Hypothesis

THE LINK BETWEEN CHARGE SEPARATION, PROTON MOVEMENT AND ATPase REACTIONS

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1. Introduction

The elegant chemiosmotic hypothesis developed by Mitchell [1,2] has done much to increase our knowledge of energy-transducing membranes.. The need to understand what happens, not only across the membrane but also within it, was pointed out by Williams [3–5]. Recent increased knowledge of membrane structure and of the components of mitochondrial, thylakoid and bacterial membranes have made this understanding more feasible. The properties of the lipophilic regions of the membrane have not been sufficiently considered in explaining the extrusion of protons, which occurs under many experimental conditions, or their passage to the ATPase where they take part in the synthetic reaction.

A characteristic of energy transducing membranes is that an electron is conducted rapidly, sometimes through, sometimes across, the membrane away from the proton which previously accompanied it in a hydrogen atom. The problems of how the electrons move in mitochondrial and thylakoid membranes are fairly well understood but what happens to the protons has been less investigated and less well interpreted.

In this paper, three novel features are suggested: (1) that protons, whatever their origin, become part of a small lipophilic molecule which allows them to diffuse in the region of the lipophilic chains of the phospholipids; (2) that the small, fast-diffusing, lipophilic, proton carrier will move laterally in the fluid region of the hydrocarbon chains in the centre of the bilayer. It would not diffuse readily through the outer region of the hydrocarbon chains where the CH_2 groups, adjacent to the polar heads, have very restricted movement and are almost in the solid state. The carrier within the membrane is postulated as conveying the dry protons to the ATPase to bring about ATP synthesis; (3) that protons from water outside the membrane are carried into the lipophilic region without their water of hydration by the polar groups of the quinones, and passed to the proton carrier.

2. The injection of protons into the lipophilic region

The principles of the hypothesis are best understood by considering the probable role of ubiquinone at Site II in the inner mitochondrial membrane. Ubiquinone presumably works on the inner (matrix) side of the membrane to bring protons, without water of hydration, from matrix water, into the dry lipophilic region, as part of the dihydroquinone. When ubiquinone in the quininoid form receives two electrons from the non-haem iron and sulphydryl proteins in the electron transport chain, it will temporarily carry two negative charges on its polar group which will be aligned with the polar groups of the lipid membrane. The strong negative field on this polar group will result in its movement towards the water, with repulsion of the negative groups on lipids in the immediate vicinity, loosening the bilayer molecules. Thus ubiquinone, then protruding its polar group into the aqueous phase, will react with two hydrogen ions which attach to the negative oxygens as protons to form dihydro-ubiquinone and leave their water of

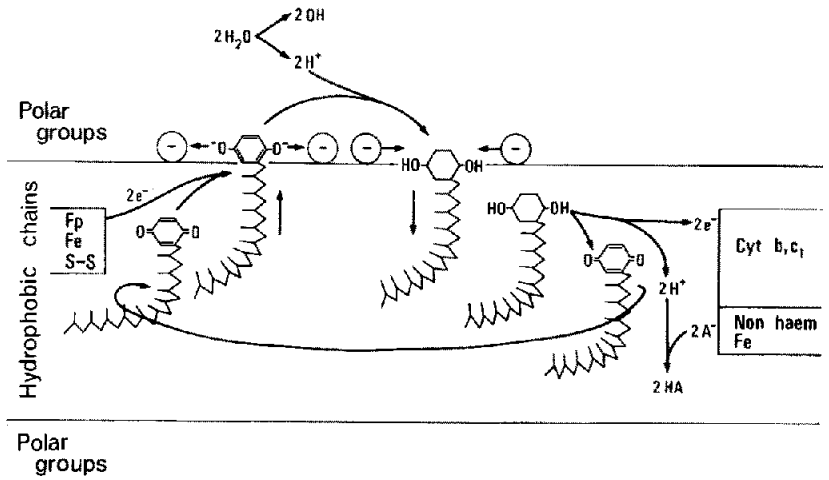

Fig. 1. The suggested function of ubiquinone in bringing protons into the lipophilic region of the membrane: Fe, iron; Fp, flavoprotein; S–S, sulphydryl groups; ⊖, negative charges of polar groups of phospholipids.

hydration behind. The consequent loss of charge on the ubiquinone results in its sinking back into the lipophilic region. When it loses its electrons to cytochrome *b*, the next member in the chain, thought to be buried in the lipophilic region, a proton acceptor will be required in the reaction; it is suggested that the protons combine with a small lipophilic anion (A^-). The abundant non-haem iron near cytochrome *b* undergoes oxidation and reduction; when oxidised, Fe^{3+} with net positive charge would attract any small anion (A^-) in its vicinity. When the Fe^{3+} became reduced by an electron, the A^- could move to the proton liberated at the same time. Thus, non-haem iron would act as a temporary storage site for A^-. The whole cycle of proton injection is shown in fig. 1. The hypothetical proton carrier, HA, can either move through the membrane to take part in the reactions in the ATPase or leak from the membrane to the outside where the protons would become hydroxonium ions. This hypothesis is thought to be more probable than the alternative, that ubiquinone carries protons across the membrane by flip-flop; such mechanisms are known to be very slow in lipid membranes.

The hypothesis may be described as the 'bobbing up and down' of ubiquinone to pull protons from water on the matrix side of the membrane and to donate them to a hypothetical small lipophilic anion. It is possible that the double bonds of the lipid hydrocarbon chains may play a part since a proton might add to form a carbonium ion which would also require the mobile A^-; protons in carbonium ions might also move laterally in the membrane by passing from double bond to double bond of adjacent lipid hydrocarbons.

The 'bobbing-up-and-down' hypothesis can also be applied to the ubiquinones of bacterial membranes and plastoquinone in thylakoids.

It is also envisaged that protons are injected into the lipophilic region of the energy-transducing membrane at other sites, e.g. during the oxidation of NADH in mitochondria and the oxidation of water in thylakoid membranes. It seems likely that water oxidation, which might be associated with a higher oxidation state of a Mn–protein complex, occurs in the lipophilic thylakoid membrane rather than in the aqueous phase on the inside (or the outside) of the thylakoid.

3. Protons in the lipophilic region

It is not proposed to enter into the difficult debate on the nature of the reactions involved in the phosphorylation reaction but there seems to be general agreement that protons are the means by which the energy from the charge separation is transmitted to the ATPase. If so the movement of dry protons within the lipophilic region of the mem-

brane would have considerable advantages. If they leave the membrane and enter the aqueous phase, they become hydrated and energy is required to dehydrate them again to enter into the proton-requiring reaction in a lipophilic environment. As Williams [5] has pointed out, the reaction could be in the nature of a condensation for which a non-hydrated proton has an advantantage.

Other phenomena can be explained by the participation of protons in the lipophilic region.

(a) Mid-point potentials: When excess ATP is added to coupled mitochondria, there is a shift in the mid-point potentials of cytochrome a_3, cytochrome *b* and of the iron sulphur associated with the flavoprotein of Site I (Wilson et al. [6]). The high mid-point potential of cytochrome *b* in the presence of ATP is almost unchanged by external pH between 6.0 and 8.1, so such shifts in the potential cannot be induced by changes in external hydrogens ions [7], indicating that these enzymes are inaccessible to protons from the aqueous phase. The presence of protons in the lipophilic region affecting these enzymes would explain the result and a flow of protons back from ATP through the ATPase could be maintained by the diffusible proton carrier in the non-aqueous phase.

(b) Uncouplers: Uncouplers are lipid soluble substances and the more powerful, e.g. S 13 (5-chloro-3-tert-butyl, 2′-Cl, 4′-NO_2-salicylanilide), are characterised by a preponderance of lipophilic groups. On this hypothesis, uncouplers would be capable, not only of conducting protons from one side of the membrane to another, but also of stripping the lipids of their protons.

(c) Calcium loading: The presence of an anionic proton carrier in the lipophilic region of the membrane might also explain mitochondrial membrane loading with calcium [8]. If the calcium salt of the anion had some lipophilic properties, calcium could compete with the protons for the anion in the membrane. The rapid release of H^+ from the membrane would be due to the displacement of H^+ by Ca^{2+}. Though this release of H^+ is dependent on the respiration, it would not be stoichiometric with electron transport and be complete before the calcium-stimulated respiration occurs; the calcium, having replaced the reserve of H^+, would prevent its passage to the ATPase and thus uncouple respiration [9].

(d) Proton movements: The presence of protons ir the lipophilic region is quite consistent with the observed proton extrusions. If protons were being liberated in the lipophilic region, and the ATP synthetic reaction, which normally uses the protons is slow or blocked (e.g. by the absence of ADP), increased extrusion of the protons to the outside of the mitochondrial membrane or to the inside of the thylakoid membrane would be expected. The establishment of such a gradient in the water across the membranes could lead to other ion movements which are observed in such systems, e.g. phosphate exchanging for OH^- in mitochondrial membranes or in the presence of ionophores, monovalent cations crossing the membrane to balance OH^- or $H_2PO_4^-$.

(e) Artificial proton gradients: Acid on the outside of a membrane in which the lipophilic portions are accessible to protons, though for reasons which will be discussed protons are prevented from passing right through, would result in protons partitioning into the lipophilic region, accompanying their appropriate carriers. Once in the lipophilic region, the protons would be able to move by the proposed mechanism to the ATPase. Thus either artificial gradients of pH, or protons extruded in electron transport in excess of those entering into the ATPase reaction, will move into the lipophilic region and hence to the ATPase, which, using them in a synthetic reaction, forms a sink.

4. The lipophilic proton carriers

Various small lipophilic molecules might act as proton acceptors and proton carriers in the lipophilic region of the membrane, depending on the circumstances and on the conditions of the experiment. Thus, in the average experiment on isolated mitochondria, the weak acid supplied as substrate might be the proton acceptor. Artificial lipophilic proton acceptors are used experimentally [10]: oxidised diamino-durene (i.e. in the presence of excess ferricyanide) is able to partition to the hydrophobic region of the thylakoid membranes and react with the plastohydroquinone in competition with the endogenous electron acceptor, cytochrome *f*. The reduced diamino-durene carries two electrons and two protons from the plastoquinone back to the outside aqueous phase where it

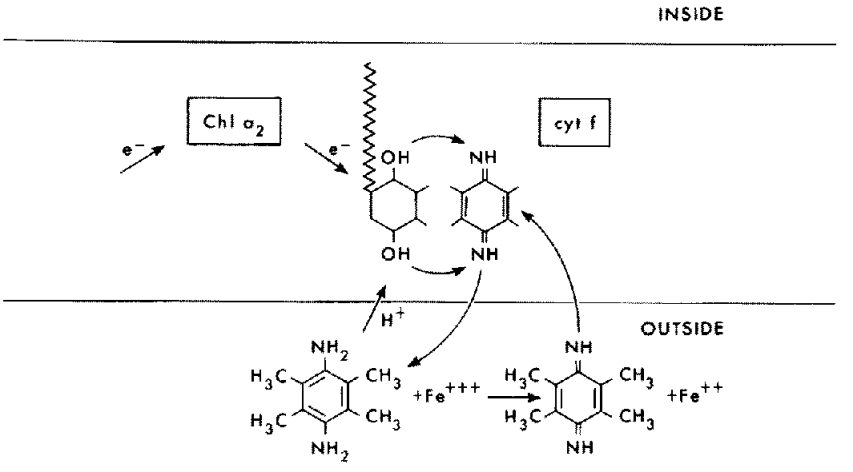

Fig.2. Reoxidation of plastohydroquinone in the lipophilic region of the thylakoid membrane by the artificial electron acceptor, oxidized diamino-durene.

is reoxidised by ferricyanide. In this cycle as shown in fig.2 electron flow from chlorophyll *a* to plastoquinone results in protons being taken in from water by the bobbing up of plastoquinone to the outside of the thylakoid membrane, entry into the lipophilic region by the bobbing down of plastoquinone, loss of both protons and electrons to the diamino-durene which takes them back into the water, where the electrons go to the ferricyanide, and the protons are liberated as hydrogen ions.

It is suggested that under some natural conditions the small lipophilic anion may be chloride so that hydrogen chloride becomes the proton carrier. In the chloroplast, chloride is known to be essential for electron flow from water [11]. In plant mitochondria, chlorides have been shown to restore the oxidation rates of NADH and cytochrome *c* which are low in sucrose in the absence of ions [12,13]. Rat liver mitochondria have oxidation rates in sucrose two or three times lower than the rates in KCl [14,15].

Other lines of evidence suggest that chlorides may play an important role in the properties of these membranes.

(a) Hydrogen chloride in membranes: Chloride in artificial membranes shows the 'chloride paradox' which means that the isotopic flux of Cl^- may be as great as 1000 times that of the cation, though the electrically determined transference numbers of the two ions are closely similar. Bangham [16] suggested that the enhanced Cl^- diffusion probably involves covalent association of Cl^- ions and protons at the lipid–water interface. Since HCl is quite soluble in non-polar, particularly unsaturated, solvents, its presence in the lipophilic region of the membrane is to be expected.

In some circumstances, the packing of the phospholipids may be such as to prevent its ready escape into the water on either side. It is well known that the motional freedom of the CH_2 chains near the polar groups is very restricted. McConnell and McFarland [17] found that this movement can approach that of a crystalline hydrocarbon, while, in the same chain near the terminal methyl groups, the motional freedom is that of an isotropic liquid hydrocarbon. High motional freedom, distant from the polar groups, will be expected, for example, in the inner mitochondrial membrane, aided by the double bonds of the hydrocarbons with phosphatidylcholine (20% arachidonic) and diphosphatidylglycerol (70% linoleate). We do not know how the different phospholipids are distributed between the bilayers of the inner mitochondrial membrane but on the matrix side, the presence of the divalent cations, Ca^{2+} and Mg^{2+}, will tend to result in closer packing, particularly if the diphosphatidylglycerol content is high on that side. The outer surface of the bilayer, possibly with different composition, and not exposed to such high divalent ion concen-

trations, might have quite different properties and be relatively permeable, particularly under in vitro conditions; there, HCl could readily re-enter the water, and dissociate immediately to H^+ and Cl^- which diffuse away in the outside solution, accounting for the rapid appearance of H^+ on the outside, during an oxygen pulse experiment for example.
(b) Cytochrome *c* in mitochondrial membranes: Another piece of evidence is the special properties of cytochrome *c* in the mitochondrial membrane. Dickerson and his colleagues [18,19] have shown that chloride ions are necessary for the crystallization of oxidized cytochrome *c*, but not for crystallization of reduced cytochrome *c*. They suggest that chloride is normally accommodated in the haem pocket in the oxidized molecule, held in position by the FeIII porphyrin with its net positive charge. On reduction, the FeII porphyrin having no net positive charge, the Cl^- would leave. Another Cl^- would be picked up when the electron from the FeII porphyrin is passed on to the cytochrome oxidase and the cytochrome regains FeIII.

This behaviour of cytochrome *c* may be the clue to injection of chloride into the lipophilic environment of the bilayer. The oxidised cytochrome *c* molecule is likely to attach itself to the lipid bilayer by the lipophilic residues surrounding the haem pocket making contact with lipophilic parts of the membrane. With oxidised cytochrome *c* in this position and Cl^- in the pocket, reduction might be expected to expel the chloride into the lipophilic environment of the membrane, i.e. inside the polar groups. The hydration state of Cl^- in the pocket is not known; but it seems likely that the strong attraction to the FeIII of the iron porphyrin in the highly lipophilic part of cytochrome *c* may result in a non-hydrated chloride. The chloride on leaving the cytochrome *c* and entering the lipophilic region of the membrane may be attached to the non-haem iron in its reduced state, so abundant in Sites I and II. Chloride would then be available to pick up the protons from ubiquinone when it loses its electrons to cytochrome *b*.
(c) Oligomycin and trialkyltins: Both oligomycin and trialkyltins seem to have quite specific reactions with Cl^-. For example, the uncoupling effects of oligomycin are not observed when Br^- or NO_3^- is used instead of Cl^- [20]. In sucrose medium trialkyltin compounds inhibit respiration coupled to phosphorylation or arsenate stimulated respiration but do not inhibit respiration stimulated by uncouplers [14]. In KCl medium, the trialkyltins, which are highly lipophilic, and similar oligomycin-like effects and also showed some uncoupling at concentrations which produce a Cl^-–OH^- exchange. A Cl^-–OH^- exchange in the lipophilic region would directly neutralize the HCl in that region and explain the uncoupling action of the trialkyltins. These observations can be interpreted as showing not only that Cl^- has a role, but also that it is normally trapped within the lipophilic region.

5. Possible experiments

This hypothesis can be tested in several ways. Further evidence for the presence of protons in the lipophilic region would come from experiments with artificial lipophilic proton carriers. The presence of the natural proton carriers in different membranes should be investigated, including the possibility that chloride is involved in some. NMR techniques can be used to see whether the properties of the lipophilic region are consistent with the hypothesis. In particular, it should be possible to detect the polar groups of the quinones in different parts of the bilayer; the lipophilic forms (the dihydroquinone and the oxidised quininoid) should be down in the lipophilic region, and the hydrophilic form (the reduced quininoid) should be up in the aqueous phase. Some experiments will be carried out in our laboratories, but it is hoped that others may be stimulated to test the hypothesis with techniques not available to us.

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