyl residues, other polar residues and/or hydrogen-bonded H_2O molecules also participate in H^+ transfer.

The arginine-45 and other basic residues in the central loop or in the *N*-terminus could be involved in the access of H^+ at the entry mouth (Pansini *et al.*, 1979; Guerrieri and Papa, 1981). Modification by phenylglyoxal of basic residues at the entry mouth will depress proton conductivity. Interestingly enough, butanedione, which is even more specific than phenylglyoxal for arginine, has been found to cause stimulation of H^+ release instead of inhibition as found with phenylglyoxal (Pansini *et al.*, 1979; Guerrieri and Papa, 1981). This could be due to specificity of attack of arginines of different subunits of F_0 with differential accessibility to the two reagents or modification of residues other than arginine. In any case, modification by butanedione of basic residues at the exit mouth could enhance proton conductivity.

The basic residues at the mouth of the F_0 channel can be engaged in salt bridges with acidic residues of the connection polypeptides of F_1. As clearly revealed by the elegant studies on cooperative proton transfer in hemoglobin (Perutz, 1976; Kilmartin, 1976), formation of salt bridges can shift the pK of the residues engaged. Furthermore pK shifts caused by hydro–anhydro bond exchange catalyzed by F_1 could play a role in H^+ pumping.

Conclusion

After 20 years of intensive scrutiny the concept (Mitchell, 1966; 1979) that anisotropic organization of the primary chemical reaction at the catalytic center can represent the central step in the coupling between metabolism and proton transport appears to remain valid. This, however, seems insufficient to account for the net transport of the protons across the membrane. Evidence has been discussed which shows that proteins can play a direct role here, in two ways (Fig. 6). First, they can provide a network of hydrogen-bonded polar residues, properly intercalated in hydrophobic transmembrane helices of membrane integral proteins, so as to constitute a proton-conducting pathway. Second, weak electrostatic interactions, like salt bridges between ionizable residues, linked to protonmotive covalent catalysis and resulting in the proper adjustment in space and time of the proton accepting and proton donating capacity of ionizable residues, can be directly involved in proton transport. They can probably play a direct role in the coupling and gating process of the pump.

Vectorial metabolism, i.e., diffusion of substrates and/or chemical groups along specific conduction pathways in catalytic proteins or lipoproteins (Mitchell, 1966; 1979) and protolytic reactions at other sites in the same proteins (or different polypeptides of the same oligomer), cooperatively linked to primary bond exchange at the catalytic center (Papa, 1976), are proposed to result together in proton pumping (Papa *et al.*, 1981a, 1981b). Neither of the two processes would be sufficient by its own to accomplish active proton transport. Their combination would optimize the efficiency of energy coupling and biological regulation.

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