Proton mobility in biological membranes: the relationship between membrane lipid state and proton conductivity

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It is demonstrated that phospholipid vesicles and mitochondria are relatively permeable to ionised water with a permeability coefficient of approx. 10^{-4}–10^{-3} cm/s. The H^+/OH^- conductance is further shown to depend in a non-linear fashion upon an applied electric field and in a linear fashion upon applied pH gradients. In an attempt to characterise the molecular basis of these phenomena, the effects of electric fields of various magnitudes upon the relative dynamic state of the membrane were studied using the technique of fluorescence polarisation. It was found that the steady-state fluorescence polarisation of 1,6-diphenyl-1,3,5-hexatriene (DPH) was dependent upon the applied field in a similar manner to the proton conductance. Whereas the effect of a thermodynamically equivalent pH gradient had a negligible effect. There was thus a correlation between the 'state' of the membrane and the rate of permeation of H^+/OH^-.

Proton conductivity *Lipid microviscosity* *Order parameter* *Membrane fluidity* *Permeability coefficient* *Fluorescence polarisation*

1. INTRODUCTION

In recent years, changes of membrane structure have been implied to play roles in the mediation or regulation of many biological processes. Although it is clear that the membrane is not just a simple insulatory device, the primary role of the membrane must be insulatory and hence facilitate compartmentalisation. Such a permeability barrier was considered particularly significant in [1], in the chemiosmotic hypothesis, for the process of oxidative phosphorylation. In support of this hypothesis it was later found [2] that the penetration by protons of biological membranes appeared to be a relatively slow process. Lately, however, it has been clearly demonstrated [3] that phospholipid membranes are in fact relatively permeable to protons with a H^+/OH^- permeability coefficient (P_{net}) of approx. $10^{-4} - 10^{-3}$ cm/s. The proton permeability coefficient of such membranes is thus several orders of magnitude larger than those of other monovalent cations. We have carried out similar studies [4] to those in [3] and concur with their evaluation of the P_{net} for ionised water. Such observations are not, however, inconsistent with the chemiosmotic hypothesis because although the P_{net} is relatively high, actual concentrations of protons in subcellular fluids are very small (~0.1 μM) the futile dissipation current would therefore be very small. Thus, whereas the above described phenomena are not significant with respect to the efficiency of oxidative phosphorylation, the origin of the high proton conductance is of very real interest in itself. We have therefore attempted to further investigate which factors may influence proton conductance through membranes. It is conceivable that if we could ascertain the structures of the relatively simple membrane which facilitate a high proton conductance, then it may be possible to extrapolate such features as may be necessary to the so-called proton-specific channels associated

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with proteins, which would be of fundamental interest.

To account for their observation of a high value of P_{net} for H^+/OH^-, authors in [3] have speculated that a fraction of the water flux across the membrane may be in the form of metastable strands or slow flickering clusters. The high proton flux was envisaged to occur via an ice-like proton conduction process. For a discussion of these latter points, review [5].

We would tend to agree with the suggestion in [3] that the rapid proton mobility may have its origin with the state of the membrane-associated water. In principle, the amount of water associated with a membrane may be influenced by the fluidity/order of the membrane (due to the 'hydrophobic effect'). It is possible that an imposed electric field may alter the ordered state of the membrane such that the H^+/OH^- permeability is affected. We report here, therefore, preliminary experiments whereby the 'fluidity' state of the membrane has been altered by the imposition of a membrane potential and correlated with likewise dependent changes of the H^+/OH^- permeability. The 'state' of the membrane was monitored using the technique known as fluorescence polarisation.

1.1. Measurement of the ordered state of a membrane by the fluorescence polarisation of 1,6-diphenyl-1,3,5-hexatriene (DPH)

The original interpretations [6] of the observed steady-state fluorescence polarisation, of the membrane soluble fluorescence probe, DPH, indicated that the behaviour of DPH in the membrane was isotropic. Classical hydrodynamic expressions could thus be applied. The fluorescence polarisation was therefore considered [6] to be proportional to the lipid 'microviscosity' or fluidity. This approach, however, has been shown to be a gross simplification due to the hindered rotation of DPH in the membrane [13,14]. Fortunately DPH may still be applied and due to the valuable treatments of [13] and [14] the steady-state fluorescence polarisation also yields, in addition to dynamic quantities (i.e., 'fluidity'), information with respect to the order of the membrane. From our point of view such treatments may be especially useful as we may be able to assign more exactly structures which facilitate proton conductivity and those which do not.

Fluorescence polarisation measurements were carried out with a Perkin-Elmer fluorescence spectrofluorimeter, model MPF-2A, equipped with a magnetic stirring device, thermostat and polarisation filters.

The treatment previously applied to obtain the membrane microviscosity from the observed fluorescence anisotropy has been the Perrin equation (eq.1) for rotational depolarisation [6].

$$\frac{r_0}{r} = 1 + C(r)\frac{T\tau}{\eta} = A \qquad (1)$$

where r is the measured value of the fluorescence polarisation, r_0 is the value of the limiting fluorescence anisotropy at the excitation wavelength 355 nm and is taken to be 0.39 [14]. The lifetime of the excited state (τ) is taken to be 8.8 ns at 25°C. The values of $C(r)$ together with the fluorescence lifetime were considered to be constant in [6–8]. Thus the Perrin equation was further simplified (eq.2) and applied to give a direct relationship between the fluorescence anisotropy and the lipid microviscosity.

$$\left(\frac{r_0}{r} - 1\right)^{-1} = \eta K \quad \text{where } K \text{ is a constant)} \qquad (2)$$

It is clear that eq.2 does not differentiate between the dynamic characteristics of the membrane (viscosity) and the static (order) properties (as briefly described above). Such a treatment, therefore, may possibly lead to very significant interpretative errors [13,14]. Using the nomenclature of [13] and [14] the above equation should be ammended such that the parameter which accounts for the static (order) characteristics of the membrane must be retained as a variable. The structural parameter (r_∞) is thus included and is related to the steady-state fluorescence polarisation (r_s) by the following expression:

$$r_s = \frac{r - r_\infty}{1 + \tau/\phi} + r_\infty \qquad (3)$$

Where $\phi = \eta V/(KT)$ with V equivalent to the volume of the fluorophore. The microviscosity (η) thus remains as envisaged in [6] and eq.3 is equivalent to the Perrin equation (eq.1).

In [14] it was suggested that τ/ϕ may be con-

sidered to be equal to 8.0 when DPH is located in a lipid phase. With this latter case, eq.3 simplifies to eq.4.

$$r_\infty = 9/8 r_s - 1/20 \quad (4)$$

The validity of the relationship described by eq.4 was evaluated in [14] and was found to give a good approximation to the value of r_∞ obtained by other methods. Furthermore, in [13] it was pointed out that the relationship between the fluorescence lifetime and the rotational correlation time will determine the relative contributions to the observed fluorescence polarisation.

The problems and solutions raised in [13] and [14] with respect to the interpretation of the steady-state fluorescence polarisation of DPH require that within certain limits the membrane microviscosity remains constant. As a result of the points raised in [13,14] together with the observed tentative relationship between the steady-state fluorescence polarisation, the proton conductivity and the membrane potential (see below), it is clear that at the present time it is premature to differentiate between the relative contributions of the dynamic and static (order) parameters to the measured fluorescence polarisation. In consequence, we have presented our results as a plot of the measured fluorescence polarisation, which we shall consider to reflect the membrane 'state', against the various imposed membrane potentials (fig.3).

2. MATERIALS AND METHODS

All biochemical reagents purchased commercially were of the highest purity available. DPH was obtained from Serva (Heidelberg). Pyranine was obtained from Kodak-Eastman.

Phospholipid vesicles (PLVs) and rat-liver mitochondria (RLM) were prepared as in [4]. The RLM were labelled for fluorescent depolarisation measurements by the addition of 40 μl of a 2 mM ethanolic solution of DPH to each of the 50-ml centrifuge tubes used for the last of two centrifugation washes of the RLM. The RLM protein concentration in each centrifugation tube was approx. 4 mg/ml. A short incubation period (~20 min) was allowed for DPH labelling before the RLM were finally centrifuged.

Forty μl of a solution of 20 mM DPH was added to 1 ml suspensions of PLVs (40 mg lipid/ml), this mixture was vortex-mixed and then dialysed for 1 h against the last dialysis medium used for the preparation of the vesicles (see [4]). All treatments were carried out at 4°C.

Any changes of r_s induced by perturbation of the reaction mixture were resolved within about 5 s. The excitation wavelength was 355 nm and the emission wavelength was 430 nm, polarisation filters were changed manually.

After labelling with the various probes the vesicles and RLM were assayed for semipermeability to ions as in [4]. Membrane potentials were induced across the mitochondrial and vesicular membranes by imposing potassium ion diffusion potentials. This was achieved by making use of the high (100 mM) intramitochondrial K^+ concentration and the low (0.1 mM) intravesicular K^+ concentrations. Thus suspension of the respective particles in media of different K^+ concentrations would immediately impose a K^+ gradient. With the addition of valinomycin a diffusion potential was generated. By varying the K^+ concentrations of the various suspension media, at constant osmolarity, it was possible to vary the imposed membrane potentials, which were deduced by application of the Nernst equation. Further details of such techniques may be found in [4].

The proton flux across the membranes at various membrane potentials and pH gradients were assayed as in [4]. This method involved the assumption that the initial rate of relaxation of pH either after a pH jump or by the transient imposition of a membrane potential would reflect the membrane permeability to protons at the particular imposed gradient. In the case of PLVs both intra- and extravesicular pH were measured, achieved by preparing the PLVs in the presence of pyranine [4], a pH sensitive fluorescent probe. The extravesicular pyranine was then dialysed away. Pyranine is almost completely impermeant and therefore it was relatively simple to measure intravesicular pH with the aid of the spectrofluorimeter. The excitation wavelength was 460 nm and the measuring emission wavelength was 510 nm. Thus when the vesicles were suspended in media of low external buffering capacity and a pH electrode introduced both the intra- and extravesicular pH could be monitored continuously. In the cases of RLM,

pyranine was not used and pH measurements were made solely with an electrode.

Fluorescence polarisation measurements were performed upon the various membrane systems after membrane potentials were induced as described above.

3. RESULTS AND DISCUSSION

It is shown in figs 1 and 2 that membrane proton conductance was observed to be a linear function of pH gradients up to 200 mV and electrical gradients up to about 140 mV. If, however, larger electrical gradients were applied the protonic conductance was observed to increase manifold. These effects were apparent in both artificial phospholipid membranes and mitochondria. In both cases

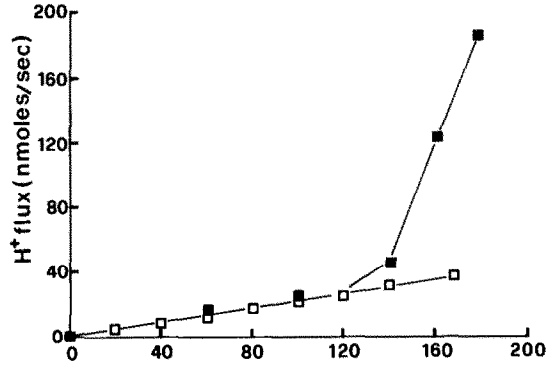

Fig.1. The effect of a pH gradient and an electric field upon proton flux across the membranes of phospholipid vesicles. PLVs with an intravesicular medium consisting of 96 mM choline chloride, 5 mM Li–Hepes, 0.5 mM pyranine, 0.1 mM EDTA and 0.1 mM KCl (pH 6.8) were suspended in media of various KCl concentrations at the same pH and with the same concentration of buffer and chelator. Iso-osmolarity was maintained with choline chloride. The KCl concentrations used were calculated to generate the membrane potential (mV) (■---■), on addition of 1.0 μM valinomycin, shown by the abscissa (scale: mV). At this time the initial rate of change of both the intra- and extravesicular pH values were measured with pyranine fluorescence and an electrode, respectively. Similar measurements were taken after making acid or alkaline pH jumps of differing magnitudes by the addition of quantities of 0.1 M HCl or 0.1 M NaOH, respectively. The H^+ flux across the membrane may thus be calculated and is plotted on the ordinate at the different imposed gradients. (□---□) pH gradient (mV).

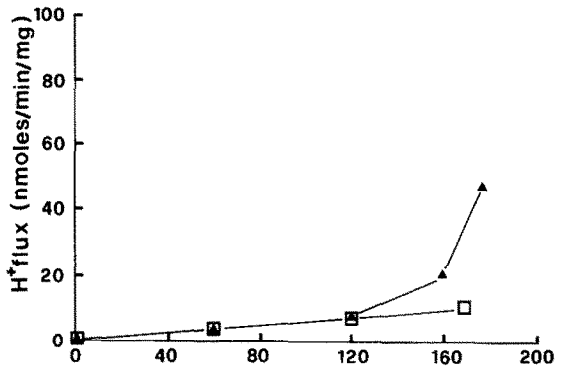

Fig.2. The effect of a pH gradient and an electric field upon proton flux across the membranes of rat-liver mitochondria. The experimental conditions were as follows: 2 mg/ml protein RLM suspended in 2 mM Hepes, 2 mM cyanide, 0.5 mM EGTA, 1.0 μM oligomycin and various KCl–choline chloride mixtures such that the various media were iso-osmotic with the intramitochondrial space. The KCl concentration was calculated to produce, on addition of 1.0 μM valinomycin, the indicated membrane potentials (mV) (▲---▲). Acid and alkaline pH jumps were initiated in the presence of 1.0 μM valinomycin by the addition of quantities of 0.1 M HCl and 0.1 M NaOH, respectively. The initial rates of the resultant proton fluxes were then measured with an electrode. (□---□) pH gradient. Abscissa: scale in mV.

the protonic current was, therefore, not a linear function of the total voltage range studied. Rather, the relationship approximated to an exponential function. Thus although there was an equivalence in thermodynamic terms of the driving forces for proton flux the respective effects of a pH gradient and an electrical gradient on the flux were not equivalent. We may infer from these observations that due to the nature of the electric field some feature of the membrane structure was altered to facilitate a more rapid conduction process; such an effect was absent when a chemical pH gradient was applied.

To equate the state of the membrane (viscosity or order) and conductance (protonic or otherwise) similar experiments were performed to those described in figs 1 and 2 with fluorescence polarisation as the measured independent variable.

Membrane fluidity may be altered in several ways, for example by increasing the ambient temperature or changing the pressure on the membrane

whether it be osmotic, atmospheric, or otherwise. It is conceivable, therefore, that when the membrane fluidity or order changes, the amount of ordered water is also affected. The latter may be due to a change in the total amount of water in the membrane or to a change of the existing ordered water.

It has been reported [7] that an electric field may affect the lipid microviscosity of vesicles made from a single type of phospholipid. We have extended these studies to the lipid systems used for protein reconstitution (e.g., cytochrome oxidase vesicles) and mitochondria and found that indeed the 'lipid microviscosity' (cf eq.3) appeared to be affected by the imposition of a transmembrane electrical potential difference [8]. Although, as we have discussed above, such conclusions assume the isotropic behaviour of DPH. Therefore, although there was a significant change of either/both the order and the viscosity, it was not possible to resolve their relative contributions to the observed fluorescence polarisation.

Fig.3 indicates the effect of various applied membrane potentials on the relative membrane

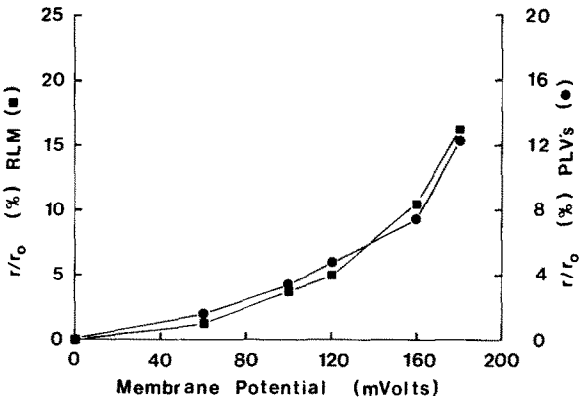

Fig.3. The effect of a membrane potential upon the observed fluorescence polarisation of DPH labelled RLM and COVs. Fluorescence polarisation is expressed as a percentage change of r with respect to r_0 (r/r_0). This is considered to reflect the 'ordered state' of the membrane. Membrane potentials (mV) were generated as in figs 1 and 2. The reaction conditions were similar, but RLM were suspended at 0.1 mg protein/ml and the media included 20 mM Li–Hepes. The fluorescence polarisation was measured as soon as possible after the induction of the membrane potential (~5–10 s). The ambient temperature was 20°C.

state as measured by fluorescence polarisation of the hydrophobic membrane probe, DPH. It is clear that there is not a linear dependence of the membrane 'fluidity' on the applied field. Furthermore, it was found using the same technique (not shown) that pH gradients did not affect the membrane in the same way.

There is a striking resemblance between the figures. Both the membrane order/viscosity and the proton conductance bear an exponential relation to an applied electric field. Furthermore, the region of the current/voltage curve, whereupon the conductance was seen to begin to increase rapidly (~140–160 mV), is in the same voltage region as that of the break from linearity exhibited by the fluidity/voltage curve (fig.3). It seems that there is an empirical relationship between the fluidity or ordered state of a membrane and the proton conductance.

The molecular phenomena leading to changes of membrane permeability are no clearer in the system we have investigated than the more general system whereby other ions are considered. A recent review [9] gives a useful overview of the various events which are evident when other solutes are considered. Moreover, in many ways the elementary nature of the proton makes it a less than ideal species on which to model ion transport processes in general. In fact, even the manner of proton movement in aqueous media is quite different from that of other cations. However, as proton movements are of particular interest in studies of oxidative phosphorylation, it would be useful to consider how an electrical field may initiate changes of the membrane integrity such that the higher conductance state may be attained.

In an attempt to model the effects of electric fields upon membranes, it has been [10] proposed that an electromechanical compression of the membrane takes place with the imposition of an electrical field. This model thus attempted to relate changes of the membrane, in response to the application of an electric field, to similar changes of the membrane due to other treatments. It is conceivable, therefore, that the effects of hydrostatic and osmotic pressures upon lipid fluidity could be directly related to those of an electrical field. It may be expected therefore that the well established increase of lipid fluidity attributed to anaesthetics [12] may be reversed by an increase of a membrane

potential as in the cases of the other pressures [12].

Presumably the exponential nature of the current-voltage curve indicates that a certain activation energy is necessary to initiate the higher conductance state or initiate a change of the inertial fluidity of the membrane. Thus if we assume that the measured fluorescence polarisation reflects the membrane fluidity state, a Boltzmann distribution would be expected to respond to such thermodynamic parameters as temperature and pressure. At constant temperature and applying the Crowley model [10] it is feasible, therefore, to consider the membrane potential as a pressure upon the membrane and therefore the activation energy/mol may be expressed as a function of the membrane potential. Using this approach it was possible to calculate an activation energy for the inertial change of the membrane 'fluidity' to be of the order of 7–15 kJ/mol depending on the temperature at which the measurements were made. It is probable therefore that the applied electric field causes a change in the order of the lipid which in turn affects the state of any associated water. It is even possible that the dielectric constant of the membrane is then altered to make proton permeation more probable.

The mechanisms of proton movement in ice and aqueous water are the same, thus similar mechanisms would probably be responsible for proton mobility in membranes. Such mechanisms, i.e., the so-called Bjerrum fault propagation or tunnelling, are responsible for the anomalously high mobilities of protons in water media ($\sim 5.4 \times 10^{-6}$ mho/m) [5]. Thus, although the various states of water are capable of transporting protons very rapidly, transport is far less probable through membrane-associated water. There are many reasons why this latter condition prevails but it remains unclear as to how the membrane potential dependent ordering of the membrane initiates the higher conductance condition.

Hydrogen bonded chains of water molecules have been suggested to be responsible for proton transport within hydrophilic domains of transmembrane proton pumps [5]. It is interesting, however, that even the hydrophobic components of membranes may provide relatively rapid proton conduction devices, presumably by similar mechanisms.

Obviously the tentative relationship between the field induced change of the fluidity state of a membrane and the associated permeability to H^+/OH^- will require further investigation, and it is currently in progress in our laboratory.

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