Surface characteristics of phosphatidylglycerol phosphate from the extreme halophile *Halobacterium cutirubrum* compared with those of its deoxy analogue, at the air/water interface

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The relationship between area per molecule and surface pressure of monolayers of phosphatidylglycerol phosphate from extreme halophile *Halobacterium cutirubrum* and its deoxy analogue, deoxyphosphatidylglycerol phosphate, spread at an air/water interface was examined. The effect of ionization of the primary and secondary acidic functions of the phosphate groups of the two lipids on surface characteristics of compression isotherms was determined by spreading monolayers on subphases with pH values ranging from below the apparent pK_a of the primary ionization (pH 0) to greater than that of secondary ionization (pH 10.9). The limiting molecular area increases with decreasing pH below 2. Ionization of the primary phosphate functions of both phospholipids (with bulk pK_1 values close to 4) is associated with a marked expansion of the films, as judged by values of limiting molecular area. Ionization of the secondary phosphate functions causes further expansion of the films, with the apparent pK_2 of deoxyphosphatidylglycerol phosphate slightly less than that indicated for phosphatidylglycerol phosphate. Values of surface-compressibility modulus calculated from the surface characteristics of the phosphatidylglycerol phosphate monolayers showed that films spread on subphases with a pH of about the apparent pK_1 of the primary phosphate functions were the least compressible. Increasing or decreasing subphase pH caused an increase in compressibility; this effect on compressibility was much less with monolayers of deoxyphosphatidylglycerol phosphate at high pH. The effect of inorganic counter-ions on monolayer characteristics of phosphatidylglycerol phosphate was examined by using subphases of NaCl concentrations varying from 0.01 to 1 M. The limiting molecular area was found to increase exponentially with respect to the subphase NaCl concentration.

INTRODUCTION

The lipids of the membranes of the extremely halophilic bacteria are markedly different from those of other organisms, and this difference is believed to be related to their requirement for growth conditions that consist of saturated or almost saturated solutions of inorganic salts (Bayley & Morton, 1978; Grant & Kogut, 1986). The characteristic features of these lipids are the substitution of saturated phytol (phytanyl) chains for fatty acyl groups and the linkage of these groups via ether bonds to the carbon atoms 2 and 3 of the glycerol backbone. It has been argued (Kates, 1972) that this structure and configuration of the lipid results in resistance to peroxidative damage and protection against hydrolysis by phospholipases of other organisms, which possess specificity in general for ester bonds at the C-1 and C-2 positions of the glycerol moiety.

The dominant lipid species of both red and purple membranes of *Halobacterium cutirubrum* is the 3'-sn-glycero-1'-phosphate derivative of 2,3-di-O-phytanyl-sn-glycerol designated 'phosphatidylglycerol phosphate' (Kates, 1978). The chemistry and metabolism of these lipids has been reviewed in detail (Kates, 1978; Paltauf, 1983; Kamekura & Kates, 1988).

A number of studies have been reported on the structural features of the phytanyl ether lipids of *Halobacterium cutirubrum* when dispersed in aqueous

systems. It was found, for example, that stable bilayer structures of these lipids were only formed in solutions containing salt concentrations of less than 2 M (Chen *et al.*, 1974). At higher salt concentrations, shielding of the negative charges on the polar head groups of the lipids was thought to cause a decrease in surface area occupied by the molecules at the interface between the lipid and water, resulting in the development of strong cohesive interactions between the phytanyl chains that prevented formation of stable bilayer structures.

The causes of the loss of permeability-barrier properties observed in dispersions of lipids of *Halobacterium cutirubrum* at high salt concentrations have been identified in freeze-fracture studies of dispersions prepared in 5 M-NaCl (Quinn *et al.*, 1986). These showed that phosphatidylglycerol phosphate forms small liposomal-like structures in water, consisting of relatively few layers. On increasing the salt concentration of the dispersion the lipid is transformed into non-lamellar phases, such as tubular inverted micelles, which dominate the phase behaviour, particularly at temperatures of 70 °C or greater. The two charged phosphate groups of phosphatidylglycerol phosphate (apparent pK_1 2.4; Stewart *et al.*, 1988) appear to be highly hydrated and at low salt concentrations form lamellar structures of a continuously hydrating system. It is noteworthy that the third ionizable P-OH has an anomalously high apparent pK_2 (> 11; Stewart *et al.*, 1988). Under the temperature

and salt conditions experienced by the living organism from which the lipids were extracted, it was concluded that phosphatidylglycerol phosphate would tend to form a mixed lamellar and non-lamellar phase.

^{31}P -n.m.r.-spectroscopic studies (Ekiel *et al.*, 1981) and differential-scanning-calorimetric experiments (Chen *et al.*, 1974) have suggested that the phytanyl chains do not undergo thermal transitions at temperatures above 0 °C and remain in a disordered configuration. Transitions in the polar-head-group region can, however, be detected in lipid dispersed in 4 M-NaCl or 0.1 M-MgCl₂ over the temperature range -11 °C to 45 °C using spin-label spectroscopy and specific-volume measurements (Plachy *et al.*, 1974).

We have undertaken a study of the monomolecular film characteristics of phosphatidylglycerol phosphate spread at the air/water interface to study further the properties of this lipid. Particular emphasis has been placed on the role of the electrostatic charges and intramolecular hydrogen bonding in phosphatidylglycerol phosphate on stability and compressibility of the monolayer. Comparative studies have also been performed on the deoxy derivative of phosphatidylglycerol phosphate, 2,3-diphytanyl-*sn*-glycero-1-phospho-1'-(propane-1',3'-diol 3'-phosphate), in which intramolecular hydrogen bonding is greatly decreased or absent (Stewart *et al.*, 1988).

MATERIALS AND METHODS

The extraction, purification and preparation of phosphatidylglycerol phosphate from *Halobacterium cutirubrum* has been documented elsewhere (Kates, 1978; Quinn *et al.*, 1986). The deoxy analogue of phosphatidylglycerol phosphate was synthesized as described previously (Stewart *et al.*, 1988).

Compression isotherms were obtained at the air/aqueous interface at 17 °C in a temperature-controlled environment using a Wilhelmy dipping-plate method to measure surface pressure (π , in mN·m⁻¹) of the monolayers. The lipids were spread from a micropipette in a solvent mixture consisting of chloroform/n-hexane (1:9, v/v). The presence of surface-active impurities in the solvent-spreading system was excluded by observing no change in interfacial tension after application and evaporation of solvent from a clean aqueous subphase. Water used to prepare subphases was twice-distilled (pH 5.65) and the pH was adjusted by adding appropriate volumes of HCl (pH 0-2) or 10 mM-potassium phosphate buffer. All electrolytes were of Analar purity and used without further purification. The absence of surface-active agents was also checked by observing changes in the surface tension of the aqueous interface over prolonged periods before spreading the lipid monolayers.

Before commencing compression isotherms the monolayers were equilibrated for between 10 and 60 min to allow complete evaporation of the spreading solvent and for internal equilibration of the molecules within the film. No effects of additional equilibration times of up to 120 min were observed, as judged from monolayer characteristics or the performance of compression isotherms on any of the subphases used.

The compression isotherms were obtained after equilibration of the monolayers by compressing the film at rates of between 0.01 and 0.06 nm²/min per molecule.

Reproducibility of the surface-pressure measurements was within 0.5 mN·m⁻¹, and for determination of molecular areas it was within 0.02 nm²/molecule.

The limiting molecular area (A_0) of lipid molecules spread on various subphases was obtained by extrapolating the linear portion of the high-surface-pressure region of the compression isotherm to zero surface pressure ($\pi = 0$). The collapse pressure (π_c) and the collapse molecular area (A_c) were obtained as the coordinates of the point on the isotherm where an abrupt change in slope of the isotherm is observed on decrease in surface area per molecule. The surface compressional modulus, C_{s0}^{-1} , is related to the monolayer characteristics by the equation:

$$C_{s0}^{-1} = A_0 \pi_c / (A_0 - A_c)$$

RESULTS

The relationship between surface pressure and molecular area obtained by compression of monolayers of phosphatidylglycerol phosphate and deoxyphosphatidylglycerol phosphate, i.e. compression isotherms, on subphase of unbuffered water (pH 5.65) are shown in Fig. 1. Monolayers of the two phospholipids are seen to have similar compression isotherms, with films of deoxyphosphatidylglycerol phosphate occupying slightly greater areas than phosphatidylglycerol phosphate monolayers at equivalent pressures, except at very low surface pressures (< 2 mN·m⁻¹). The surface characteristics of these films, including limiting molecular area and area per molecule at the collapse pressure, were found to differ slightly from films spread on subphases in

Fig. 1. Compression isotherm of (a) phosphatidylglycerol phosphate and (b) deoxyphosphatidylglycerol phosphate on subphases of water (pH 5.65)

A_0 values obtained from the intersection of the broken lines with the abscissa. A_c is indicated by arrows.

Fig. 2. Relationship between the limiting molecular area (A_0) of phosphatidylglycerol phosphate (●) and deoxyphosphatidylglycerol phosphate (○) and subphase pH

which the pH was buffered to a designated value. The differences reflect the dependence of surface characteristics on the electrostatic charge carried by the lipid and its modification by adsorbed gegenions from the subphase.

The effects of subphase pH on monolayer characteristics was examined by constructing compression isotherms on subphases adjusted to pH values above and below the apparent pK_1 (2.4) of the first ionizable functions of the two phosphate groups. Values of limiting molecular area, collapse molecular area and collapse pressure were measured and used to calculate the surface compressional modulus of the lipid monolayer.

The effect of subphase pH on the limiting molecular area of phosphatidylglycerol phosphate and its deoxy derivative is shown in Fig. 2. The most compressed films are formed at pH values just below the apparent pK_1 of the first acidic functions of the two phosphate groups when the lipids would be expected to be largely un-ionized. On very acid subphases (pH 2) there is a progressive increase in limiting molecular areas of both phospholipids. This cannot be accounted for by penetration of the counter-ions into the film, since at these pH values no interaction is possible between cations and phosphate anionic sites (Lakhder-Ghazal *et al.*, 1983). Rather, a more likely possibility would be perprotonation of the phosphate group, since it has been demonstrated to occur for phosphoric acid in water at about and below pH 0 (Zwick *et al.*, 1989). With increasing pH associated with the ionization of these two phosphate groups, the limiting area increases in a manner indicating apparent pK_1 values of 4.2 and 4.0 for phosphatidylglycerol phosphate and deoxyphosphatidylglycerol phosphate respectively, compared with values of 3.5 and 3.6 re-

Fig. 3. Effect of subphase pH on the surface compressional modulus (C_{s0}^{-1}) of monolayers of phosphatidylglycerol phosphate (●) and deoxyphosphatidylglycerol phosphate (○)

spectively obtained by bulk titration in methanol/water (1:1, v/v) systems (Stewart *et al.*, 1988). Ionization of these two phosphate groups causes a greater expansion of deoxyphosphatidylglycerol phosphate monolayers compared with films of phosphatidylglycerol phosphate. Further ionization of the terminal phosphate group produces further expansion of both monolayers, and the apparent pK_2 of these appears to be greater than 9.7. In comparison, bulk titration of phosphatidylglycerol phosphate and deoxyphosphatidylglycerol phosphate shows apparent secondary pK_2 values of > 11 and 8.6 respectively in both methanol/water and aqueous dispersions (Stewart *et al.*, 1988).

The subphase pH was also found to affect the compressibility of the phospholipid monolayers. The relationship between surface compressibility modulus and the pH of the subphase of monolayers of phosphatidylglycerol phosphate and deoxyphosphatidylglycerol phosphate are shown in Fig. 3. Phosphatidylglycerol phosphate monolayers spread on subphases with pH values above or below the apparent pK_1 and pK_2 of the primary and secondary ionization produced relatively compressible films, possibly because of the modulation of counter-ion adsorption on the lipid phosphate groups during compression of the film (Sacré & Tocanne, 1977). This cannot provide a complete explanation, as the compressibility of deoxyphosphatidylglycerol phosphate monolayers is not greatly altered on changing the bulk subphase pH from 5 to 10.8. The least compressible monolayers were formed at pH values intermediate between the apparent pK of the primary and secondary ionizations of the phosphate residues.

The effect of inorganic-ion concentration on film characteristics was examined by spreading phosphatidylglycerol phosphate films on subphases of NaCl with concentrations varying from 0.01 to 1 M. The effect of NaCl concentration on the limiting molecular area of the phospholipid is presented in Fig. 4(a). The best fit to the data suggests that the limiting molecular area of phosphatidylglycerol phosphate increases approximately according to the logarithm of the NaCl concentration

Fig. 4. Effect of NaCl concentration at pH 7 (●) and pH of the subphase below phosphatidylglycerol phosphate monolayers (○) on (a) limiting molecular area, A_0 , (b) ΔA_0 and (c) ΔA_e .

See the text for the derivation of values on the ordinates.

over the range of salt concentrations examined. To compare the effect of pH with NaCl concentration (expressed as pNaCl), values of ΔA_0 and ΔA_e were plotted as a function of pH and pNaCl respectively (Figs. 4b and 4c). Values of ΔA_0 and ΔA_e were calculated as the difference between the observed values of limiting molecular areas or collapse molecular areas on a particular subphase and the minimum values obtained on the respective subphase (pH = 2 = pNaCl). It can be seen that the differences between effects of pH and NaCl on A_0 only become apparent where pH = 0 = pNaCl. The values for A_e appear to be the same, within experimental error, for pH and pNaCl and are consistent with expulsion of electrolytes from the monolayer at pressures approaching collapse of the film (Tomoaia-Cotisel *et al.*, 1988).

DISCUSSION

The lipids of the red and purple membranes of *Halobacterium* spp. are distinctive in that the hydrocarbon domains are ether-linked phytanyl chains, the polar groups are highly charged, either as phosphate groups or, in the case of glycolipid sulphate, sulphated glycosyl groups. The organism requires inorganic-salt concentrations exceeding 3 M for growth and stability of their constituent membranes (Lanyi, 1971; Kushner, 1978). This implies that the charged polar group may be an essential feature of these lipids that is necessary in their role in membrane function and integrity.

The electrostatic status of phosphatidylglycerol phosphate at neutral pH is in the form of a dianion, with each of the phosphate groups carrying a single negative charge (Kates *et al.*, 1965; Kates & Hancock, 1971; Stewart *et al.*, 1988). The apparent pK_1 of these two ionic groups was reported to be about 3.5–3.6 in methanol/water (1:1, v/v), which is in close agreement with the value of about 4.0 that can be deduced from the monolayer characteristics, especially the limiting molecular areas of films spread on subphases of different pH

(Fig. 2). It is noteworthy that sonicated dispersions in water were found to give an apparent pK_1 of 2.4 for phosphatidylglycerol phosphate, a value which is somewhat lower than that obtained in the monolayer. The pK_2 of the second ionizable function located on the terminal phosphate residue was originally reported to be about 8, but more recently Stewart *et al.* (1988) obtained a value of > 11, and this is more consistent with the values recorded for limiting molecular areas. Furthermore, the greater increase in A_0 for deoxyphosphatidylglycerol phosphate than for phosphatidylglycerol phosphate in the pH range 6–11 may be attributed to the greater degree of ionization of the third P–OH group in the deoxy derivative at high pH values, and hence a greater negative charge repulsion between deoxyphosphatidylglycerol phosphate molecules than for phosphatidylglycerol phosphate. For both phospholipids, further increase in A_0 with increasing subphase pH could result from penetration of counter-ions into the film. Indeed, studies of cation- and pH-dependence of charge neutralization in dispersions of total polar lipid extracts of *Halobacterium cutirubrum* have indicated that the penetration of counter-ions into the Stern layer requires a spacing out of the molecules with uncharged lipids (Lanyi *et al.*, 1974). These conclusions were based on a co-dispersion of polar-lipid extracts with squalene, the major neutral lipid of these membranes, which, together with other neutral lipids, represent about 8–10% by weight of the total membrane lipids.

In a wider context, the possibility of hydrogen-bonding between the C–OH and P–OH groups of phosphatidylglycerol phosphate may give rise to proton-translocation pathways of the type that are contemplated in localized proton coupling across energy-transducing membranes (Williams, 1974; Ernster, 1977; Kell, 1979; Ferguson, 1985). The role of acidic, and particularly amino, phospholipids, in such proton-conduction pathways have been described (Lange *et al.*, 1975; Haines, 1983; Boggs, 1987) and demonstrated in lipid monolayers (Teissie *et al.*, 1985). Accordingly, in the case of the purple

membrane, light-driven trans-membrane proton translocation mediated by bacteriorhodopsin (Stoeckenius, 1980) may be locally coupled to the ATPase of the red membrane by conduction of these protons along the hydrogen-bonded network formed by the polar lipid head groups, largely of phosphatidylglycerol phosphate, resident at the membrane surface.

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