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X-ray diffraction demonstrates that phosphatidyldiacylglycerol and phosphatidylcholesterol are not lamellar above the main transition temperature

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X-ray diffraction was used to investigate the lattice structure of aqueous dispersions of two phosphatidyldiacylglycerols and of a phosphatidylcholesterol above and below the chain melting transition temperature. Previously, Noggle et al. (Biochim. Biophys. Acta (1982) 691, 240–248) had investigated these lipids and had concluded on the basis of electron microscopy that the lipids were in a lamellar state above the transition temperature. However, they found the ^{31}P-NMR signals were not characteristic of lamellar phases. It was, therefore, concluded that these lipids were yielding unexpected ^{31}P-NMR spectra. The present X-ray results demonstrate that, in fact, the lipids are not in a lamellar state above the transition temperature and that the ^{31}P-NMR and X-ray data are not necessarily in disagreement. Characteristics of the phases both above and below the chain melt temperature are discussed.

Introduction

^{31}P-NMR has become an important tool in investigating the structure of of phospholipid aggregates [1,2]. Three characteristic lineshapes, ('lamellar', 'tubular' and 'isotropic') have been found to correlate well with the X-ray diffraction determined structures of a wide variety of phosphodiester lipids [1,3]. The ^{31}P-NMR lineshape results from the diffusional averaging of the phosphate chemical shift anisotropy tensor over the time scale of the NMR experiment [4–6]. In so far as the conformation of the phosphate region is invariant, the anisotropy tensors involved can be related to the spectral shape, which in turn is related to the geometry limited diffusion of lipid in the aggregate. However, it has been shown theoretically that if the phosphate region conformation is changed, then the resultant tensor averages to yield spectra which may be incorrectly identified [7,8]. Although the general good agreement of ^{31}P-NMR and X-ray diffraction [1] suggests that the phosphate conformation does not vary widely, it is important to identify phospholipids in which the ^{31}P-NMR assignment disagrees with a phase assignment by other probes. Recently, using calorimetry, diphenylhexatriene fluorescence depolarization, electron microscopy, and ^{31}P-NMR, Noggle et al. [8] reported that a series of synthetic phosphatidyldiacylglycerols and phosphatidyl cholesterols ** were bilayers above the chain melt temperature but did not yield lamellar ^{31}P-NMR spectra. We now report on an X-ray diffraction study of some of these lipids. It is demonstrated that the phases above the chain melt temperature are not simple lamellar phases but exhibit diffraction characteristic of a hexagonal lattice.

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** Phosphatidyldiacylglycerols consist of two diacyl lipids joined via a single phosphate linkage. Phosphatidylcholesterol (PCh) is a diacyl phospholipid with cholesterol attached to the phosphate. See Ref. 8, and references therein, for more information.

Methods

The sodium salts of 1′,2′-di-*O*-acyl-3′-*O*-(1,2-di-*O*-acyl-*sn*-glycerol-3-*O*-phosphoryl)-*sn*-glycerol (abbreviated as 4R-bis-PA, where R = L or M for 12 or 14-carbon chains, resp.) and the calcium salt of *O*-(1,2-diacyl-*sn*-glycerol-3-phosphoryl) cholesterol (abbreviated as DPPCh, where P indicates 16-carbon chains) were synthesized as described previously [8] and were kindly provided by Professor Fausto Ramirez. Lipids were dispersed by sonication in a bath type (Sonicor) sonicator in an excess of buffer (100 mM KCl, 100 mM Pipes, pH 7.0). Lipid dispersions were loaded into 1.5 mm glass X-ray capillaries. X-ray diffraction patterns were obtained on an image-intensifier X-ray apparatus and reduced to graphs of intensity vs. scattering angle as described elsewhere [3,9,10]. No correction was made for the sensitivity non-uniformities of the detector. This affects the X-ray peak intensities (approx. 20%, max.) but not the peak position. Specimens were investigated over the temperature range of 10 to 80°C in roughly 5 Cdeg steps.

Results

The small-angle diffraction (SAXS) from 4M-bis-PA at 54°C is shown in Fig. 1a. Note the presence of three equally spaced peaks. Although a definitive lattice assignment on the basis of so few peaks is subject to uncertainty, it is consistent with a lamellar organization with a 83 Å repeat. This general kind of pattern is observed over the investigated range of 10 to 54°C. The corresponding wide-angle pattern (Fig. 1b) (WAXS) shows a sharp peak at 4.2 Å, indicative of frozen acyl chains [11,12]. If, however, the temperature was raised to 59°C, the low-angle pattern changes dramatically (Fig. 1c) to indicate peaks spaced in the ratio $1:\sqrt{3}:2$, consistent with a hexagonal lattice symmetry with a 45 Å basis. This pattern cannot arise from a single lamellar phase. The corresponding wide-angle pattern Fig. 1d shows a broad peak at about 4.5–4.6 Å, indicative of melted acyl chains [11,12]. Qualitatively similar diffraction patterns are seen with 4L-bis-PA, with the phase transition occurring between 39 and 44°C (data not shown).

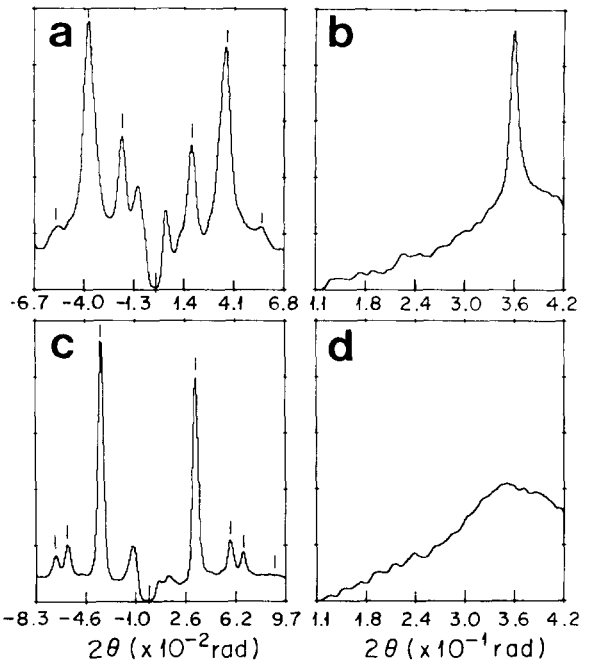

Fig. 1. (a) The X-ray diffraction intensity (arbitrary units) vs. scattering angle (2θ in radians) is shown in the low-angle region for 4M-bis-PA at 54°C. The expected peak positions of an 83 Å lamellar lattice are indicated by tic marks above the graph. In all the low-angle data to be shown, the extra long mark on the abscissa indicates zero scattering angle. (b) The wide-angle diffraction at 54°C. The sharp peak is at 4.2 Å and indicates frozen chains. The beam stop shadow would be off the graph to the left. (c) At 59°C, the low-angle diffraction has changed dramatically. The peak positions of a hexagonal lattice with a 45 Å basis are shown by the tic marks above the graph. The specimen to detector distance is different from that in (a). (d) At 59°C, the sharp 4.2 Å peak in (b), above, has been replaced by a broad hump at 4.5–4.6 Å, indicative of melted chains.

These transition temperatures agree with the calorimetry data of Ref. 8. Also, in agreement with the diphenylhexatrience fluorescence data of Ref. 8, the X-ray diffraction indicates that acyl chains melt at the transition. Moreover, the relative spacings between the orders of the SAXS data places rigorous constraints on the lipid lattice. Below the transition, the symmetry is consistent with a lamellar lattice. Additionally, the WAXS indicates the presence of frozen acyl chains. Thus, below the transition, the current X-ray data are consistent with the data of Noggle et al. [8]. However, above the transition temperature, the symmetry of the

SAXS cannot arise from a single lamellar phase. Rather, the SAXS is consistent with a lattice exhibiting hexagonal symmetry, such as an H_{II} phase. Many phospholipid phases which are known to exhibit such hexagonal symmetry also yield ^{31}P-NMR spectra with an effective chemical shift anisotropy value roughly half that of the same lipids when these lipids are in a lamellar form. The spectra characteristically have a down-field peak with an up-field shoulder [1]. This spectral shape was observed by Noggle et al. [8] for 4L-bis-PA above the phase transition temperature, consistent with the present X-ray data. However, Noggle et al. [8] concluded that the lipid was really in a bilayer phase on the basis of freeze-fracture electron microscopy of the dispersions quenched from approx. 10 Cdeg above the phase transition temperature. Note that interpretation of the electron micrographs is dependent on the assumption that the quenching of the specimen exceeded the transition rate back to the lower temperature lamellar phase. The required quench rate is not known for these lipids. Thus, the electron micrographs may have been misleading.

The X-ray data indicate that the transition simultaneously involves the melting of acyl chains and the transition from a lamellar to a hexagonally symmetric lattice. There is precedent for this behavior in other lipid systems. For example, L_β to H_{II} transitions have been observed (Ref. 13, and possibly Ref. 14). Note that measurement of the transition enthalpy for the tetra-acyl lipid 4M-bis-PA (data not shown) yields a value of 22.6 kcal · mol^{-1}. This is considerably in excess of twice the 6.6 kcal · mol^{-1} value observed in the L_β to L_α transition of the diacyl lipid dimyristoylphosphatidylcholine [15], suggesting that the transition involves more energy than normally associated with only melting the chains. Such high enthalpies are also observed for other chain homologs. It is noted that the ^{31}P-NMR lineshape of these dispersions in lamellar phase below the phase transition [8] is consistent with restricted rotational motion, such as is generally observed in the pseudocrystalline L_c phase.

X-ray diffraction data were also obtained from DPPCh. On the basis of calorimetry and diphenylhexatrience fluorescence depolarization, Noggle et al. [8] concluded that DPPCh underwent a chain melting transition at 47°C. As seen in Fig. 2a, at 44°C, DPPCh exhibits a sharp peak at 4.2 Å. At 54°C, (Fig. 2a) the peak has disappeared and has been replaced by slightly more X-ray scatter broadly distributed at lower angles (Fig. 2a). The position of the peak at 4.1 Å is slightly displaced from the 4.2 Å peak normally seen with L_β phases [11,12]. Also, the broad diffraction at 54°C differs from most L_α phase diffraction in that there is not a well defined broad peak at 4.5–4.6 Å [11,12]. However, these features are observed to vary slightly from lipid to lipid (unpublished observations) and are, still, suggestive of the melting of acyl chains.

DPPCh also gave unusual low-angle diffraction.

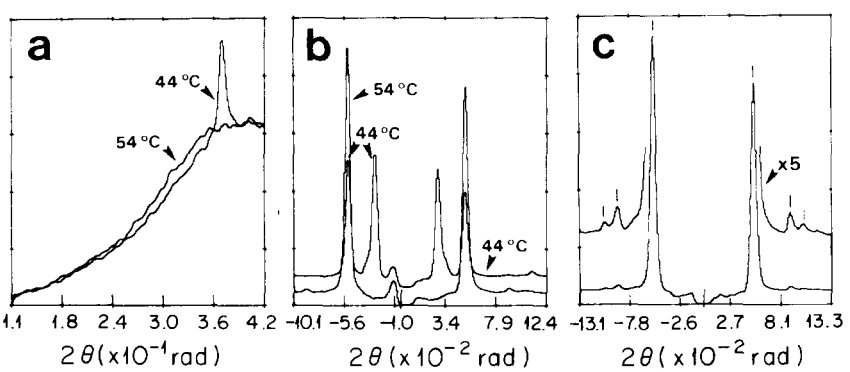

Fig. 2. (a) The wide-angle diffraction from DPPCh is shown at 44 and 54°C. The sharp peak is at 4.1 Å. At 54°C, this peak has disappeared. (b) the low-angle diffraction is shown for 44 and 54°C. The 44°C curve, which has been displaced upward for clarity, does not index as a single simple lamellar lattice. The 54°C curve does index on a hexagonal lattice with a 34 Å basis. This is more readily seen in (c), in which the detector to specimen distance has been decreased. The tic marks above the graph are for the 34 Å hexagonal lattice.

At 44°C, the low-angle pattern (Fig. 2b) showed definite peaks at radii in the ratio of 1 : 1.86 : 3.2 : 4.0, with the first orders occurring at $(29.7\ \text{Å})^{-1}$. This is not readily indexed as any simple, single lamellar lattice. At 54°C, the pattern has changed markedly (Fig. 2b). The three peaks that were detected were indexed on a hexagonal lattice with a 34 Å lattice basis, as more clearly seen in Fig. 2c. Note that this is a very small value for a typical 16-carbon H_{II} phase. For instance, even the 12-carbon dialkyl didodecylphosphatidylethanolamine a H_{II} basis length of 52 Å [16]. The unusual nature of the DPPCh diffraction makes us reluctant to suggest what either the 44 or 54°C structures are in terms of orientation of the constituent molecules. Further work is needed to define the structures. But the data does show that a single lamellar phase does not characterize either temperature.

Comparison of the ^{31}P-NMR lineshape of DPPCH (Fig. 4b and c of Ref. 8) to the isotropic signal of many other phospholipids (eg., see Fig. 3b of Ref. 5 and Fig. 5a of Ref. 17) indicates the DPPCH lineshape to be very broad and somewhat asymmetric. A sharp, symmetric lineshape is generally taken as consistent with isotropic lipid motion on the NMR time scale, as might be experienced with fluid micelles, small vesicles and complex phases such as cubic phases [5]. However, caution is urged in interpreting lineshapes as broad as those seen with DPPCH.

In summary, the X-ray data do not support the conclusion [8] that DPPCh is lamellar at 54°C. Both the X-ray and NMR data exhibit puzzling features which have yet to be understood.

Conclusion

Noggle et al. [8] concluded that dispersions of the sodium salts of 4L-bis-PA and 4M-bis-PA and the calcium salt of DPPCh were in a bilayer phase above the transition temperature. These lipids did not yield characteristic 'lamellar' ^{31}P-NMR spectra. The X-ray data presented here demonstrate that the lipids are not in a lamellar phase above the transition temperature. Thus, on this basis, one should not expect to see characteristic 'lamellar' ^{31}P-NMR spectra. It is important to note that, of all the experimental data presented by Noggle et al. [8], only the freeze-fracture electron microscopy is inconsistent with the X-ray data shown here. It was on the basis of the electron microscopy that a lamellar organization was assigned to the lipids above the transition temperature. the quench rates required to avoid freeze-fracture artefacts with these novel lipids are not known. Thus, it is possible that high temperature quenches, fast as they may have been, were insufficiently rapid.

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