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CHARGE-INDUCED TILT IN ORDERED-PHASE PHOSPHATIDYLGLYCEROL BILAYERS

EVIDENCE FROM X-RAY DIFFRACTION

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X-ray diffraction studies have been performed, as a function of water content, on dipalmitoyl phosphatidylglycerol bilayers, both in the charged state at pH 8.0 and in the protonated state at pH 1.5, using buffers of 1.5 M salt concentration. Measurements were made at 20°C, and the high-angle reflections indicated that the bilayers were in the ordered phase at both pH values. Lamellar diffractions were observed under all conditions studied. The lamellar repeat reached a limiting value of 62.4 Å (6.24 nm) at a water/lipid ratio of 0.24 at pH 8.0, and a limiting value of 67.3 Å (6.73 nm) at a water/lipid ratio of 0.22 at pH 1.5. The area per lipid molecule in the plane of the bilayer, deduced from the bilayer thickness and the lipid partial specific volume, is 48 Å^2 (0.48 nm^2) at pH 8.0 and 37 Å^2 (0.37 nm^2) at pH 1.5. The area per molecule in the plane perpendicular to the chain axes, deduced from the X-ray short spacings, is 40.5 Å^2 (0.405 nm^2) at pH 8.0 and 39.2 Å^2 (0.392 nm^2) at pH 1.5. Thus the lipid molecules are tilted by approx. 30° relative to the bilayer normal at pH 8.0, but are essentially untilted at pH 1.5.

Introduction

Phosphatidylglycerol is a negatively-charged phospholipid found at high concentrations in the plasma membranes of microorganisms, in the chloroplast membranes of plants and to a lesser extent in mammallian systems, notably in the lung surfactant [1,2]. Negatively-charged phospholipids are of particular interest from the point of view of a possible functional role of phospholipids in membranes, since both their phase transition behaviour [3–6] and bilayer structure [4,7] are responsive to changes in the ionic environment. This provides a potential membrane trigger mechanism initiated by changes in the surface pH or concentration of monovalent and divalent ions [3,8].

In a previous study [5] we used spin labels to determine the pH-induced titration of the ordered-fluid phase transition in dipalmitoyl (DPPG) and dimyristoyl (DMPG) phosphatidylglycerol bilayers. As the phosphate group becomes protonated, on decreasing the pH from the fully charged state at pH 8.0, the transition temperature increases by 18° for DMPG, and by 15° for DPPG, on going down to pH 1.5. Similar results have subsequently been obtained by differential scanning calorimetry [6,18].

Perhaps more interesting was the finding that pH can affect the state of the bilayer both in the ordered and the fluid phase, above and below the bilayer transition [5]. This provides a possible isothermal triggering mechanism which does not require the mediation of an ordered-fluid phase transition. In fluid bilayers, above the main transition temperature,

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Abbreviations: DMPG, dimyristoyl phosphatidylglycerol; DPPG, dipalmitoyl phosphatidylglycerol.

the fluidity was found to be greater when the phosphatidylglycerol molecules were in the charged state than when they were in the protonated state at pH 1.5. In the ordered phase the bilayers in the charged state were found to display bilayer structures in freeze-fracture electron microscopy similar to those observed for phosphatidylcholines, viz. a defect pattern and a rippled pattern for bilayers quenched from below and above the pretransition, respectively. On the other hand, the bilayers in the protonated state at pH 1.5 gave rise to exclusively smooth fracture faces, independent of the temperature from which the bilayers were quenched. This lead to the suggestion that the lipid molecules were tilted relative to the bilayer normal in the charged state, but were not tilted in the protonated state. Subsequently we have shown that this structural difference between the charged and protonated bilayers in the ordered phase is also accompanied by a change in the molecular mobility [9]. Using saturation transfer ESR we were able to demonstrate the occurrence of a rapid rotation about the long axes of the lipid molecules in the ordered phase of the charged bilayers, whereas the long axis rotation was much slower and was completely non-cooperative in the ordered phase of the protonated bilayers.

In the present study we investigate the structural effects of pH titration on ordered DPPG bilayers in more detail. Using X-ray diffraction it is shown that the lipid molecules are tilted with an angle of 30° relative to the bilayer normal in the charged state, whereas in the protonated state at pH 1.5 the tilt angle is less than 5°. These results verify our original prediction regarding the molecular tilt and demonstrate that the bilayer can be switched from a tilted to a non-tilted structure, solely by manipulation of the external pH.

A bilayer structure with interdigitated lipid chains has recently been suggested on the basis of X-ray diffraction measurements on ordered-phase DPPG bilayers in the charged state [10]. We find no evidence for such a structure in our bilayer preparations. Our results are consistent with a conventional bilayer structure of two apposed, non-interdigitated monolayers. The reduction in bilayer thickness relative to that expected for two monolayers with all-*trans* chains can be accounted for completely by the molecular tilt.

Materials and Methods

Dipalmitoyl L-α-phosphatidylglycerol (DPPG) was synthesized by phospholipase D-mediated headgroup exchange from dipalmitoyl L-α-phosphatidylcholine (Fluka, Buchs) in the presence of excess glycerol, as previously described [5]. The purity of this preparation has been characterized previously [5,16]. Sample purity was rechecked after the X-ray measurements, using thin layer chromatography with the solvent system $CHCl_3/CH_3OH/25\%$ NH_4OH (65 : 15 : 1, v/v) [5]. The buffers used were 1.0 and 1.5 M KCl/HCl at pH 1.5 and 1.5 M KCl/50 mM Tris at pH 8.0. Lipid dispersions at pH 1.5 were prepared using the acid form of DPPG, and dispersions at pH 8.0 were prepared with the salt form. A high salt concentration was used in the buffers to ensure that the X-ray long spacing reached a limiting value. In low salt, negatively-charged phospholipid bilayers tend to swell indefinitely on hydration [7].

X-ray diffraction measurements were performed using a Guinier camera (operating under vacuum) with a bent quartz crystal monochromator (R. Huber, Rimsting. F.R.G.). The monochromator was set to isolate the $CuK\alpha_1$ line ($\lambda = 1540.5$ nm). Further details of the experimental set-up have been reported elsewhere [11]. For the measurements with excess water, the lipid samples were prepared by adding 50 μl of buffer to approx. 5 mg of dry lipid. The samples were then sealed with teflon between mica plates and equilibrated above the transition temperature for a least 10 min. For the water-uptake measurements, the desired amounts of lipid and buffer were weighed into small polythene tubes. After sealing the tubes the lipid was mixed with the water by repeated contrifugation and heating above the transition temperature. In some cases the mixing could be facilitated by inserting a glass bead into the polythene tube. The lamellar reflections of the lipid were then measured while the lipid was still in the polythene tube. (The use of the polythene tubes for the water-uptake measurements had the advantage that the lipid samples did not have to be transferred to the usual sample holders after the lipid had been mixed with the buffer. The lipid/water ratio could therefore not change during the sample preparation. As polythene does not show reflections in the small angle region, its presence does not disturb the detection of the lamel-

lar repeat distances of the lipid. Polythene does, however, give rise to strong wide angle reflections and therefore these lipid reflections could not be studied with polythene tubes.) The exposure times of the photographic films (Kodak, 'Kodirex, une face') varied between 15 min and 2 h. The density of the reflections was scanned with a Joyce-Loebl microdensitometer type 3 CS.

Density measurements at room temperature were made using a 5 ml pyknometer. A dispersion of DPPG was made at a concentration of 100 mg/ml by shaking dry lipid with the required buffer at a temperature above the main transition. Small amounts of 1 N HCl were required to adjust the pH of the acid dispersion. The pyknometer was weighed on a Sartorius balance to within ±0.01 mg immediately after filling.

Results

The X-ray diffraction patterns from fully hydrated DPPG bilayers at 20°C and at both pH 8.0 and pH 1.5 are given in Fig. 1. The low-angle reflections indicate a lamellar structure at both pH values. At pH 1.5 the lamellar repeat did not change with temperature, but at pH 8.0 the lamellar spacing increased with increasing temperature from 59.7 Å (5.97 nm) at 3°C, 62.4 Å (6.24 nm) at 20°C, to 64.1 Å (6.41 nm) at 38°C.

The high-angle reflections are indicative of the lateral chain packing within the bilayer. At pH 1.5 there is a single sharp reflection at 4.12 Å, characteristic of hexagonal packing of the lipid chains in the plane perpendicular to the chain axes. Even at low temperature a splitting of the wide-angle line could not be seen at pH 1.5. At pH 8.0 a sharp reflection at 4.21 Å with a broad shoulder at approx. 4.13 Å is observed in the high-angle region. The lateral packing at pH 8.0 thus corresponds to a distorted hexagon in the plane perpendicular to the chain axes. (The actual lattice type has been described in Ref. 11) The line-splitting becomes more pronounced, as does also the hexagonal distortion, at lower temperature. At 3°C, for example, the sharp line is at 4.28 Å and the diffuse line at 4.05 Å. With increasing temperature these two lines come closer together: at 38°C the sharp line is observed at 4.25 Å and the diffuse line at ca. 4.20 Å. The chain-chain separations and the cross-sectional area occupied by the lipid chains can be calculated from these short spacings. The area per chain is given

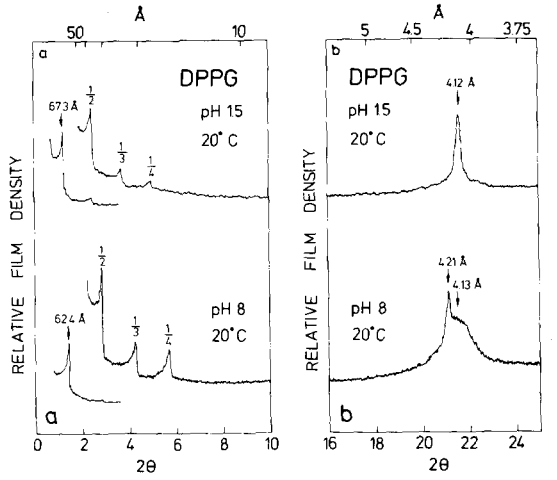

Fig. 1. Densitometer traces of powder X-ray diffraction intensities from fully-hydrated DPPG multibilayers at pH 8.0 (1.5 M KCl/50 mM Tris) and pH 1.5 (1.5 M KCl/HCl), at 20°C. (a) Low-angle, and (b) high-angle reflection regions, respectively. The diffraction intensity is plotted against the diffraction angle 2θ and the corresponding Bragg spacings.

by:

$$f_0 = s_1 \cdot s_2/\sqrt{1 - (s_2/2s_1)^2} \quad (1)$$

where s_2 is the short spacing corresponding to the diffraction peak which has twice the intensity of that corresponding to s_1 [12]. The values for f_0 are given in Table I, from which it can be seen that the chains are more closely packed at pH 1.5 than they are at pH 8.0.

The dependence of the long spacing, derived from the low-angle reflections, on water content is given in Fig. 2. It is seen that the bilayers reach their limiting hydration at much the same water content at both

TABLE I

X-RAY DIFFRACTION PARAMETERS OF DIPALMITOYL PHOSPHATIDYLGLYCEROL BILAYERS IN EXCESS WATER AT pH 8.0 AND pH 1.5, T = 20°C

	s (Å)	f_0 (Å^2)	d (Å)	$(1 - c)/c$ *	d_l (Å)
pH 8.0	4.22, 4.13	20.3	62.4	0.24	51.1
pH 1.5	4.12	19.6	67.3	0.22	53.3

* Value at limiting hydration.

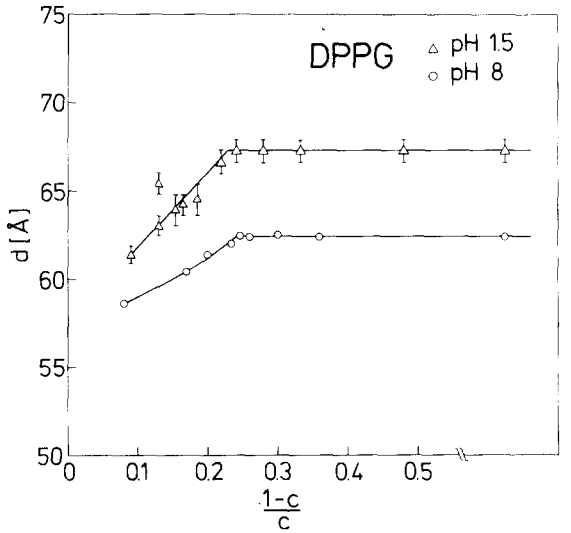

Fig. 2. X-ray long spacings at 20°C as a function of water/lipid weight ratio, $(1 - c)/c$. Derived from the low-angle diffraction of DPPG multibilayers at pH 8.0, 1.5 M KCl/50 mM Tris (○———○) and pH 1.5, 1.5 M KCl/HCl (△———△).

pH values: at a water/lipid ratio of 0.24 at pH 8.0 and 0.22 at pH 1.5 (Table I). The long spacing at limiting hydration is considerably greater, however at pH 1.5 that at pH 8.0. This suggests a smaller tilt of the lipid molecules relative to the bilayer normal at pH 1.5 than at pH 8.0. The measured long spacing, or lamellar repeat, can be divided into an interlamellar water layer of partial specific volume $\bar{v}_w$ and the lipid bilayer of thickness d_l and partial specific volume, $\bar{v}_l$:

$$d = d_l[1 + (\bar{v}_w/\bar{v}_l)(1 - c/c)_{lim}] \quad (2)$$

where c is the weight fraction of lipid at limiting hydration. The values of the lipid partial specific volume measured by pyknometry are given in Table II. These were calculated from the density measurements using the following expression:

$$\bar{v}_l = (1 - (\rho_d - \rho_b)/c_l)/\rho_b \quad (3)$$

where ρ_d, ρ_b are the densities of the lipid dispersion and the buffer respectively, and c_l is the lipid concentration in gm/ml. The values for the density of the buffer $\rho_b = 1/\bar{v}_w$, were measured to be 1.044 g · ml^{-1} and 1.068 g · ml^{-1} for the pH 1.5 and pH 8.0 buffers at 20°C, respectively. The values for d_l, the thickness of the lipid bilayer, calculated from Eqn. 2 are given in Table I.

The area per lipid molecule in the plane of the bilayer can be calculated from the bilayer thickness, d_l:

$$F = \frac{2M_l\bar{v}_l}{N \cdot d_l} \quad (4)$$

where M_l is the lipid molecular weight and N is Avogadro's number. These values of F are given in Table II, where they are compared with the area/molecule, $F_0 = 2f_0$, measured in the plane perpendicular to the lipid chain axes. The difference between the two is a measure of the angle of tilt, θ, of the lipid molecules relative to the bilayer normal:

$$F = F_0/\cos\theta \quad (5)$$

The values for the tilt angle are given in Table II from which it can be seen that the DPPG bilayers have a pronounced tilt, 32°, at pH 8.0, but no tilt at pH 1.5. In fact, the area/molecule at pH 1.5 deduced from the bilayer thickness and partial specific volume, is less than that obtained from the cross-sectional area of the lipid chains. This indicates a high-density molecular packing in the headgroup region also, of the bilayers at pH 1.5, and certainly no indication of a chain tilt. Ranck et al. [10] estimated a value of $\bar{v}_l$ = 0.895 ml/g for ordered phase DPPG, based on component molecular volumes. Although this corresponds to less dense molecular packing than our measurements at pH 1.5, it nonetheless would give rise to a molecular area of only 39.5 Å^2, deduced from the X-ray long spacings.

TABLE II

MOLECULAR AREAS AND TILT ANGLES IN DIPALMITOYL PHOSPHATIDYLGLYCEROL BILAYERS IN EXCESS WATER, AT pH 8.0 AND pH 1.5, T = 20°C

	$\bar{v}_l$ (ml/g) *	F_0 (Å^2)	F (Å^2)	θ
pH 8.0	1.01	40.5	48	32°
pH 1.5	0.82	39.2	37	0°

* Lipid partial specific volume determined by pyknometry.

Discussion

The high-angle reflections in Fig. 1a show a clear difference in the lipid chain packing between DPPG bilayers at pH 8.0 and pH 1.5, when both are in the ordered phase at 20°C. This parallels the difference in surface structure observed previously by freeze-fracture electron microscopy [5], for samples quenched from temperatures within the ordered phase. The high-angle reflections and symmetry of the chain packing of DPPG at pH 8.0 are very similar to those observed for dipalmitoyl phosphatidylcholine under similar conditions [12], as is the temperature dependence. This further strengthens the homology found previously [5] between phosphatidylcholines and phosphatidylglycerols in the charged state.

The molecular areas measured from the short spacings can be used to estimate the difference in chain-chain interaction energies, arising from the closer packing in the bilayers at pH 1.5. Using the model with which Salem [13] successfully estimated the heat of sublimation of crystalline hydrocarbons (see also Refs. 14 and 15), the attractive interchain dispersive energy is given by:

$$W_{disp} = -1.24 \cdot 10^3/D^5 \text{ (kcal/mol per } CH_2) \quad (6)$$

where D is the separation of the two chains (in Å), and the chain-chain repulsive interaction is approximated by the empirical expression based on experimental molecular potentials:

$$W_{H\text{-}H} = +33.2/d^{6.18} \text{ (kcal/mol per } CH_2) \quad (7)$$

where d is the distance (Å) between the centres of the two interacting hydrogen atoms. The net difference in intermolecular interaction energy between the two different charge states, per CH_2 group, per chain pair, is then given by:

$$\delta H_{int} = \delta W_{disp} + \delta W_{H\text{-}H} \quad (8)$$

where δ signifies the difference between the values at pH 8.0 and pH 1.5. For the 15-CH_2 groups per chain, and using the values for the chain areas in Table I, it is found that $\delta H_{int} \sim -2.2$ kcal/mol, corresponding to a weaker attractive chain-chain interaction in the bilayers at pH 8.0 than at pH 1.5.

This can be contrasted with the bilayer electrostatic repulsive energy in the fully charged state at pH 8.0. Within the framework of Gouy-Chapman electrostatic double layer theory, the electrostatic surface energy (see Ref. 4) is given by:

$$G_{el} = 2RT[\sinh^{-1}(\sigma/c) - 1/(\sigma/c) \cdot \{\sqrt{(\sigma/c)^2 + 1} - 1\}] \quad (9)$$

where σ is the surface charge density and $c = \sqrt{2\epsilon kTn/\pi}$, where ϵ is the dielectric constant within the double layer, and $n = c_M N/10^3$ is the ionic strength of the assumed 1 : 1 electrolyte. For $F = 48$ Å^2 (Table II), $c_M = 1.5$ M, $\epsilon = 80$ and $T = 293$ K, the net electrostatic surface free energy for fully charged phosphatidylglycerol headgroups becomes $G_{el} = 1.1$ kcal/mol. Thus it appears that the total electrostatic surface energy at pH 8.0 is of the same order of magnitude or less than, the net decrease in dispersive chain interactions between pH 1.5 and pH 8.0. Hence the lateral expansion of the pH 8.0 bilayers relative to the pH 1.5 bilayers cannot be accounted for simply in terms of an electrostatic repulsive surface pressure, but must include contributions from other headgroup interactions.

The results of Table II clearly indicate that the lipid molecules are tilted relative to the bilayer normal in the bilayers at pH 8.0, but not in the bilayers at pH 1.5, at 20°C in the gel phase. Additional evidence for the tilt comes from the relative widths of the high-angle reflections. At pH 8.0 the 4.13 Å reflection is much broader than the 4.12 Å reflection at pH 1.5, because the tilt reduces the number of coherently scattering chain planes [12,17,7]. This difference in tilt between bilayers at pH 8.0 and pH 1.5 confirms our previous prediction [5] made on the basis of surface morphology in the freeze-fracture electron microscopy of samples quenched from within the gel phase. The molecular tilt at pH 8.0 is again consistent with the homology between phosphatidylcholines (which also have a tilt in the gel phase [12]) and phosphatidylglycerols in the charged state. The phosphatidylglycerol tilt could possibly arise from the repulsive electrostatic surface pressure between the headgroups. As pointed out by Jähnig et al. [7], this is a way of decreasing the surface electrostatic repulsive energy (by increasing the area per headgroup), without increasing the chain-chain spac-

ing. The calculations from Eqns. 6–9 above indicate that the repulsive electrostatic energy can be appreciable, but the distance dependence of the chain-chain interactions is so strong that the electrostatic repulsion cannot be appreciably alleviated by direct chain-chain expansion below T_t. Chain tilt on the other hand, can cost relatively little in chain-chain interaction energy provided that end effects are small or are compensated across the two halves of the bilayer.

It has also been suggested by Jähnig et al. [7] that a difference in tilt between protonated and unprotonated bilayers in the gel phase could contribute to the shift in phase transition temperature on titration. The shift arises from end effects: the Van der Waals interactions between the chain ends are less well optimized in tilted structures, possibly giving rise to a preferential fluidization of the chain ends. The maximum value for the mismatch between chain ends is: $d_f \sim s_l \cdot \tan \theta$; although the mismatch could be considerably less, as a result of overlap between the two halves of the bilayer. This gives a maximum mismatch of 2.0 CH_2 groups at pH 8.0 and zero at pH 1.5. Using a value of $dT_t/dCH_2 = 9°C$ [14], gives a maximum contribution to the titration shift of the transition temperature of $\Delta T_t^{tilt} = 18°C$. This is to be compared with the experimentally measured shift of $\Delta T_t^{max} = 15°C$ at 0.1 M ionic strength [5]. Clearly the tilt effect could possibly make a sizeable contribution to the total shift. It has recently been shown that the electrostatic contribution to the shift is $\Delta T_t^{el} = 5°C$ at 0.1 M ionic strength [19]. This leaves a non-electrostatic contribution of $\Delta T_t^{non\text{-}el} = 10°C$, at least part of which could be accounted for by the tilt contribution.

Finally it should be noted that the present results are completely consistent with a conventional bilayer structure consisting of two apposed monolayers. There is no evidence for a structure involving interdigitated chains as suggested by Ranck et al. [10]. In the present study, unlike that of Ref. 10, the first order lamellar reflection was always greater than 55 Å, even at low water content. The reduction in bilayer thickness at pH 8.0 can be satisfactorily accounted for in terms of the chain tilt, independent evidence for which comes from the high angle reflections.

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