Ubiquinones have Surface-Active Properties Suited to Transport Electrons and Protons Across Membranes

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Surface-active properties of ubiquinones and ubiquinol have been investigated by monomolecular-film techniques. Stable monolayers are formed at an air/water interface by the fully oxidized and reduced forms of the coenzyme; collapse pressures and hence stability of the films tend to increase with decreasing length of the isoprenoid side chain and films of the reduced coenzymes are more stable than those of their oxidized counterparts. Ubiquinone with a side chain of two isoprenoid units does not form stable monolayers at the air/water interface. Mixed monolayers of ubiquinol-10 or ubiquinone-10 with 1,2-dimyristoyl phosphatidylcholine, soya phosphatidylcholine and diphosphatidylglycerol do not exhibit ideal mixing characteristics. At surface pressures less than the collapse pressure of pure ubiquinone-10 monolayers (approx. $12 \text{ mN} \cdot \text{m}^{-1}$) the isoprenoid chain is located substantially within the region occupied by the fatty acyl residues of the phospholipids. With increasing surface pressure the ubiquinones and their fully reduced equivalents are progressively squeezed out from between the phospholipid molecules until, at a pressure of about $35 \text{ mN} \cdot \text{m}^{-1}$, the film has surface properties consistent with that of the pure phospholipid monolayer. This suggests that the ubiquinone(ol) forms a separate phase overlying the phospholipid monolayer. The implications of this energetically poised situation, where the quinone(ol) is just able to penetrate the phospholipid film, are considered in terms of the function of ubiquinone(ol) as electron and proton carriers of energy-transducing membranes.

One of the central tenets of the chemiosmotic hypothesis of energy coupling (Mitchell, 1961, 1966*a,b*, 1968, 1973; Greville, 1969) is the fundamental role of the protonmotive force. How electrochemical proton gradients are created in various biological systems and the way these are coupled to endergonic processes remains unclear. The prevailing theory to explain how respiration by mitochondria generates an electrochemical proton gradient postulates the existence of 'loops' in the respiratory chain (Mitchell, 1976*a*) in which the proton and/or electron carriers representing an 'energy-coupling site' are disposed within the energy-coupling membrane so that two protons and two electrons pass from a site located on one side of the membrane in the first arm of the loop to the next component of the electron-transport chain situated on the opposite side of the membrane. The loop is completed by the vectorial transfer of electrons only from this carrier to an electron acceptor on the first side of the membrane thereby releasing two protons into the aqueous phase. Operation of the loop in mitochondria therefore results in the extrusion of

protons from the matrix and flow of electrons from the outer to the inner surface of the membrane, thereby generating the electrochemical proton gradient.

The role of ubiquinone as a redox carrier in the respiratory chain has been characterized by reconstitution studies (Ernster *et al.*, 1969) and kinetic measurements (Kröger & Klingenberg, 1973). Ubiquinone, in addition to its catalytic function, appears to regulate the activity of succinate dehydrogenase (Gutman *et al.*, 1971*a,b*) and the cytochrome *b-c*₁ complex (Nelson *et al.*, 1972), but how this effect is mediated is unknown. Mitchell (1975*a,b*, 1976*b*) has proposed that ubiquinone acts as the electron and proton carrier at site II in the mitochondrial membrane. A protonmotive ubiquinone cycle has been described to explain how the coenzyme fulfils this role. Briefly the cycle relies on the translocation of ubiquinone or ubiquinol by a flip-flop process from one side of the membrane to the other. Similar movements of the partially reduced ubisemiquinone species, on the other hand, are prohibited in this scheme. When such intermediates form they are

presumed to remain at the surface of the membrane.

One of the major criticisms of the proposed protonmotive Q cycle is that, by analogy with transmembrane movements of phospholipids, the rate of flip-flop of ubiquinone(ol) would not be fast enough to sustain observed rates of electron transport (DePierre & Ernster, 1977). We have undertaken experiments to investigate the surface-active properties of ubiquinones and their fully reduced quinol analogues. The behaviour of these molecules in mixed phospholipid systems was examined to provide information about the orientation and likely mobility of ubiquinones(ols) in energy-transducing membranes.

Materials and Methods

Surface-pressure–area curves of monomolecular films were determined on an apparatus consisting of a Perspex trough (16 cm × 4.5 cm in surface area and 0.8 cm deep) with a capacity of about 65 ml. The subphase was stirred during measurements by means of a glass-coated metal bar drawn magnetically along the bottom of the trough at a constant rate. Surface pressure was determined by measuring the vertical force exerted on a glass rod (approx. 2 mm diameter) dipping into the surface film. To ensure zero contact angle with the subphase surface the rod was coated with carbon particles by passing it through a benzene flame and subsequently rendering it completely hydrophilic by immersion for several hours in water (Cheesman, 1946). The force was measured continuously by an electronic micro-force balance (C.I. Electronic, Slough, Bucks, U.K.) and recorded on a pen recorder. Surface-tension measurements performed on a clean water surface at 23°C gave a value of 72.4 mN·m⁻¹. The surface potential was measured with an ²⁴¹Am air-ionizing electrode (Quarles & Dawson, 1969). Conventionally ΔV represents the difference between the interfacial junction potential of a surface monomolecular film and a clean substrate surface on which the film is spread. The subphase in these experiments was 10 mM-NaCl prepared from water distilled freshly each day from alkaline KMnO₄ solution immediately before use. The interfacial junction potential of a clean substrate surface remained relatively constant over a period of 30 min with stirring, suggesting that the distillation process had substantially removed surface-active materials from the subphase.

Monolayers of ubiquinones and ubiquinol were spread on the surface by delivering a portion of a solution of the lipid in light petroleum (b.p. 40–60°C) to the surface with an Agla microsyringe (Burroughs Wellcome and Co., Beckenham, Kent, U.K.). Mixed monolayers of ubiquinones and phospholipids or pure phospholipid monolayers were

spread in a similar manner from a solvent of light petroleum/chloroform, (4:1, v/v). Some experiments using benzene as a spreading solvent indicated that there was incomplete evaporation of the solvent from the surface layer and this solvent was avoided for this reason. Measurements were performed on at least two separate films (usually three or more) of appropriate composition. Reproducibility of surface-pressure measurements in molecular area isotherms was within ± 2 mN·m⁻¹ and the surface area per molecule was within ± 0.05 nm². Surface-potential measurements were reproducible within ± 10 mV.

Ubiquinones were a gift from Eisai Co., Tokyo, Japan. Where required, ubiquinones were reduced with NaBH₄ and complete reduction was confirmed by spectroscopic examination. 1,2-Dimyristoyl phosphatidylcholine was obtained from Sigma, London, U.K. Soya phosphatidylcholine was purified from crude preparations supplied by Sigma using methods described by Vigo *et al.* (1978) and bovine heart diphosphatidylglycerol was supplied by Lipid Products, South Nutfield, Surrey, U.K.

Results and Discussion

Monolayer properties of ubiquinones

Ubiquinone-10, when spread on an aqueous subphase, forms stable monolayers at low surface densities. Fig. 1 shows surface-pressure–area and surface-potential–area curves of ubiquinone-10 and ubiquinol-10. Ubiquinone-10 occupies a slightly smaller surface area than the corresponding reduced quinol derivative and the latter forms stable mono-

Fig. 1. Surface-pressure–area (—) and surface-potential–area (---) curves for monomolecular films of ubiquinone-10 (●) and ubiquinol-10 (O) spread on a subphase of 10 mM-NaCl

layers at higher pressures. This could be explained by a more substantial polar interaction of the quinol with the aqueous subphase compared with the quinone. Thus in the reduced form there is a shift in the balance of hydrophobic to hydrophilic character of the molecule in favour of greater polarity. This is consistent with the surface-potential measurements, which represent the sum of the vertical dipole moments arising from the reorientation of water molecules about the adsorbed lipid (μ_1), the characteristic dipole moment of the ubiquinones or so-called group dipole moment (μ_2) and finally the dipole moment about the terminal bond of the isoprenoid unit at the upper limit of the monolayer (μ_3). The various dipoles are related to the measured ΔV by the Helmholtz formula for an array of n dipoles per unit area. Where the dipole moments are vectorially additive in the vertical direction then:

$$\Delta V = 4\pi n(\mu_1 + \mu_2 + \mu_3)$$

Since even the fully reduced form of the coenzyme is almost entirely uncharged at neutral pH (pK_a approx. 9.45) no term is required to account for any electrostatic potential. Significantly higher values for surface potentials of ubiquinol-10 compared with ubiquinone-10 are most likely due to an increase in μ_1 and possibly a small change in μ_2 , but it should be emphasized that an alteration in one dipole invariably affects the orientation of the remaining dipoles and unequivocal assignment to one or other dipole cannot be made with precision.

Additional information concerning the amphipathic character of these molecules can be obtained from studies of the monolayer properties of ubiquinones and ubiquinols with isoprenoid chains of various lengths. Surface-pressure–area curves were constructed for a homologous series of ubiquinones varying in the number of isoprenoid units from 3 to 10 and for a corresponding series of their fully reduced counterparts. Similar surface-pressure–area curves to those illustrated in Fig. 1 were obtained but the pressure at which the monolayer became unstable and collapsed was directly related to the length of the isoprenoid side chain between 4 and 10 isoprenoid units. This is illustrated in Fig. 2, where the collapse pressure of the monolayer is plotted as a function of the length of the isoprenoid side chain. Again it can be seen that ubiquinols form more stable monolayers than the corresponding ubiquinones. Moreover, as the length of the isoprenoid chain decreases the monolayers become more stable to higher pressures, which is consistent with an alteration of the amphipathic balance within the molecule. The ability of ubiquinone derivatives to penetrate phospholipid monolayers at surface pressures of $5 \text{ mN} \cdot \text{m}^{-1}$ has also been shown to increase with increasing isoprenoid chain length (Maggio *et al.*, 1977), consistent with the surface-active char-

Fig. 2. Relationship between the length of the isoprenoid substituent of ubiquinones (●) and ubiquinols (▲) and the collapse pressure of monomolecular films spread on subphases of 10 mM-NaCl

acter of the ubiquinones. Ubiquinol-3 was unstable at high surface pressures and appeared to partition into the aqueous phase; ubiquinone monolayers had a stability consistent with the length of the isoprenoid side chain in the homologous series presented in Fig. 2. Attempts to prepare monomolecular films of ubiquinone-2 on subphases of 10 mM-NaCl were unsuccessful, since this compound was not sufficiently insoluble in the subphase to form stable films. The best estimate of the cross-sectional area of the ubiquinones that we could obtain from these monolayer studies was about 0.45 nm^2 . This is consistent with the van der Waals dimension of a methyl-branched hydrocarbon chain oriented in close-packed array.

Mixed monolayers of ubiquinones and dimyristoyl phosphatidylcholine

To obtain information about the properties of ubiquinones in mixed phospholipid systems we determined the surface-pressure–area relationships for monomolecular films consisting of various molar ratios of ubiquinones and 1,2-dimyristoyl phosphatidylcholine. The curves for mixed monolayers of ubiquinone-10 and ubiquinol-10 are presented in Figs. 3(a) and 3(b) respectively. It is obvious from both sets of curves that the films deviate markedly from an ideal mixture in which the mean area per molecule is directly related to the particular proportion of each molecule in the monolayer. Thus in an ideal mixture the mean area per molecule would fall on a straight line joining the area per molecule in monolayers of the pure compounds as illustrated in Fig. 4(a). It can be seen from this Figure that at a surface pressure of $10 \text{ mN} \cdot \text{m}^{-1}$ the molecules occupy a volume in the film that is greater than would

Fig. 3. Surface-pressure-area curves of ubiquinone-10 (a) and ubiquinol-10 (b) and mixtures of the respective coenzymes with 1,2-dimyristoyl phosphatidylcholine. The ubiquinone(ol)/phospholipid molar ratio was: ○, 0:1; △, 25:75; □, 50:50; ■, 75:25; ●, 1:0. Monolayers were spread on subphases of 10 mM-NaCl.

be expected from the pure compounds alone. The reason for this may be appreciated by considering the likely arrangements of the molecules in the film.

Fig. 4. Mean-molecular-area-mole-fraction plots of (a) ubiquinone-10/1,2-dimyristoyl phosphatidylcholine mixtures and (b) ubiquinone-4/1,2-dimyristoyl phosphatidylcholine mixtures at 10 (○), 20 (●) and 30 $\text{mN}\cdot\text{m}^{-1}$ (□).

The limiting area of ubiquinones at pressures of 20 and 30 $\text{mN}\cdot\text{m}^{-1}$ was taken as 0.45 nm^2 .

Dimyristoyl phosphatidylcholine is an amphipathic molecule that orients strongly with the hydrated phosphocholine head group immersed in the underlying aqueous phase and the fully saturated hydrocarbon chains directed vertically into the air. Ubiquinone-10, however, contains an isoprenoid chain that when fully extended is more than twice the length of the fatty acyl residues of the phospholipid molecules and, assuming that the quinone ring is located at or near the polar-hydrocarbon interface, at least at pressures less than 10 $\text{mN}\cdot\text{m}^{-1}$, the isoprenoid chain is unlikely to be oriented vertically along its entire length. That the deviation from ideal mixing becomes greater as the proportion of ubiquinone-10 in the monolayer increases suggests that at surface pressures of 10 $\text{mN}\cdot\text{m}^{-1}$ or less the isoprenoid chain is interpolated in the hydrocarbon chain region of the phospholipids in the film. At higher pressures, judged from Figs. 3(a) and 3(b) to be between 10

and $15 \text{ mN} \cdot \text{m}^{-1}$, there appears to be a marked condensing effect indicated by the curves at surface pressures of 20 and $30 \text{ mN} \cdot \text{m}^{-1}$ respectively plotted in Fig. 4(a). The effect is most noticeable when the proportion of ubiquinone-10 to phospholipid increases. Two interpretations are possible to explain this effect. The first is that there is a condensing effect brought about by the interaction between the molecules of the film in an analogous manner to that of cholesterol on expanded lipid monolayers (Chapman *et al.*, 1969). Alternatively, ubiquinone molecules may be progressively squeezed out of the film.

Further experiments with mixed monolayers of ubiquinone-4 and dimyristoyl phosphatidylcholine were undertaken to provide information on this point. Ubiquinone-4 was chosen because the length of the fully extended molecule is approximately the same as the phospholipid when both molecules are oriented vertically in the film. The curves shown in Fig. 4(b) are the mean molecular areas of these mixed films. In this case no 'condensing' effect is observed at a monolayer pressure of $20 \text{ mN} \cdot \text{m}^{-1}$, which is consistent with the fact that the shorter isoprenoid chain alters the amphipathic balance so that mixed monolayers are stable at higher surface pressures. Nevertheless, if this interpretation is correct, the ubiquinone-4 molecules may be squeezed out of the film at higher pressures, e.g. $30 \text{ mN} \cdot \text{m}^{-1}$. It is noteworthy that at low pressures the mean molecular area is still greater than that of ideally mixed components, suggesting that the ubiquinone molecule does not pack comfortably between the hydrocarbon chains of the phospholipids.

To confirm that ubiquinone-10 molecules are squeezed out from between the phospholipid molecules of the film we recalculated the force-area curves for mixed monolayers of ubiquinone-10 and dimyristoyl phosphatidylcholine and plotted the area occupied by the phospholipid alone. These curves are illustrated in Fig. 5; similar curves were also obtained for mixed monolayers of ubiquinol-10 and dimyristoyl phosphatidylcholine (results not shown). This Figure shows that at lower pressures, i.e. less than the collapse pressure of the ubiquinone, the area occupied by the phospholipid depends on the molar ratio of ubiquinone-10 and dimyristoyl phosphatidylcholine in the film. At surface pressures greater than about $15 \text{ mN} \cdot \text{m}^{-1}$ the composition of the mixed component of the monolayer becomes constant irrespective of the overall molar ratio of the film constituents and the proportion of ubiquinone-10 in the mixed compartment decreases with increasing surface pressure. When a surface pressure of about $35 \text{ mN} \cdot \text{m}^{-1}$ is reached the two components of the monolayer appear to be completely separated into two phases with the surface characteristics, with respect to surface pressure,

Fig. 5. Surface-pressure curves of mixed monolayers of ubiquinone-10 and 1,2-dimyristoyl phosphatidylcholine plotted as a function of the area occupied by the phospholipid only

The ubiquinone-10/phospholipid molar ratio was:
○, 0:1; △, 25:75; □, 50:50; ■, 75:25.

identical with that of a monolayer of pure dimyristoyl phosphatidylcholine. Because of the hydrophobic character of ubiquinone-10 it may be assumed that these molecules have been squeezed out from between the hydrocarbon chains of the phospholipid and are present as a disordered phase overlying the phospholipid monolayer.

To check whether the effects observed with mixed monolayers of the fully saturated phospholipid were the same as that for unsaturated phospholipids of natural origin, the experiments reported above were repeated with purified soya phosphatidylcholine. Similar results were obtained, i.e. ubiquinone-10 and ubiquinol-10 occupied a greater area at pressures in mixed monolayers less than about $10 \text{ mN} \cdot \text{m}^{-1}$, but were progressively squeezed out from between the phosphatidylcholine molecules, so that again a two phase system was created at about $30 \text{ mN} \cdot \text{m}^{-1}$. It may be concluded that the behaviour of mixed monolayers of phosphatidylcholines and ubiquinones(ols) are not affected by the degree of saturation of the fatty acyl residues.

Mixed monolayers of ubiquinones and diphosphatidylglycerol

Experiments were undertaken to see if mixed monolayers containing dimyristoyl phosphatidylcholine behaved similarly to mixed monolayers containing phospholipids of the type found in energy-transducing membranes containing ubiquinone. Diphosphatidylglycerol was selected for this study because not only is it a phospholipid found predominantly in ubiquinone-containing membranes, but it has polyunsaturated fatty acyl residues and negatively-charged phosphate groups, which together lead to a gaseous-expanded type of monolayer. This is illustrated in Fig. 6 were surface-pressure–area curves of mixed monolayers containing different molar ratios of ubiquinone-10 (Fig. 6a) and ubiquinol-10 (Fig. 6b) are also presented. Plots of mean molecular areas of films containing different molar ratios of ubiquinone-10 and ubiquinol-10 to diphosphatidylglycerol at 10, 20 and 30 $\text{mN}\cdot\text{m}^{-1}$ as a function of the film composition (results not shown) show similar effects to those noted with mixed monolayers of dimyristoyl phosphatidylcholine (Fig. 4a). Nevertheless, it can be seen from a comparison of Figs. 6(a) and 6(b) that ubiquinol-10 forms much more stable monolayers when mixed with diphosphatidylglycerol than does ubiquinone-10. This is especially apparent when the molar ratio of the coenzyme is high relative to phospholipid and at surface pressures greater than 20 $\text{mN}\cdot\text{m}^{-1}$. When the area per molecule is calculated on the basis of diphosphatidylglycerol alone at a pressure corresponding to that in bilayer membranes (32 $\text{mN}\cdot\text{m}^{-1}$) ubiquinone-10 is almost entirely excluded from the interfacial region, whereas ubiquinol-10 is largely oriented at the water/hydrocarbon interface. This behaviour markedly contrasts with that observed with mixed monolayers containing phosphatidylcholines. The effect observed may be due to the particular shape of the diphosphatidylglycerol molecule, which differs considerably from the diacylphospholipids, or to the surface-charge properties of the monolayers. We investigated the latter point by modifying the electrostatic charges of the phosphate groups of diphosphatidylglycerol and observing the effects in mixed monolayer systems. Plots of mean-molecular-area curves of mixed monolayers of diphosphatidylglycerol and ubiquinone-10 or ubiquinol-10 spread on subphases of either 10 mM- or 150 mM-NaCl showed that over this range of salt concentration there was no significant effect on the properties of the films. Mixed monolayers of ubiquinol-10 and diphosphatidylglycerol spread on subphases of 1 M-HCl, however, showed similar behaviour to the system containing oxidized coenzyme. Thus adjusting the subphase pH to below the pK_a of the phosphate groups of diphosphatidylglycerol causes the ubiquinol-10 to be squeezed

Fig. 6. Surface-pressure–mean-molecular-area curves for mixed monolayers of ubiquinone-10 (a) and ubiquinol-10 (b) with diphosphatidylglycerol

Symbols: $\square$, pure ubiquinone(ol)-10; $\blacksquare$, 75 mol% ubiquinone(ol)-10; $\bullet$, 50 mol% ubiquinone(ol)-10; $\circ$, 25 mol% ubiquinone(ol)-10; $\blacktriangle$, pure diphosphatidylglycerol. The points indicating molecular areas at 32 $\text{mN}\cdot\text{m}^{-1}$ are calculated for diphosphatidylglycerol alone in the respective mixed monolayers with oxidized and reduced coenzyme respectively. All monolayers were spread on subphases of 150 mM-NaCl.

out from between the lipid molecules. This effect is most likely due to the close packing of the diphosphatidylglycerol molecules, which is permitted once electrostatic charge repulsion between neighbouring molecules is decreased rather than

ionization of the ubiquinol-10, which would increase the stability of mixed monolayers.

Ubiquinone(ol) as a protonophore and redox carrier

The two questions of paramount importance in explaining the molecular mechanisms associated with the function of quinones in biological electron-transport systems are (1) how are the protons and electrons shuttled between the various compartments and redox carriers of the chain and (2) what provides the vectorial driving force for these movements? The studies reported here provide some indication of how ubiquinones and ubiquinols may be oriented and move in the energy-transducing membrane without invoking the participation of specific binding proteins. Thus from the behaviour of mixed monomolecular films at the air/water interface, it is possible to predict the likely orientation of ubiquinone(ol)-10 in lipid bilayer membranes. To relate the two systems, however, it is necessary to equate the behaviour of mixed monomolecular films at surface pressures equivalent to that which exists in natural bilayer membranes. This is particularly important in the case under consideration because the characteristics of mixed monolayers have been found to depend markedly on the surface pressure of the monolayer. The most reliable data on the equivalent pressure in phospholipid bilayer membranes are the studies of phospholipase susceptibility of monomolecular films of phospholipids compared at different surface pressures with bilayer structures reported by van Deenen *et al.* (1976). They concluded in the case of the erythrocyte membrane that the packing density of phospholipids at the exterior layer of membrane was equivalent to a lateral surface pressure of between 31 and 34 mN·m⁻¹. Examination of our results show that in mixed monolayers of phosphatidylcholines and ubiquinone(ol) at surface pressures of about 32 mN·m⁻¹ the quinone(ol) residue was poised at the interface. This would indicate that in phospholipid bilayer membranes the bulk of the oxidized and reduced coenzyme would reside in the centre of the structure where the terminal methyl residues of the hydrocarbon chains are located. A small proportion of the polar region of the molecules, however, would be oriented at the hydrocarbon/water interface. Furthermore, because of the poised situation there is likely to be only a small difference in free energy of molecules oriented at the interface and that of molecules in a largely hydrophobic environment. The same situation would also seem to apply to bilayers of cardiolipin containing ubiquinone-10, but the corresponding reduced quinol appears to orient somewhat more favourably at the charged interface. The preferential surface orientation was reduced by adjusting the subphase to pH 0. This suggests that when the phospholipid

carries a surface charge the more polar quinol residue is able to occupy a position between the lipid molecules which are less densely packed as a consequence of charge-repulsive effects. The alternative explanation that ubiquinol was partially dissociated at neutral pH seems unlikely since the pK_a for the reaction:

is about 9.45, assuming an acidity comparable with that for 1,4-naphthoquinol (Kortüm *et al.*, 1961), and similar effects were not observed with the zwitterionic phosphatidylcholine films.

We may conclude from our monolayer studies that in phospholipid bilayers the quinone(ol) ring system of ubiquinone and ubiquinol would be capable of sufficient transbilayer motion to account for an efficient proton conduction across lipid bilayers. Thus the movement of hydrogen atoms across the hydrocarbon region of the membrane could be accomplished with a relatively low activation energy. This conclusion is consistent with that of Hauska (1977), who demonstrated transbilayer transport of reducing equivalents and protons across liposomes consisting of mixtures of soya phosphatidylcholine and plastoquinone-9 or ubiquinone-10. Furthermore although the scheme proposed by Robertson & Boardman (1975) provides a plausible explanation of the reduction of ubiquinone and its subsequent protonation to form ubiquinol at the water/hydrocarbon interface of the lipid bilayer membrane, there seems, on the basis of our experiments, no thermodynamic reasons why ubiquinol could not act as a protonophore and deliver protons at the opposite side of the membrane on subsequent oxidation.

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