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INDUCTION OF THE LAMELLAR-INVERTED HEXAGONAL PHASE TRANSITION IN CARDIOLIPIN BY PROTONS AND MONOVALENT CATIONS

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The effect of pH and sodium ion concentration on the polymorphic structure of cardiolipin aqueous dispersions has been investigated by X-ray diffraction. A transition from the fluid bilayer phase, L_α, to the inverted hexagonal phase, H_{II}, is induced either upon lowering the pH to below 2.8, or on increasing the NaCl concentration to above 1.6 M at pH 7.

Introduction

Since the pioneering investigations of lipid polymorphism by Luzzati and co-workers [1], it has been clear that this phenomenon could be directly involved in a variety of biological membrane functions, although this has only recently become widely recognized [2].

The tetraacyl phospholipid cardiolipin (for reviews see Ref. 3) is a particularly interesting candidate for playing an active physiological rôle. In mammalian cells it is located primarily in the inner mitochondrial membrane constituting approx. 33 wt% of the total lipids. It is normally found to have a very homogeneous fatty acid composition, consisting predominantly (60–80%) of linoleic acid chains.

Following the observation of a hexagonal H_{II} phase in a lipid extract from mitochondria either at low water content [4] or in the presence of ferricytochrome *c* [5], it was subsequently shown that cardiolipin adopts the H_{II} phase in the presence of calcium and other divalent ions or at low water content [6,7]. More recently, it has been shown that the H_{II} phase is induced in cardiolipin by ferricytochrome *c* [8] or by the cationic local anaesthetics dibucaine and chlorpromazine [9]. On the other hand, the induction of the H_{II} phase in cardiolipin by calcium is blocked in the presence of poly(L-lysine) [10] or by the antineoplastic drug adriamycin [11].

^{31}P-NMR studies have provided evidence for the possible existence of other non-lamellar phases of cardiolipin. After extensive dialysis of hexagonal-phase calcium-cardiolipin, an isotropic ^{31}P-NMR signal was observed, which was interpreted to indicate the formation of a cubic phase [12]. Isotropic ^{31}P-NMR signals have also been observed in cardiolipin/phosphatidylcholine mixtures upon addition of calcium [13]. In addition, unilamellar vesicles formed from such mixtures fuse within seconds of addition of more than 9 mM calcium [14,15], implying a possible rôle of non-lamellar phases in the fusion process.

Because of the possible involvement of cardiolipin in mitochondrial function, and since these functions involve protonic and other ionic gradients, it is important to characterize the effects

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of protons and ions on the structure of cardiolipin. This is particularly so, since it has been demonstrated that cardiolipin is an extremely powerful ionophore, able to transport cations across an apolar barrier between two aqueous phases, this capacity probably being related to the formation of inverted micellar structures by the cation-lipid complex [16].

Materials and Methods

The sodium salt of bovine heart cardiolipin in ethanolic solution was obtained from Sigma, stored in the dark at $-20°C$, and used within 2 weeks of receipt. Thin-layer chromatography revealed minimal degradation. Chemicals were Analar grade and the water was double-distilled. The buffers employed were $(HOCH_2CH_2)_3N$ at values of pH greater than 4, and NaCl/HCl at pH values less than 4, and also contained 10 μM EDTA.

The lipid (approx. 5 mg) was dried on the bottom of glass vials under a stream of pure N_2 gas, then stored in vacuo overnight. 400 μl of buffer was then added and the lipid was dispersed with a spatula. The pH of the dispersion was directly measured (± 0.1 pH units). After spinning in a bench centrifuge (N.B., the lipid floats at $J > 0.8$ M), the pellet was removed and sealed with 10 μl of the supernatant either in 1-mm thin-walled glass capillaries (K. Hilgenberg, Malsfeld, F.R.G.) or between thin mica sheets in specially built metal holders.

X-ray diffraction experiments were performed at $23 \pm 2°C$ using either a Franks double-mirror focusing camera with a sample-film distance of 140 mm, or a Guinier camera with a quartz crystal monochromator to isolate the Cu $K\alpha_1$ ($\lambda = 0.15405$ nm) radiation. Because the Franks camera required lengthy exposure times (over 20 h) and the lipid is liable to degradation, the detailed salt and pH dependences were investigated using the Guinier camera, for which exposure times for the CEA REFLEX 15 x-ray film (Ceaverken AB, Stragnäs, Sweden) were typically 30 min.

Lipid purity was checked by TLC at the end of the experiment and the data from samples showing more than minimal degradation were rejected.

Results

Typical low-angle diffraction patterns of cardiolipin in excess buffer at 22°C are shown in Fig. 1. At pH 7 (1 M KCl) three orders of a lamellar periodicity of $d = 5.3$ nm are observed (Fig. 1a). With the long exposure times required for the Franks camera, additional very faint reflections were sometimes observed, probably due to slight lipid degradation. At low pH, a white precipitate was formed which gave diffraction lines with reciprocal spacings in the ratio 1, $\sqrt{3}$, 2, characteristic of the hexagonal phase (Fig. 1b). The spacing of 5.9 nm (in 0.1 M KCl) corresponds to an axial separation of lipid/water cylinders of 6.8 nm. A preliminary report of this finding has appeared [17].

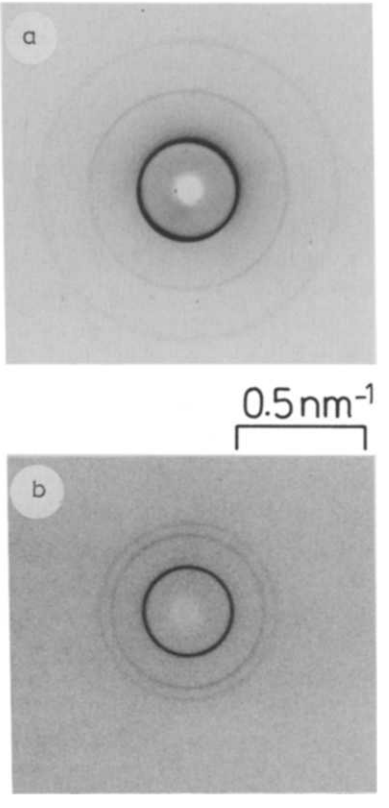

Fig. 1. X-ray diffraction patterns of cardiolipin in: (a) the lamellar phase (pH 7, 1 M KCl); and (b) the hexagonal phase (pH ≈ 2.2, 0.1 M KCl). The patterns were obtained with a Franks camera.

Freeze-fracture electron microscopy of a sample quenched at pH 2.4 (data not shown) showed large areas of striations, which are characteristic of the hexagonal phase [7]. The axial separation of the lipid cylinders estimated from the micrographs was 7 nm, in good agreement with the X-ray results.

The results of varying the pH on the lipid polymorphism are shown in Fig. 2. A salt concentration of 1 M NaCl was chosen in order that the lamellae did not exhibit indefinite swelling (cf. Fig. 3). Between pH 7 and pH 3, a pure lamellar phase with a pH-independent spacing of 5 nm is observed. The absence of sharp diffraction lines in the wide-angle region identifies the structure as the fluid bilayer phase, L_α. Upon further lowering the pH below 2.8, a hexagonal phase with a pH-independent spacing of 6.5 nm appears. It was frequently observed that a small proportion of the sample remained in the L_α phase. Upon heating one such sample to 37°C, a pure hexagonal phase was observed, with a spacing of 6 nm. The region below pH 2 was not investigated due to problems of lipid degradation. The formation of the hexagonal phase below pH 2.8 was also observed at low salt concentrations (0.1 M; cf. Fig. 1b).

The effect of varying the monovalent ion concentration on the lipid structure at pH 7 was also investigated, the results being given in Fig. 3. At low salt concentration ($J < 0.5$ M), the lipid lamellae take up large amounts of water and swell to very large spacings. Diffraction lines from the L_α phase were first observed at salt concentrations greater than 0.5 M NaCl, the long spacings then falling steeply from 6.25 nm to a value of 5.1 nm at 0.8 M NaCl. The spacing then falls more slowly with increasing salt, a limiting value of 4.9 nm being reached at 1.6 M NaCl. At this point, a hexagonal phase with a spacing of 6.9 nm appears. In the range $1.5\ M < [NaCl] < 2.5\ M$ the L_α and H_{II} phases coexist, the spacing of the latter phase decreasing to a value of 6.2 nm at 2.5 M NaCl. At higher salt concentrations a pure H_{II} phase is observed, the long spacing falling above 4 M NaCl to an approximate value of 4.5 nm in saturated NaCl. At these very high salt concentrations, the lines became quite blurred and sometimes doubled, and so the identification of the H_{II} phase was less certain.

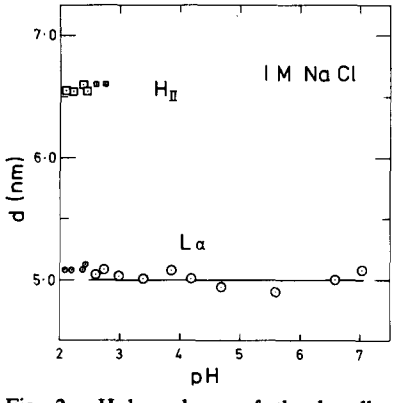

Fig. 2. pH-dependence of the lamellar (⊙———⊙) and hexagonal (⊡———⊡) X-ray long spacings of cardiolipin in 1 M NaCl.

The structural parameters of the phases may be approximately estimated by comparison with previous work. As before, the basic structural unit is taken to be one half of a cardiolipin molecule, with a molecular weight of 750 and a partial specific volume of 1 $ml \cdot g^{-1}$ [6]. We assume that in L_α the lipid layer thickness is constant * and has the value of 3.5 nm previously determined at high water content [6]. This gives a constant value for the area per molecule of 0.71 nm^2. The water layer thickness in L_α is then calculated from Fig. 3 to decrease from 2.8 to 1.4 nm on increasing the salt concentration from 0.5 to 2 M. In the hexagonal phase, we assume that the headgroup-headgroup distance has the value of 2.8 nm previously measured at the maximum water content (14 wt.%) of the pure H_{II} phase [6] *. The diameter of the aqueous cylinder calculated either at $pH < 2.8$ from Fig. 2 or at 2.5 M NaCl from Fig. 3 then has a value of approx. 4.5 nm, corresponding to a water content of the H_{II} phase of approx. 32 wt.%, and the area per molecule at the lipid/water interface has a value of approx. 0.57 nm^2. The corresponding values in the presence of calcium are 1.5

* The lipid layer thickness, as well as the total repeat distance, varies with water content up to limiting hydration, but in excess water it then remains constant. Thus, since we are always working in excess water, it is justified to assume that the lipid layer thickness remains approximately constant.

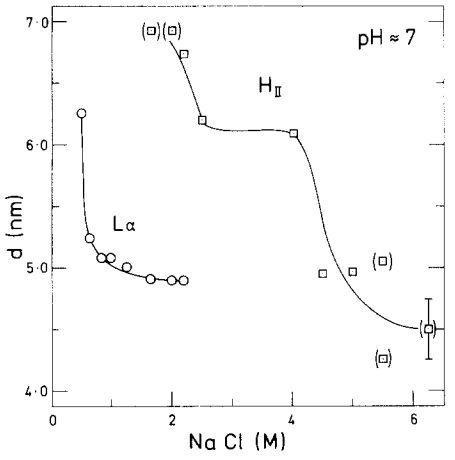

Fig. 3. Dependence on NaCl concentration of lamellar (⊙———⊙) and hexagonal (⊡———⊡) X-ray long spacings of cardiolipin at pH 7.

nm for the diameter of the aqueous cylinder, 7.4 wt.% water content, and 0.27 nm^2 for the area per molecule [6].

Discussion

The present work shows that the effect of protons or monovalent ions on cardiolipin is qualitatively similar to that of divalent ions such as calcium in inducing the hexagonal phase above a certain threshold concentration. Protons are effective in the millimolar concentration range, similar to calcium, whereas sodium ions are only effective in the molar concentration range. These differences reflect the different binding affinities and/or electrostatic shielding capabilities of the different cations for the cardiolipin headgroup.

The formation of the hexagonal phase in cardiolipin by calcium has been attributed to an effective dehydration [6] and charge neutralization [9] of the headgroups, both effects tending to increase the 'inverted-wedge' shape of the molecule (c.f. Ref. 33). The results reported here may be similarly understood, but it should be noted that in attempting to explain the effect on the lipid polymorphism of varying any parameter such as pH or salt concentration, it is essential that all significant interactions that are affected by varying this parameter be taken into account. For example, the hexagonal phase is favoured on titrating phosphatidylethanolamines to low pH, although a net charge is induced on the lipid headgroups in this state [18]. The probable explanation is that the unfavourable effect of lateral repulsion between charges is outweighed by the effect of dehydration of the phosphate groups upon protonation, which favours the hexagonal phase.

The indefinite swelling of charged phospholipids in water is a consequence of electrostatic repulsion between adjacent bilayers [19]. In the case of phosphatidylserine from bovine brain 0.5 M NaCl was sufficient to screen the charged surfaces, such that multibilayers with a repeat spacing of 6.0 nm were observed [20]. This value is similar to that which we observe with cardiolipin under these conditions (cf. Fig. 3).

In a study of phosphatidylserine by electron microscopy, the absence of the hexagonal phase at all degrees of hydration was attributed to the negative charge on the headgroup [21]. This suggestion was confirmed by the observation of the hexagonal phase in phosphatidylserines of natural origin upon lowering the pH below 3.5, thereby removing the charge on the carboxyl group [22]. Similar behaviour has been observed with phosphatidic acid upon lowering the pH to below 5 [23,24]. Our results on the effect of pH on cardiolipin are similar to these findings. As with phosphatidic acid, the formation of the hexagonal phase may be attributed to protonation of the phosphate group; however, with cardiolipin we observe this effect at a considerably lower pH than was found with phosphatidic acid. Our results indicate significant protonation of the phosphate groups by pH 2.8, which implies that their apparent pK_a in 1 M NaCl is not far from this value, rather than the previously reported much lower value of 1.05 [25]. For comparison, the apparent pK_a of phosphatidylglycerol bilayers in 0.1 M NaCl has been found to be $pK_a \approx 2.9$ [26]. Correction to 1.0 M NaCl would give $pK_a \approx 2.1$, i.e., a similar value for this structurally closely related lipid.

The spacings observed at pH values or NaCl concentrations sufficient to induce the H_{II} phase have values in the range of 6–7 nm (cf. Figs. 2, 3), which is considerably larger than the value of 4.6 nm observed with calcium [6]. This implies that

the hexagonal phase in the presence of protons or sodium ions is much more hydrated than in the presence of calcium. This is confirmed by the estimates of the structural parameters given in the Results section, which shows that the diameter of the aqueous cylinders, and concomitantly the area per molecule, is much larger in the presence of protons or sodium ions than in the presence of calcium. On going to very high NaCl concentrations, the spacing of the hexagonal phase decreases to a value of 4.5 nm, implying an equivalent dehydration to that produced by calcium.

The biological relevance of lipid polymorphism is still an open question, but various suggestions for possible functional roles of non-bilayer structures in the inner mitochondrial membrane have been made [27]. It was observed by freeze-fracture electron microscopy of freshly isolated mitochondria that 5 mM manganese ions causes the appearance of regions having the morphology of the hexagonal phase [28]. However, a ^{31}P-NMR study of intact functional rat liver mitochondria at 37°C indicated that more than 95% of the lipid is organized in bilayers [29], emphasizing that non-bilayer structures in biological membranes must be highly localized and/or transient.

It has been suggested that non-bilayer phospholipid structures are the mediators of ion and solute transport in biological membranes [16,30], and this idea has been developed further [31]. If this proposal is correct, then our finding that proton-binding to cardiolipin in excess buffer induces the formation of the hexagonal phase suggests that cardiolipin could carry protons across a non-polar region such as a bilayer membrane, this transport occurring by the formation of inverted micellar structures by the protonated cardiolipin.

It has been proposed that localized proton flows within the mitochondrial membrane couple electron transport to phosphorylation [32]. This proposal depends upon the possibility that the proton concentrations in localized regions of the membrane may be much greater than that implied by the bulk pH. It is conceivable that in localized regions of the mitochondrial membrane, some of the cardiolipin headgroups are protonated, and this raises the possibility that nonbilayer structures of cardiolipin, perhaps associated with proteins, might play an active role in proton translocation.

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