A Subtransition in a Phospholipid with a Net Charge, Dipalmitoylphosphatidylglycerol†

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ABSTRACT: Suspensions of dipalmitoylphosphatidylglycerol (DPPG) have been analyzed by differential scanning calorimetry, equilibrium and differential scanning dilatometry, and X-ray diffraction techniques. After the DPPG suspensions are stored several days at 2 °C, a new phase transition is observed at a lower temperature than either the main transition or the pretransition. This subtransition has an enthalpy of about 6 kcal/mol and occurs at about 20 °C, the exact temperature depending on the buffer used. The lipid partial specific volume increases by 0.035 mL/g upon warming through the subtransition. X-ray diffraction patterns from suspensions in the subgel phase contain orders of a lamellar repeat and several additional sharp and broad wide-angle reflections between 8 and 2 Å. As the water content in the specimen is reduced, the lamellar repeat period decreases, whereas the spacings and intensities of these additional wide-angle reflections are unchanged. These data indicate that on incubation at 2 °C the lipid molecules crystallize in the plane of each bilayer. X-ray experiments also show that this subgel phase converts to the normal $L_{\beta'}$ gel phase above the subtransition.

Since the pioneering work of Chapman et al. (1967), much of the interest in phospholipid research has been focused on the acyl chain melting or main transition and the "biologically relevant", fluidlike L_α phase. In 1980, Chen et al. showed that in certain saturated phosphatidylcholines there exists in addition to the gel and L_α phases, another phase that forms slowly at low temperatures and melts into the gel phase at a well-defined temperature. This subgel phase has subsequently been characterized as more nearly crystalline and less hydrated than the gel phase (Fuldner, 1981; Ruocco & Shipley, 1982); the kinetics of formation and the thermodynamics of the subtransition in DPPC[1] have also been studied (Nagle & Wilkinson, 1982). Subgel phases are not limited to saturated phosphatidylcholines, however. Both DLPE (Chang & Epand, 1983; Seddon et al., 1983) and DMPE (Wilkinson & Nagle, 1984) form such phases, but with the major difference that the gel phase appears to be metastable with respect to the subgel phase at all temperatures. The possible biological significance of "high-temperature" crystalline phases has been discussed (Wilkinson & Nagle, 1984). It is therefore important to determine how widespread the phenomenon of subgel phases is among different lipids.

In this paper, we show for the first time the existence of such a phase in a lipid bearing a net charge—DPPG, which is negatively charged at neutral pH. The one titratable proton has an apparent pK_a of 2.9 (Watts et al., 1978). The thermodynamics of the subtransition have been studied by differential scanning calorimetry and differential scanning dilatometry. X-ray diffraction analysis indicates that in the subgel phase the lipid molecules crystallize in two dimensions, in the plane of each individual bilayer. The structure of these crystalline bilayers is analyzed in the accompanying paper (Blaurock & McIntosh, 1986).

Materials and Methods

Dipalmitoylphosphatidylglycerol (DPPG ammonium salt) was obtained from Sigma Chemical Co., St. Louis, MO and used without further purification since the narrowness of the main transition (0.3 °C half-width) is comparable to that of other synthetic phospholipids currently available. In our experience, thin-layer chromatography is a less sensitive assay of purity than microcalorimetry and was therefore not used. During the course of long-term incubation at low temperature, there was some broadening of this transition to about 0.6 °C half-width (see Figure 1). Samples were hydrated in buffer at neutral pH by heating them to 70 °C for several minutes and dispersing the lipid by vortexing (5 times, 15 s each time). Three buffers were used: 5 and 50 mM sodium phosphate, pH 7.0, and 50 mM Tris-HCl, pH 7.4. Lipid concentrations were 2–2.5 mg/mL for the calorimetry experiments and 15 mg/mL for the dilatometry. Higher lipid concentrations were used for the X-ray experiments, with the buffer content being 20–60% by weight for most experiments. For these higher lipid concentrations, the specimens were allowed to equilibrate above the lipid's main transition temperature for several hours.

The apparent heat capacity was measured on a Microcal MC-1 DSC (Amherst, MA) at a scanning rate of 12 °C/h. The calorimeter was interfaced to an Apple II+ computer system for data acquistion and computation. ΔH values were obtained by numerical integration of the digitized data. The heat capacity scale was calibrated with NaCl solutions of known specific heat values (International Critical Tables). The change in volume was determined by a differential dilatometer (Wilkinson & Nagle, 1978) using either a scanning rate of 5 °/h or the equilibrium mode. Temperature was maintained to within ±0.1 °C.

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[1] Abbreviations: DPPG, L-α-dipalmitoylphosphatidylglycerol; DPPC, L-α-dipalmitoylphosphatidylcholine; DLPE, L-α-dilauroylphosphatidylethanolamine; DMPE, L-α-dimyristoylphosphatidylethanolamine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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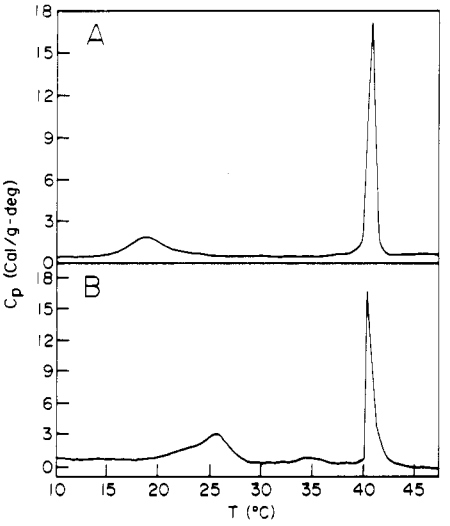

FIGURE 1: Specific heat vs. temperature for DPPG in (A) 50 mM Tris buffer, pH 7.4, incubated for 52 days at 2 °C and (B) 5 mM sodium phosphate buffer, pH 7.0, incubated for 34 days at 2 °C.

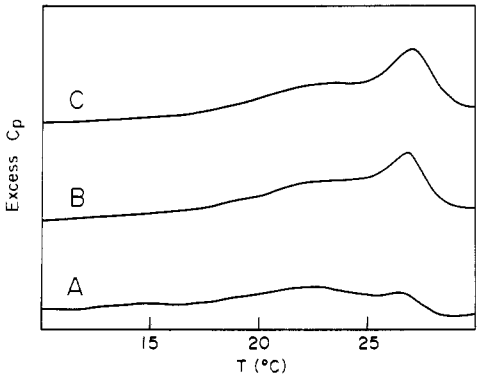

FIGURE 2: Excess heat capacity vs. temperature for DPPG in 50 mM phosphate buffer, pH 7.0, after (A) 4, (B) 12, and (C) 31 days at 2 °C.

For the X-ray experiments, lipid suspensions were drawn into X-ray capillary tubes (0.7-mm diameter) by suction. The capillary tubes were sealed and mounted in a temperature-regulated (±2.0 °C) specimen holder in a pinhole collimation X-ray camera containing three sheets of Kodak DEF-5 X-ray film in a flat film cassette. Specimen to film distances were from 5 to 10 cm, and exposure times were from 5 to 24 h.

RESULTS

Calorimetry and Dilatometry. Figures 1 and 2 show differential scanning calorimetry heating curves of suspensions of DPPG in Tris and phosphate buffers after low-temperature incubation of the specimens for various times. In Figure 1A, DPPG in 50 mM Tris, pH 7.4, was incubated at 2 °C for 52 days, and in Figure 1B, DPPG in 5 mM sodium phosphate buffer, pH 7.0, was incubated 34 days at 2 °C. In both thermograms there is a sharp main transition at 41 °C, which has an enthalpy of 9.1 ± 0.5 kcal/mol for Tris and 8.8 ± 0.5 kcal/mol for phosphate buffer. In the phosphate buffer only, a pretransition with an enthalpy of 0.7 ± 0.1 kcal/mol is observed at about 35 °C. In both buffers, another thermal event, the subtransition endotherm, is observed. For Tris buffer, a single subtransition endotherm with an enthalpy of 4.3 ± 0.5 kcal/mol is centered at 17.7 °C. In phosphate buffer, a somewhat broader subtransition is centered at about 25 °C and has an enthalpy of 6.0 ± 0.6 kcal/mol. The slower rate of formation and lower transition temperature observed in Tris buffer may be indications of a slightly less stable subgel phase in that buffer. No subtransition endotherms are observed on second or subsequent heating scans in either buffer.

Table I: Thermal Characteristics of the Subtransition of DPPG

buffer	T_{mid} (°C)	ΔH (kcal/mol)	ΔV (mL/g)
5 mM sodium phosphate, pH 7.0	24.8	6.0 ± 0.6	
50 mM sodium phosphate, pH 7.0	27.0[a]	5.7 ± 0.6	0.035 ± 0.003
50 mM Tris-HCl, pH 7.4	17.7	4.3 ± 0.5	

[a] Shoulder at 22.0 °C.

The temporal evolution of the subtransition endotherm is shown in Figure 2. Suspensions of DPPG in 50 mM sodium phosphate buffer, pH 7.0, were incubated at 2 °C for 4 (Figure 2A), 12 (Figure 2B), and 31 days (Figure 2C). The thermograms in Figure 2B,C are nearly identical, indicating that equilibrium is reached within 12 days of incubation at 2 °C. Under these conditions, the subtransition contains two endotherms, a broad one centered at 22 °C and a sharper one centered at 27 °C. The total enthalpy of the two endotherms shown in Figure 2C is 5.7 ± 0.6 kcal/mol. No evidence was obtained for the progressive growth of one endotherm at the expense of the other in these experiments. However, from equilibrium dilatometry experiments, we find that the volume change associated with the subtransition is complete by 22.5 °C. Double-peaked endotherms have been noted previously in subtransitions of other lipids (Wilkinson & Nagle, 1984; Finegold & Singer, 1984; Mulukutla & Shipley, 1984). The subgel phase forms more slowly (by a factor of 3–4) in Tris compared to phosphate buffer. The thermodynamic parameters characterizing the subtransition are listed in Table I.

Dilatometry (Table I) shows that the partial specific volume of DPPG in 50 mM phosphate buffer increases by 0.035 ± 0.003 mL/g upon warming through the subtransition. This compares to a value of 0.009 ± 0.0005 mL/g found for DPPC (Nagle & Wilkinson, 1982).

X-ray Diffraction. X-ray diffraction experiments were performed to investigate the structural change occurring at the DPPG subtransition. Control experiments, performed on samples at 10 or 20 °C with no low-temperature incubation, gave diffraction patterns characteristic of the normal $L_{\beta'}$ gel phase, as reported by Watts et al. (1981). A typical diffraction pattern of DPPG in the $L_{\beta'}$ phase is shown in Figure 3A. In this experiment, partial orientation of the specimen occurred as it was drawn into the X-ray capillary tube. In Figure 3A the vertical or meridional axis of the X-ray film corresponds to the direction in which the bilayers are preferentially stacked. Thus, a series of sharp, arced reflections is observed centered on the meridian. The reflections in this pattern have a lamellar repeat period of 63 Å, corresponding to the center to center distance from one bilayer to the next. This lamellar spacing is variable and depends on the thickness of the fluid spaces between bilayers, which is a function of the amount of buffer added to the specimen. However, the wide-angle pattern remains the same for any water content greater than ≃10% and at 20 °C consists of a sharp reflection at 4.25 Å (arrow in Figure 3A) surrounded by a broad band at about 4.0 Å. This type of double wide-angle pattern is characteristic of the tilted gel chains in the $L_{\beta'}$ phase (Tardieu et al., 1973).

A typical X-ray pattern from DPPG specimens incubated at low temperatures (4 °C) for 2 or more weeks and then exposed at 10 °C is shown in Figure 3B. The arced low-angle reflections centered on the meridian index as a lamellar repeat and vary in spacing with water content in a similar way to the

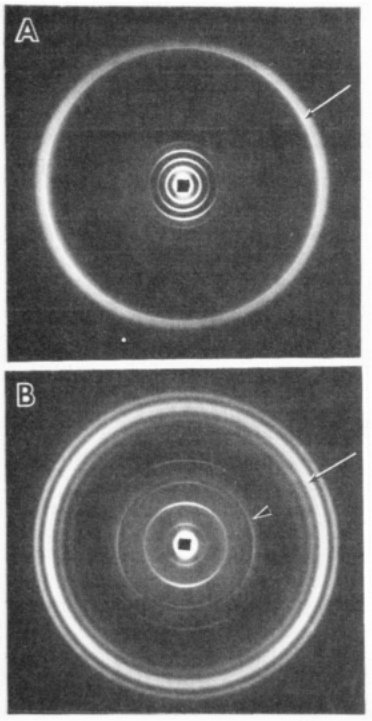

FIGURE 3: X-ray diffraction patterns for DPPG in 50 mM phosphate buffer, pH 7.0, (A) at 20 °C with no low-temperature incubation and (B) at 10 °C after being incubated for 2 weeks at 4 °C. Visible in (A) are the first five lamellar orders of a repeat period of 63.3 Å and a sharp wide-angle reflection at 4.25 Å (arrow) with a broad band at 4.0 Å. Visible in (B) are several orders of a lamellar repeat period of 51.5 Å centered on the meridian and wide-angle reflections at 7.83 (arrowhead), 4.88, 4.40, 4.11 (arrow), 4.00, and 3.75 Å.

$L_{\beta'}$ phase of DPPG. However, the wide-angle reflections are quite different than those from the $L_{\beta'}$ phase. The DPPG subgel pattern (Figure 3B) contains several sharp orders of a lamellar periodicity of 51.5 Å near the center of the pattern, and an additional series of sharp and broad wide-angle reflections not belonging to the lamellar series. Sharp reflections are observed at 7.83 (arrowhead), 5.40, 4.88, 4.11 (arrow), and 2.94 Å, and broader bands are observed at 4.75, 4.40, 4.00, and 3.75 Å. The same wide-angle reflections are observed in 50 mM phosphate or 50 mM Tris buffers. Moreover, the spacings, breadths, and relative intensities of these wide-angle reflections are independent of the water content of the specimen and therefore of the low-angle lamellar spacing. For example, panels A and B of Figure 4 show subgel diffraction patterns with lamellar repeat periods of 60.4 and 75.2 Å, respectively. The difference in lamellar spacing is apparent from the different positions and intensities of the arcs along the meridional axis of the patterns. However, the wide-angle bands from the two patterns are perfectly superimposable (Figure 4A,B). That is, the wide-angle reflections are constant and not dependent on the spacing between bilayers. This is strong evidence that the subgel structure is not a three-dimensional crystal. Rather, the lipid molecules have crystallized in the plane of each bilayer independently of the molecules in adjacent bilayers.

Wide-angle X-ray diffraction patterns were recorded to show the temporal development of the subgel phase. The sharp reflection at 4.25 Å, which is characteristic of the $L_{\beta'}$ phase, is present in patterns recorded after specimen incubation at 4 °C for 2, 4, and 7 days but is absent after incubation for 2 weeks or longer. The strong reflections at 7.83, 4.11, and 3.75 Å, characteristic of the subgel phase, are just barely visible in the patterns recorded after 4 days at 4 °C and become more

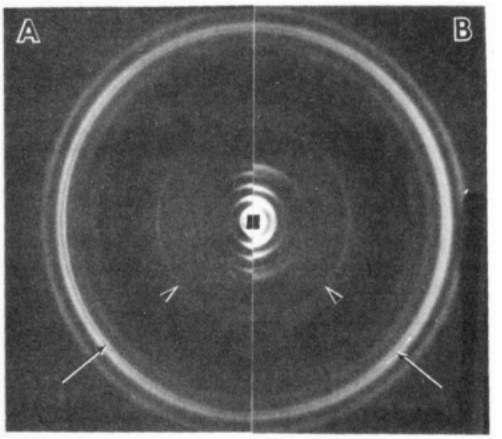

FIGURE 4: X-ray diffraction patterns for DPPG in 50 mM phosphate buffer, pH 7.0, incubated for 2 weeks at 4 °C. (A) Lamellar repeat period d = 60.4 Å and (B) lamellar repeat period d = 75.2 Å. In both (A) and (B), wide-angle reflections at 7.83 (arrowhead), 4.88, 4.40, 4.11 (arrow), 4.00, and 3.75 Å are visible.

intense in the pattern recorded after 7 days at 4 °C incubation. The wide-angle reflections recorded after 14 days or longer at 4 °C incubation are identical with those shown in Figures 3B and 4A,B. Thus, both the calorimetry (Figure 2) and X-ray diffraction results indicate that the complete conversion from $L_{\beta'}$ to subgel phase takes about 2 weeks of low-temperature incubation. When samples in the subgel phase are heated to 30 °C, a normal $L_{\beta'}$ pattern is observed, indicating that the subtransiton is a transition between two solid phases.

Discussion

The subtransitions studied so far in phospholipids appear to fall into two groups. In one group, type I, a "solid–solid" transition between subgel and gel phases occurs. This type of behavior has been found in the saturated phosphatidylcholines with chain lengths of 16, 17, and 18 carbons (Chen et al., 1980; Finegold & Singer, 1984) and now in the charged lipid DPPG. Type I transitions are characterized by having only a very small change in rotameric disordering (an average of one or fewer gauche rotamers formed per lipid molecule).

The second subtransition type (II) is the more complete melting of the subgel phase into the L_{α} phase, and it involves a greater rotameric disordering. Examples of this transition type are certain saturated phosphatidylethanolamines (Chang & Epand, 1983; Seddon et al., 1983; Wilkinson & Nagle, 1984). The case of DMPE may, however, be more complicated. Mulukutla & Shipley (1984) reported type I transition behavior for this lipid whereas Wilkinson & Nagle (1984) observed type II behavior. Perhaps the particular form the crystallization into the subgel phase takes depends on such factors as incubation temperature, lipid purity, and so on.

From the density data (i.e., specific volume either above or below the transition and the volume change at the transition), the change in the van der Waals energy (ΔU_{vdw}) that occurs due to changes in chain–chain separation may be calculated (Nagle & Wilkinson, 1978). Using the density data of D. A. Wilkinson, D. A. Tirrell, A. B. Turek, and S. D. Merajver (unpublished results) and the value of ΔV reported here, we find that ΔU_{vdw} = 5–7 kcal/mol depending on whether or not the repulsive interaction term is included in the calculation. This implies that almost all of the observed ΔH for the subtransition is accounted for by the van der Waals term, leaving almost nothing for rotameric disordering as is typical for type I subtransitions.

The X-ray diffraction patterns in the subgel phase of DPPG (Figures 3B and 4) are similar in several regards to those

recorded from other phospholipids such as DPPC (Fuldner, 1981; Ruocco & Shipley, 1982), DLPE (Seddon et al., 1983), and DMPE and its *N*-methyl derivatives (Mulukutla & Shipley, 1984). All of these patterns contain a lamellar repeat period and several additional nonlamellar sharp and broad reflections in the range of 8–2 Å. As pointed out by Ruocco & Shipley (1982), the presence of these additional wide-angle reflections indicates that the subgel phase is a more highly ordered bilayer structure than the $L_{\beta'}$ phase.

The diffraction patterns shown in Figure 4, where the wide-angle reflections are constant in spacing and intensity distribution for different lamellar repeat periods, clearly demonstrate that the lipid molecules crystallize in the plane of each bilayer independently of adjacent bilayers. Thus, all of the wide-angle reflections should index in terms of a two-dimensional lattice. The indexing of these reflections is not a trivial matter, particularly since there are more reflections in the 8–2-Å range than would index as lattice points on a simple two-dimensional lattice of reasonable dimensions. Ruocco & Shipley (1982) have also discussed the difficulties of indexing the wide-angle reflections from the subgel phase of DPPC. However, in the following paper, Blaurock and McIntosh (1986) have obtained oriented diffraction patterns from subgel-phase DPPG. These patterns show that the sharp wide-angle reflections define the two-dimensional lattice points and the broader bands are subsidiary maxima lying along the lattice lines. Thus, all of the reflections in Figures 3 and 4 can be accounted for, and it is found that the lipid molecules of DPPG crystallize in a two-dimensional oblique lattice (Blaurock & McIntosh, 1986).

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