Cubic Phases in Hydrated 1:1 and 1:2 Dipalmitoylphosphatidylcholine-Dipalmitoylglycerol Mixtures

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ABSTRACT The structures of fully hydrated 1:1 and 1:2 (mol/mol) dipalmitoylphosphatidylcholine (DPPC)-dipalmitoylglycerol (DPG) mixtures were studied by means of small-angle x-ray diffraction. The x-ray diffraction pattern of the 1:1 (mol/mol) DPPC-DPG mixture at 65°C contains three reflections with spacings in the ratio of $1:1/\sqrt{2}:1/\sqrt{3}$ in addition to reflections of an inverted hexagonal (H_{II}) phase. A possible interpretation of this result is that a cubic phase of the body-centered space group Im3m, with a lattice constant of 23.1 ± 0.6 nm, is formed. This cubic phase appears at intermediate temperatures between the lamellar and the H_{II} phases. The 1:2 (mol/mol) DPPC-DPG mixture gives an x-ray diffraction pattern at temperatures higher than the lamellar-to-H_{II} transition containing a number of reflections that index a cubic phase structure. The space group of the cubic phase was assigned a face-centered group Fd3m with a lattice constant of 16.3 ± 0.1 nm at 82°C. The possible role of cubic phases in membrane phenomena such as transmembrane signal transduction and fusion is discussed.

INTRODUCTION

The generation of diacylglycerols (DAGs) from substrate phospholipids is known to be involved in the transduction of molecular signals across biological membranes. DAGs, for example, have been shown to activate protein kinase C in the presence of calcium ions and phosphatidylserines, thus providing the link between extracellular events and intracellular protein modification (Nishizuka, 1984, 1992; Liscovitch and Cantley, 1994). The presence of DAGs is also believed to be associated with certain types of membrane fusion (Siegel et al., 1989). The induction of inverted phases, evidenced by the appearance of isotropic ^{31}P nuclear magnetic resonance signals in a predominantly lamellar phase, is closely correlated with the fusion event in model membrane systems (Ellens et al., 1989; Siegel et al., 1989). Similar structures formed by phospholipids and DAGs have also been shown to stimulate the hydrolysis of the phospholipids mediated by endogenous phospholipase A_2 and phospholipase C-type enzymes (Dawson et al., 1983, 1984) The action of these enzymes in concert with DAG kinases may serve to restore the membrane lipid matrix to a more stable state, thereby ensuring that transmembrane signaling and fusion events are transitory in nature. To obtain an understanding of the effects of DAGs on biomembrane structure and stability, it is necessary to describe the inverted phase formed in mixtures of phospholipids and DAGs.

The construction of thermotropic phase diagrams of phospholipid-DAG mixtures is a useful way of characterizing the interactions between the two lipids. Complete phase diagrams of 1,2-dimyristoylglycerol-dimyristolyphosphatidylcholine mixtures (Heimburg et al., 1992) and 1,2-dipalmitoylglycerol (1,2-DPG)-dipalmitoylphosphatidylcholine (DPPC) mixtures (López-García et al., 1994) over the temperature range 0°C to 70°C have been reported. These phase diagrams show that, in the gel phase, phosphatidylcholines and DAGs form two complexes with approximate stoichiometries of 1:1 and 1:2 (mol/mol) in phosphatidylcholine:DAG. These complexes form lamellar structures in the gel phase and undergo a chain melting phase transition on heating at temperatures greater than either of the two individual lipids. The structures formed by these complexes above the chain melting phase transition temperature, however, remain unclear. Examination of these mixed lipid dispersions by ^{31}P nuclear magnetic resonance spectroscopy has revealed the presence of an isotropic peak at temperatures about the transition between lamellar and hexagonal phases (figure 6 in Heimburg et al., 1992, and figure 9 in López-García et al., 1994). One interpretation of this result is that DAG induces a proportion of the phospholipid molecules to undergo rapid isotropic motion, which might be expected with the creation of cubic or micellar structures in the liquid-crystal phase.

The aim of the present work was to characterize the nonbilayer structures formed by 1:1 and 1:2 mol/mol DPPC-DPG mixed aqueous dispersions at temperatures above the chain melting phase transition (more than about 65°C), which give rise to the isotropic peak in ^{31}P nuclear magnetic resonance spectra. X-ray diffraction methods were used for the structure determination, and two cubic phases with different space groups were observed. There are many reports for the formation of cubic phases in unsaturated phosphatidylcholine-DAG mixtures (Das and Rand, 1984, 1986; Seddon, 1990a; Seddon et al., 1990a; Luzzati et al., 1992; Orädd et al., 1995), but so far there have been no

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reports of the formation of cubic phases in fully saturated lipid mixtures, except for a report by Seddon and Bartle (1992) in which they stated that saturated phosphatidylcholine-diacylglycerol mixtures form a face-centered Fd3m cubic phase.

MATERIALS AND METHODS

Materials

L-α-Dipalmitoylphosphatidylcholine (DPPC) and 1,2-dipalmitoylglycerol (1,2-DPG) were purchased from Avanti Polar Lipids (Alabaster, AL) and Sigma Chemical Co. (St. Louis, MO), respectively. These lipids were used without further purification. The purity of each sample was examined by thin-layer chromatography on slica gel plates. The solvent systems used were chloroform:methanol:water, 65:25:4 (v/v/v) and chloroform:methanol:30% aqueous ammonia, 10:10:3 (v/v/v) for DPPC and 1,2-DPG, respectively. Both samples showed a single spot. In addition, the thermal behavior of anhydrous 1,2-DPG was investigated by using differential scanning calorimetry. We did not observe any transition peaks due to the 1,3-isomer of DPG (Kodali et al., 1984, 1990a). Water used in this study was purified with a Milli-Q apparatus (Millipore Corp., Bedford, MA).

Sample preparation

Solutions of DPPC (2 mM) and 1,2-DPG (2 mM) in chloroform were mixed in required proportions in a small test tube. The solvent was evaporated under a stream of oxygen-free dry nitrogen, and then the sample was kept under reduced pressure overnight to remove any remaining traces of solvent. The sample was dispersed into water (5 wt%) at about 70°C for 10 min and then shaken for about 3 min on a vortex mixer at 25°C. The dispersion was then transferred to a 2-mm-diameter, fine-wall glass capillary (Hilgenberg, Malsfeld, Germany) and centrifuged at 2000 × g for about 5 min. The clear water layer was aspirated off to give a concentrated lipid dispersion of about 30–40 wt%. The capillaries were sealed with epoxy resin (Araldite; Ciba-Geigy, Basel, Switzerland) and used for x-ray diffraction studies.

X-ray diffraction

X-ray diffraction data were obtained by using a double-mirror focusing camera with nickel-filtered CuKα radiation from a rotating anode RU200BEH x-ray generator (Rigaku, Tokyo, Japan) with a power of 1.8 kW. X-ray diffraction patterns were recorded with a one-dimensional position-sensitive proportional counter with 4096 channels (PSPC; Rigaku, Tokyo, Japan). Sample capillaries were fixed to a hollow brass holder. The temperature of the sample was controlled (±0.1°C) by circulating water to the sample mount from a temperature-controlled water bath (B. Braun, Melsungen, Germany). The temperature of the sample was monitored with a chromel-alumel thermocouple set near the sample. Counts were typically accumulated for 1–20 h on samples that had been equilibrated at the required temperature for 10 min. Background scattering was reduced by introducing an evacuated path between the sample and the detector. Polyimide films with a thickness of 12.5 μm were used for the windows of the evacuated path. Diffraction patterns recorded from empty capillaries and capillaries containing water showed no artefactual features. The x-ray diffraction profiles were not, however, corrected for background scattering. The sample-to-detector distance was about 385 mm, and the diffraction spacings were calibrated by using the lamellar spacings of anhydrous cholesterol (3.39 nm at 20°C) (Shieh et al., 1977; Loomis et al., 1979).

RESULTS AND DISCUSSION

Fig. 1 shows the small-angle x-ray diffraction patterns recorded from 1:1 (mol/mol) DPPC-DPG mixtures at temperatures in the gel phase (50°C) and the liquid-crystal phase (65°C and 80°C). The diffraction pattern recorded at 50°C shows two orders of a lamellar structure with a lamellar d-spacing of 6.90 nm. This spacing is slightly less than that observed at 20°C (7.01 nm) (Quinn et al., 1995), presumably because of a lateral expansion of the molecules in the bilayer associated with increasing thermal motion of the hydrocarbon chains at higher temperatures. According to the results of López-García et al. (1994), a temperature of

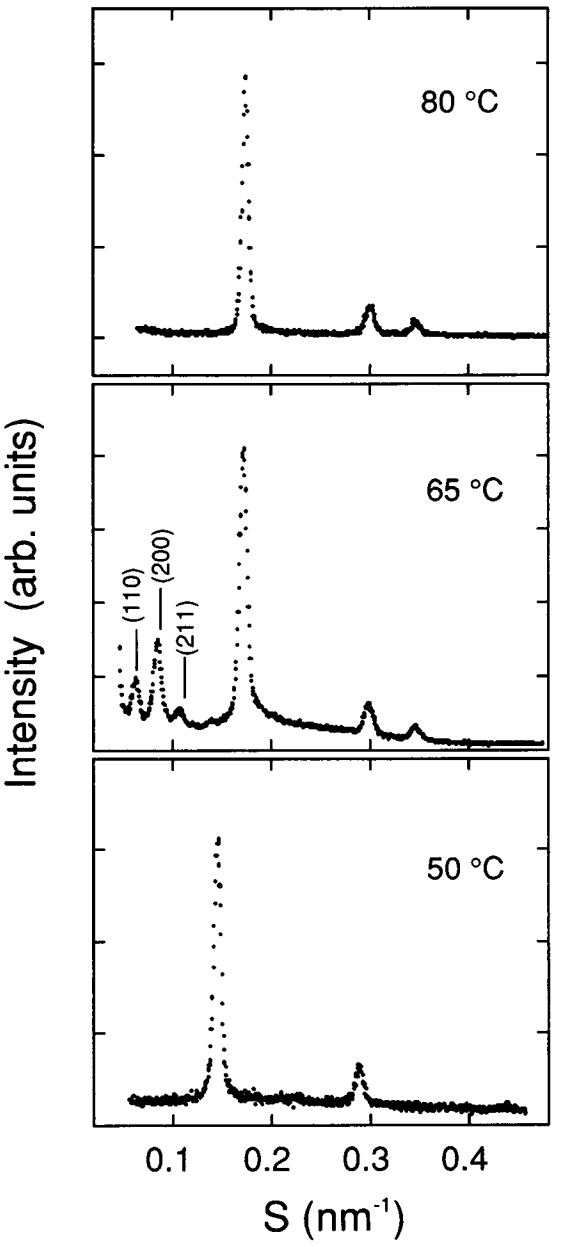

FIGURE 1 Small-angle x-ray diffraction patterns of 1:1 (mol/mol) DPPC-DPG mixtures at temperatures of 50°C, 65°C, and 80°C. The indexes of the reflections [hkl] are indicated for the pattern at 65°C. Scattering intensity, not corrected for the background, is plotted as a function of S (nm^{-1}), S is defined as 2 sin θ/λ (2θ = scattering angle, λ = wavelength of the x-rays).

50°C is below the chain melting transition of the 1:1 mixture. The diffraction pattern recorded at 80°C, by contrast, has reflections in the ratio $1:1/\sqrt{3}:1/2$, which are consistent with a lipid dispersion in an inverted hexagonal (H_{II}) phase. The lattice constant, a, of an H_{II} phase is 6.66 nm, which corresponds to the center-to-center distance of adjacent cylinders.

In the diffraction pattern at 65°C, in addition to the reflections of the H_{II} phase, three reflections were observed in the range of $S = 0.02–0.12$ nm^{-1}. The spacing ratio of these peaks is $1:1/\sqrt{2}:1/\sqrt{3}$. The space group of the lattice cannot be determined unambiguously from only three reflections, but such spacings exclude lamellar and single hexagonal lattices as well as many of the cubic space groups (see International Tables for Xray Crystallography, 1995). One cubic phase group that is not excluded is the body-centered cubic phase designated Im3m. In this case, the three peaks observed at low angle observed in the 1:1 (mol/mol) mixture of DPPC-DPG at 65°C would index the (110), (200), and (211) reflections (see Table 1). This structure gives a lattice constant, a, of 23 + 0.6 nm. However, owing to undetectable weak reflection peaks, this assignment is uncertain. The coexistence of this structure with other phases is typical of the metastability of cubic phases of this type, as has previously been pointed out by Seddon (1990b).

We examined the x-ray scattering profiles of the 1:1 (mol/mol) mixture of DPPC-DPG at other temperatures about the fluidus/liquidus line of the phase diagram and confirmed the coexistence of either lamellar or H_{II} structure with reflections at slightly wider angles than those seen at 65°C that could be assigned to a cubic phase. At 62°C coexistence of a lamellar and a H_{II} phase was observed (data not shown).

TABLE 1 X-ray diffraction spacings of the cubic phases of DPPC-1,2-DPG mixtures

hkl	d_{hkl} (nm)	a (nm)
DPPC-DPG (1:1 mol/mol) at 65°C		
110	15.98	22.6
200	11.86	23.7
211	9.42	23.1
		23.1 ± 0.6*
DPPC-DPG (1:2 mol/mol) at 82°C		
111	9.50	16.5
220	5.77	16.3
311	4.91	16.3
222	4.74	16.4
400	4.08	16.3
331	3.75	16.4
422	3.31	16.2
511, 333	3.15	16.4
440	2.87	16.3
		16.3 ± 0.1*

*Mean value and SD.

Of the many examples of cubic phases formed by lipid-water dispersions (Lindblom and Rilfors, 1989) the Im3m cubic phase has been observed in a fully hydrated dispersion of dimyristoylphosphatidylcholine-myristic acid (1:2; mol/mol) (Seddon et al., 1990b). A Pn3m cubic phase has also been reported to occur in this mixture (Heimburg et al., 1990). The bicontinuous inverted cubic phases of Im3m and Pn3m generally appear as intermediate phases between the smectic mesophase and H_{II} structure in lipid-water systems (Seddon, 1990b; Lindblom and Rilfors, 1989). Transitions between Im3m and Pn3m cubic phases in some lipid-water dispersions, such as 1-monoelaidin-water, have been suggested (Caffrey, 1987; Czeslik et al., 1995), but the relatively broad and weak reflections seen in the DPPC-DPG mixture did not provide strong evidence for the existence of a cubic phase of space group Pn3m. It is also well known that cubic phases, in general, exhibit large temperature dependences of the lattice parameters (Caffrey, 1987), so that small inhomogeneities in temperature or hydration can result in considerable broadening of the cubic reflections.

The temperature dependence of the small-angle x-ray diffraction patterns of the 1:2 (mol/mol) DPPC-DPG mixtures is presented in Fig. 2. Diffraction patterns recorded at 50°C and 70°C are consistent with lamellar and an inverted hexagonal structure, respectively. The lamellar repeat spacing and the lattice constant of the H_{II} phase are 6.96 nm and 6.42 nm, respectively. These values are in good agreement with the parameters reported by López-García et al. (1994) for this mixture. In the patterns at 82°C, many scattering peaks were observed (Fig. 2). The spacing ratio is $1/\sqrt{3}:1/\sqrt{8}:1/\sqrt{11}:1/\sqrt{12}:1/\sqrt{16}:1/\sqrt{19}:1/\sqrt{24}:1/\sqrt{27}:1/\sqrt{32}$ (see Table 1). From this spacing ratio and the identity of the cubic phase formed by dioleoylphosphatidylcholine-dioleoylglycerol mixtures (~3:~7, mol/mol) (Seddon, 1990a; Seddon et al., 1990a), we assign the reflections according to a cubic phase corresponding to a face-centered Fd3m space group. The lattice constant a for a cubic phase of this type is 16.3 ± 0.1 nm. In the diffraction pattern recorded at 82°C, the reflections of (311) and (222) cannot be distinguished with the camera configuration used in these measurements because of their close proximity. The spacing of the (222) reflection was determined by using a curve-fitting method. This consisted of fitting two Lorentzian shape functions of the same width as that of the (111) reflection to the peak in the S range of 0.18–0.24 nm^{-1}. Seddon and Bartle (1992) have already reported that fully hydrated saturated phosphatidylcholine-diacylglycerol mixtures form a face-centered Fd3m cubic phase. However, because they did not describe the detailed results such as ratio of phospholipid to diacylglycerol, lattice constant, temperature, etc., we cannot compare them with our result in detail.

To observe weak reflections from nonlamellar phases, prolonged exposure times (10–20 h) at high temperatures (>65°C) were required. It is necessary, therefore, to determine whether any degradation takes place during x-ray examination of the sample. We investigated the thermal behavior of the 1:2 (mol/mol) DPPC-DPG mixtures before

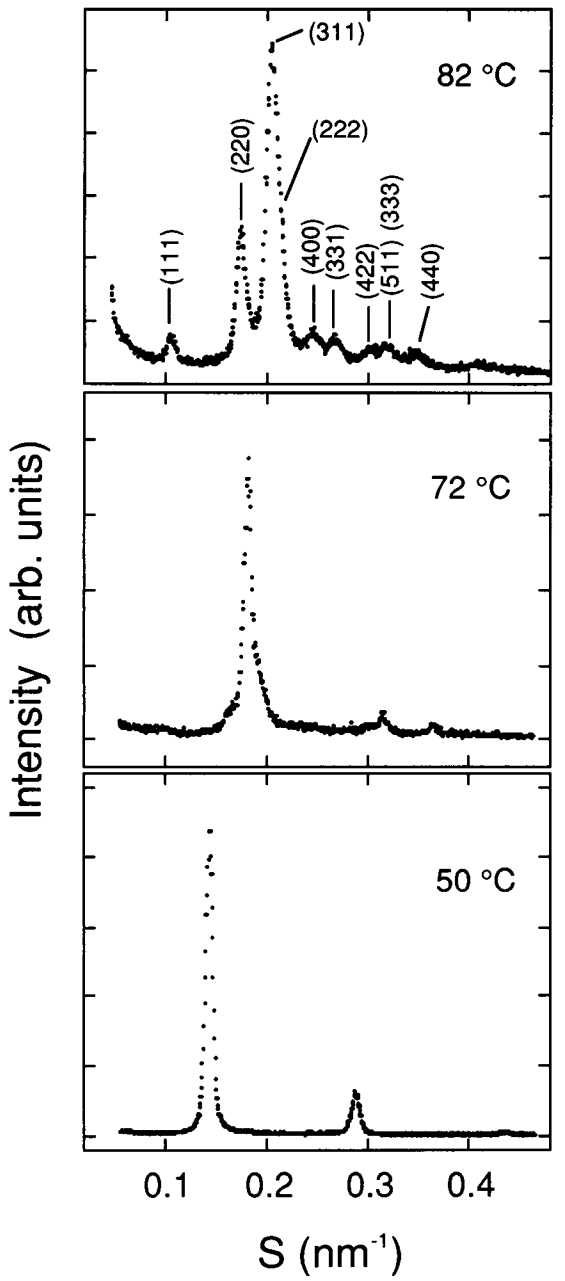

FIGURE 2 Small-angle x-ray diffraction patterns of 1:2 (mol/mol) DPPC-DPG mixtures at temperatures of 50°C, 72°C and 82°C. The indexes of the reflections [*hkl*] are indicated for the pattern at 82°C.

and after a 20-h x-ray exposure at 82°C using differential scanning calorimetry. In the freshly prepared sample, a small transition peak at 49.7°C and a large transition peak at 65.4°C were observed. This result is good agreement with the observation by López-García et al. (1994). On the other hand, in the sample after exposure to the beam for 20 h at 82°C, an additional transition peak was observed at 67.7°C. The origin of this thermal change was further investigated. So far, conversion of about 55% of 1,2-DPG to 1,3-DPG due to acyl migration in unsonicated mixed dispersions of 1,2-DPG and egg-phosphatidylcholine after incubation for 10 h at 62°C has been reported by Kodali et al. (1990b).

First to confirm directly the appearance of the conversion, we carried out following experiments. A mixture of 1,2- and 1,3-isomers of DPG appeared by incubation of a chloroform solution of 1,2-DPG for 7 days at 30°C; thin-layer chromatography on slica gel plates confirmed approximately equal amounts of two isomers. The mixed isomers were codispersed with DPPC (1:2, mol:mol). Second, the thermal behavior of this freshly prepared sample composed of DPPC, 1,2-DPG, and 1,3-DPG was investigated. As a result, the thermal behavior was identical to that of a sample with 20-h x-ray exposure at 82°C. Then, we conclude that acyl migration was responsible for the change during the x-ray measurement, and furthermore, there was no evidence of any radiation damage to the sample.

Nevertheless, acyl migration of this magnitude may not affect the type of cubic phase formed. Seddon (1990a), for example, has reported that the cubic phase formed in aqueous dispersions of DAG-phosphatidylcholine mixtures with either the 1,2- or 1,3-isomer of DAG is the same. To confirm that DPPC-DPG mixtures containing only the 1,2-isomer of DPG form a cubic phase, an experiment with a short accumulation time was performed. We obtained an x-ray diffraction pattern from a freshly prepared mixed aqueous dispersion (1:2, mol/mol) of DPPC-1,2-DPG equilibrated for 10 min at 82°C and exposed to the x-ray beam during the subsequent 10 min (pattern not shown). The resulting x-ray diffraction pattern, although noisy, closely resembles that recorded over a long exposure period at 82°C. This suggests that DPPC-1,2-DPG forms a cubic phase.

There is considerable interest in the link between the formation of diacylglycerol in biological membranes and the consequent molecular events that mediate transmembrane signal transduction and induce membrane fusion (Ellens et al., 1989). Because these processes are transient and probably restricted to local, structurally uncoordinated domains in the membrane, resolution of the lipid arrangement by diffraction methods is not practicable. Nevertheless, studies of mixtures with high DAG concentrations (>50 mol%) provide information about the structure in phase-separated DAG-rich domains within the membrane, where the ratio of DAG:phospholipid may be considerably higher than the overall molar ratio in the membrane. In this context, the amount of DAGs resulting from hydrolysis of phospholipids can reach 1–2 mol% with respect to phospholipids in biological membrane (Preiss et al., 1986). There is abundant evidence from calorimetry, freeze-fracture electron microscopy, and x-ray diffraction for the formation of phospholipid-diacylglycerol complexes (Samby and Brockman, 1985; Ortiz et al., 1988; Cunningham et al., 1989; Heimburg et al., 1992; López-García et al., 1994) and the immiscibility of these complexes with phospholipids. In agreement with earlier studies (Das and Rand, 1984, 1986; Seddon, 1990a; Seddon et al., 1990a; Luzzati et al., 1992; Orädd et al., 1995), the present results indicate that cubic

phases are formed only when equimolar or higher proportions of diacylglycerol are present in the mixture with phospholipid. In our previous studies (Quinn et al., 1995) we have shown by x-ray diffraction methods that for DPPC-DPG mixtures with less than 50 mol% DPG, the 1:1 DPPC-DPG complex is immiscible with DPPC not only in the gel phase but also in the liquid-crystalline phase. From these properties, it is considered probable that DAG, produced as the result of the hydrolysis of phospholipids, remains in a domain enriched with respect to the overall concentration in the membrane. The formation of inverted cubic phases in this DAG-rich domain might provide the molecular rearrangement required to mediate the physiological responses observed in the cell.

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