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Rapid Reversible Formation of a Metastable Subgel Phase in Saturated Diacylphosphatidylcholines

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ABSTRACT Formation of well ordered lamellar subgel (SGII) phase in aqueous dispersions of L-dipalmitoylphosphatidylcholine upon cooling from the lamellar gel phase, without low-temperature equilibration, is observed in real time using synchrotron x-ray diffraction. It has the same lamellar repeat period as the gel phase from which it was formed but differs in its wide-angle diffraction pattern. The SGII phase forms at about 7°C upon cooling at 2°C/min. In temperature jump experiments at 1°C/s from 50 to −5°C, the relaxation time of the lamellar gel-SGII transition is found to be ~15 s. The conversion between the lamellar gel and SGII phase is cooperative and rapidly reversible. Upon heating, it coincides in temperature with an endothermic event with a calorimetric enthalpy of 0.35 kcal/mol, the so-called sub-subtransition. Similar sub-subtransitions are also observed calorimetrically at temperatures ~10°C below the subtransition, without low-temperature storage, in aqueous dispersions of L-dimyristoylphosphatidylcholine and L-distearoylphosphatidylcholine, but not in racemic DL-dipalmitoylphosphatidylcholine. The formation of the equilibrium lamellar crystalline L_c phase appears to take place only from within the SGII phase.

INTRODUCTION

Aqueous dispersions of saturated diacylphosphatidylcholines with hydrocarbon chains 14, 16, or 18 carbon atoms long are known to form lamellar liquid-crystalline (L_α) phase at high temperature, two lamellar gel phases ($L_{\beta'}$ and $P_{\beta'}$) at intermediate temperatures, and lamellar subgel (L_c) phase at low temperature (NIST Standard Reference Database 34, 1993). Although the formation of the $L_{\beta'}$, $P_{\beta'}$, and L_α phases is a relatively fast process at the relevant temperatures, the formation of the crystalline L_c phase from the $L_{\beta'}$ phase requires prolonged low-temperature equilibration. The mechanisms of the L_c phase formation and the inverse L_c -$L_{\beta'}$ phase transformation (so-called subtransition) upon heating have been studied frequently (Fuldner, 1981; Ruocco and Shipley, 1982a, b; Akiyama, 1985; Akiyama et al., 1987; Lewis et al., 1987; Tenchov et al., 1987, 1989; Boyanov et al., 1983; Tristram-Nagle et al., 1987, 1994) since the discovery of the subtransition (Chen et al., 1980). Subsequently, a low-enthalpy transition has been observed calorimetrically in DPPC dispersions, at a temperature ~10°C below the subtransition, without low-temperature incubation of the sample (Slater and Huang, 1987). It has been suggested that this transition signifies the formation of a precursor of the L_c subgel phase, a so-called sub-subgel (SGII) phase.

The purpose of the present study was to identify possible structural events associated with the so-called sub-subtransition. Formation of a well-ordered lamellar SGII phase in DPPC upon cooling from the $L_{\beta'}$ phase was observed in real time at ~7°C using synchrotron x-ray diffraction. This transformation is readily reversible upon heating with a hysteresis of 2–3°C. Structural characteristics of the SGII phase were determined. The relaxation time of the lamellar gel-SGII transition was found to be ~15 s during temperature jumps at 1°C/s. Also, low-enthalpy transformations were also observed calorimetrically at temperatures well below the subtransition, without low-temperature incubation, in aqueous dispersions of DMPC and DSPC. It was established by calorimetry that racemic DL-DPPC, in which the formation of a subgel L_c phase is hindered as previously reported (Boyanov et al., 1983), does not form the SGII phase as well.

MATERIALS AND METHODS

Sample preparation

1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) (Avanti Polar Lipids, Inc., Birmingham, AL), and *rac*-1,2-dipalmitoyl-glycero-3-phosphocholine (DL-DPPC) (Fluka AG, Basel, Switzerland) were used. No chromatographic tests for purity were performed; however, the half-widths of the main phase transitions (<0.20–0.25°C for pure stereoisomers and <0.6°C for DL-DPPC) provided a guarantee that the lipid purities were comparable with the claimed value of >99%.

Multilamellar lipid vesicles were prepared by dispersing lipid in required amounts of quartz-bidistilled deionized water. The dispersions were hydrated overnight at 20°C and for 1 h at a temperature 10°C above the main transition. The samples were vortex-mixed several times at these temperatures for 1–2 min. The lipid concentrations were 2–8 mg/ml for calorimetry and 20 wt% for the x-ray measurements.

Calorimetric measurements

Calorimetric measurements were performed using high-sensitivity differential adiabatic scanning microcalorimeter DASM-1 M (Mashpriborintorg,

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Abbreviations used: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DL-DPPC, *rac*-1,2-dipalmitoyl-glycero-3-phosphocholine; DHPC, 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine.

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Pushchino, Russia) with sensitivity >4.10^{-6} cal K^{-1} and a noise level <5.10^{-7} W (Privalov et al., 1975). The samples were loaded into the calorimetric cell at 20°C, then cooled to 0°C. Heating runs were started at 0°C, with a heating rate of 0.5°C/min. The cooling step, together with the equilibration of the instrument for the heating run, takes ~30 min. Experiments with incubation times between 0 and 3 h at 0°C have been performed. The thermograms were corrected for the instrumental baseline. The calorimetric enthalpy of the transition was determined as the area under the excess heat capacity curve.

Time-resolved x-ray diffraction measurements

X-ray measurements were carried out using a monochromatic (0.15 nm) focused x-ray beam at station 8.2 of the Daresbury Synchrotron Radiation Laboratory (Daresbury, U.K.). A purpose-built camera (Lis and Quinn, 1991) allowed clear resolution of reflections between 0.35 and 10 nm. The samples were sandwiched between thin mica sheets, 1 mm apart, and were mounted on a modified cryostage (Linkam Scientific Instruments Ltd., Tadworth, U.K.). Successive cooling and heating scans were performed in defined temperature ranges with a scan rate of 2°C/min. Temperature jumps from 50 down to −5°C at 1°C/s were also performed. X-ray scattering intensities were recorded on a multiwire quadrant detector constructed at the Daresbury Laboratory. X-ray scattering data were acquired in 255 consecutive time frames of 3 s separated by a 50-μs waiting time between the data acquisition frames. Data were stored in a VAX 11/785 computer (Maynard, MA) and analyzed using the OTOKO software program (EMBL, Hamburg, Germany) (Boulin et al., 1986). Scattering intensities were corrected for detector response recorded from a ^{59}Fe source, and spatial calibrations were obtained using hydrated rat tail tendon collagen (d = 67 nm; Bigi and Roveri, 1991) and high-density polyethylene (HDPE, 0.4166, 0.3780, and 0.3014 nm) as calibration standards. Under the conditions used to record the dynamic x-ray data, no apparent radiation damage was evident either from the phase behavior of the samples or by detection of lipid breakdown products in thin-layer chromatograms of the lipid performed upon completion of the diffraction experiments. The radiation dose was well below the level where radiation damage has been observed previously (Caffrey, 1985).

RESULTS

Differential scanning calorimetry

Calorimetric scans of samples of DMPC, DPPC, and DSPC recorded on heating immediately after cooling from 20 to 0°C are shown in Fig. 1. With all three lipids, anomalies in the excess heat capacity occur at temperatures below the reported temperature of the subtransition ($L_c \rightarrow L_{\beta'}$ transformation). The calorimetric peak is at ~5°C for DMPC, ~8°C for DPPC, and ~15°C for DSPC, i.e., for all three lipids it is ~10°C below the subtransition (NIST Standard Reference Database 34, 1993). The detection of these low-enthalpy peaks on the thermograms was perfectly reproducible in the samples of DPPC and DSPC; with DMPC, these enthalpy changes were not observed in ~25% of the scans following the same temperature protocol. Incubation for up to 3 h at 0°C does not change noticeably the transition temperature. The thermodynamic characteristics of these thermal events (temperature, enthalpy change) are summarized in Table 1. With samples of DL-DPPC, no heat capacity anomalies have been detected in the temperature range 0–30°C. All four lipids exhibit pretransitions and main transitions with thermodynamic characteristics in good agreement with previously reported results (data not shown except for the DMPC pretransition included in Fig. 1) (NIST Standard Reference Database 34, 1993). No evidence of a subtransition has been detected in the thermograms after incubation at 0°C for up to 3 h.

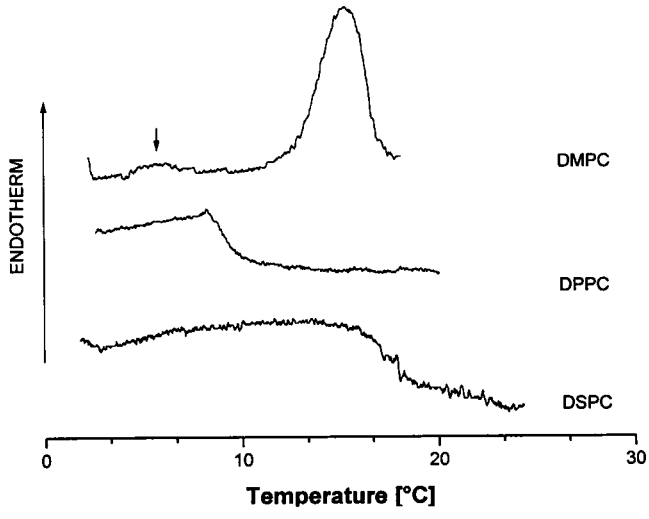

FIGURE 1 DSC heating thermograms of hydrated DMPC, DPPC, and DSPC dispersions recorded immediately after cooling from +20 to 0°C, with a heating rate of 0.5°C/min. On the DMPC thermogram, the arrow indicates the position of the sub-subtransition; the pretransition at ~15°C is also shown.

Time-resolved x-ray diffraction

Fig. 2 shows wide-angle diffraction patterns recorded in real time during cooling of hydrated DPPC sample from 20 to 0°C and then immediately reheating to 20°C at 2°C/min. At 20°C the pattern is typical of the $L_{\beta'}$ phase, with a diffraction peak at 0.419 nm and a shoulder at 0.408 nm, and does not change upon cooling down to ~10°C. In the low-angle region, up to five orders of lamellar repeat spacing are observed corresponding to d = 6.38 nm (not shown). Upon cooling in the temperature range 10–3°C, the wide-angle diffraction pattern transforms into a new one, with characteristic reflections at 0.425 nm (major) and 0.398 nm. During these changes in the wide-angle diffraction pattern, no change in the lamellar period of 6.38 nm is observed. Upon immediate reheating, the reverse transformation proceeds with a hysteresis of ~2–3°C. The last traces of the sharp reflection at 0.425 nm disappear at ~12°C.

The positions of the wide-angle diffraction peaks at different temperatures during cooling and immediate reheating

TABLE 1 Thermodynamic characteristics of the sub-subtransition in saturated phosphatidylcholines

Lipid	T (°C) ±1.0°C	ΔH (kcal/mol) ±30%
L-DMPC	5.6	0.05
L-DPPC	8.1	0.35
L-DSPC	14.8	0.82
DL-DPPC		

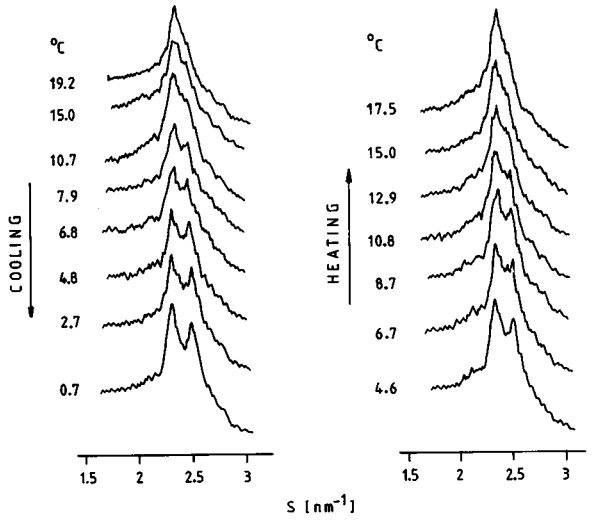

FIGURE 2 Plot of representative x-ray scattering profiles of the wide-angle reflections from hydrated DPPC recorded in real time during cooling (*left*), and immediate reheating (*right*), at 2°C/min in the temperature range +20–0°C.

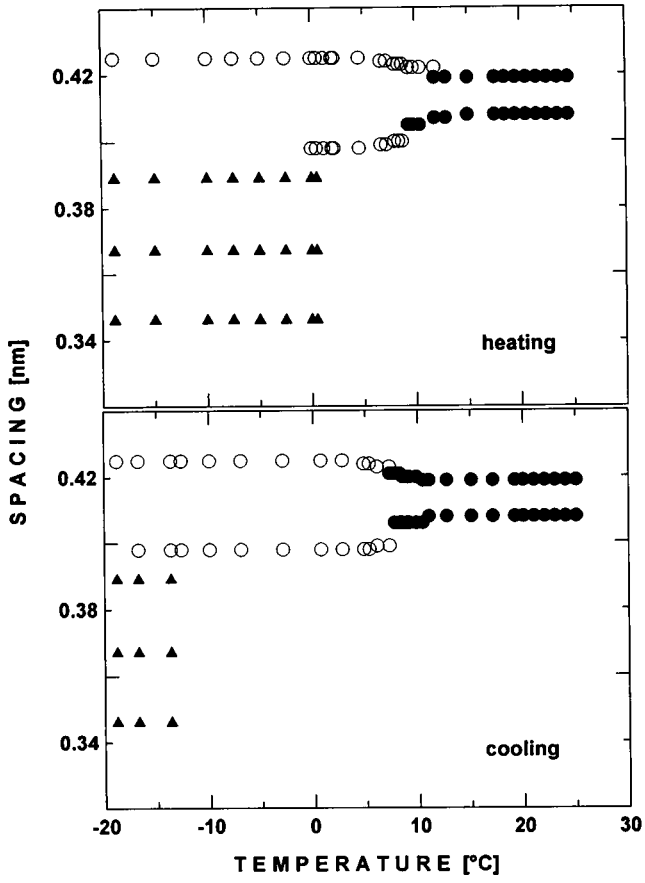

FIGURE 3 Positions of the wide-angle reflections of hydrated DPPC sample during cooling (*bottom*) and immediate reheating (*top*) at 2°C/min in the temperature range −20 to +25°C; (●) $L_{\beta'}$ phase reflections (except for the main reflection at ~0.419 nm, the position of the shoulder at ~0.408 nm is also shown); (○) SGII phase reflections; (▲) ice reflections at 0.389 nm (major), 0.367 nm, and 0.346 nm (the reflection at 0.389 nm is rather intense and possibly masks the SGII reflection at 0.398 nm).

scans at 2°C/min in the temperature range −20 to +25°C are shown in Fig. 3. The diffraction maxima at 0.389 nm (major), 0.367 nm, 0.345 nm, appearing at −13.6°C upon cooling and, finally, disappearing at 1.0°C upon heating correspond to the diffraction bands of the hexagonal ice (Dowel et al., 1962) and reflect the freezing of water (Sanderson et al., 1993). Their emergence in the wide-angle diffraction pattern coincides with a decrease of the low-angle lamellar repeat period to 5.87 nm. The SGII band at 0.398 nm supposedly is masked by the rather intense 0.389-nm band of ice. Thus, upon cooling to temperatures below −13°C, the observed phase sequence is $L_{\beta'}$-SGII-frozen SGII. This sequence is reversible upon immediate reheating, with temperature hysteresis of ~2–3°C for the first transition ($L_{\beta'}$-SGII) and ~14°C for the second one (SGII-frozen SGII).

Temperature jumps from 50°C (L_α phase) down to −5°C at 1°C/s followed by storage at −5°C were also performed to establish the relaxation processes as recorded by x-ray diffraction in real time. A $L_\alpha \rightarrow L_\beta$ transition is seen at ~35°C. The wide-angle peak of the lamellar gel phase first appears at 0.430 nm and shifts progressively to 0.420 nm as the temperature decreases between 35 and ~12°C. Patterns of a well ordered lamellar phase, identical to that formed upon cooling at 2°C/min (SGII phase), first begin to appear at ~7°C. Remaining traces of the lamellar gel phase disappear in ~3 s after reaching −5°C, i.e., the final formation of the SGII phase takes place in ~15 s (Fig. 4). The registration of these changes in the wide-angle diffraction pattern of DPPC both in temperature scan and temperature jump experiments was fully reproducible.

DISCUSSION

In a study of Slater and Huang (1987), a new low-temperature, low-enthalpy phase transformation in fully hydrated DPPC has been observed calorimetrically. Unlike the subtransition, no low-temperature equilibration of the sample was required to detect this so-called sub-subtransition. It has been found to appear at 6.8°C immediately upon cooling. Low-temperature storage, which results in the appearance of the subtransition, eliminated the sub-subtransition from the thermograms (Slater and Huang, 1987). The SGII phase formed below the sub-subtransition has been supposed to be a precursor of the subgel phase.

Our calorimetric results concerning the DPPC sub-subtransition agree with those of Slater and Huang (1987). Additionally, we observe sub-subtransitions in DMPC and DSPC, with no low-temperature storage. Thus, a rapid and reversible formation of an intermediate metastable subgel (SGII) phase appears to be a common mechanism for these saturated diacyl phosphatidylcholines. For racemic DL-DPPC, where the formation of the L_c phase is hindered (Boyanov et al., 1983), we find that, by contrast with L-DPPC, it does not display a sub-subtransition either. This observation indicates that formation of SGII is an intermediate step in the $L_{\beta'}$-L_c transition, and that the crystallization of DL-DPPC is

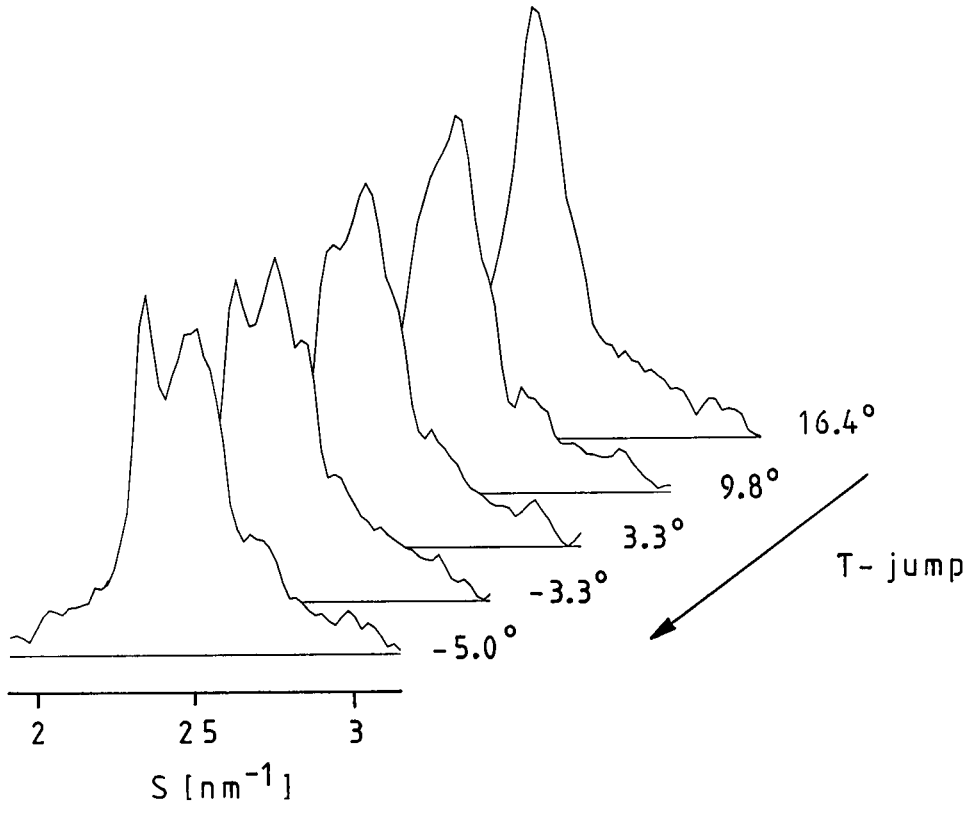

FIGURE 4 Plot of representative x-ray scattering profiles of the wide-angle reflections from hydrated DPPC recorded in real time during a temperature jump from +50 to −5°C at 1°C/s.

obstructed at the stage of formation of the SGII phase. DHPC, an ether-linked analog of DPPC, is known to display fully reversible low-temperature, low-enthalpy transition from a lamellar interdigitated gel to a highly ordered lamellar interdigitated subgel phase, which does not evolve to the L_c phase (Ruocco et al., 1985; Laggner et al., 1987), i.e., the formation of the L_c phase might be obstructed at the step of the SGII-L_c conversion as well.

The x-ray diffraction pattern of the SGII phase formed in DPPC below 7°C is typical of lamellar lipid bilayers with highly ordered hydrocarbon chains. The wide-angle bands at $d_1 = 0.425$ nm and $d_2 = 0.398$ nm differ from those reported for the subgel L_c phase formed upon prolonged low-temperature equilibration (cf. $d_1 = 0.440$ nm and $d_2 = 0.386$ nm as reported by Ruocco and Shipley, 1982b). The dimensions of an orthorhombic hybrid subcell of the SGII phase giving these reflections might be calculated as $a_s = 1.005$ nm and $b_s = 0.850$ nm. The lamellar repeat period of the SGII phase is also different from that for the L_c phase (values between 5.91 and 6.00 nm have been reported in different studies (Ruocco and Shipley, 1982a, b; Tenchov et al., 1987, 1989)) and coincides with that of the $L_{\beta'}$ phase. Thus, the structural characteristics of the SGII phase formed in DPPC samples below 7°C upon cooling typify it as an intermediate lamellar phase between the L_c and $L_{\beta'}$ phases.

In our calorimetric scans, the sub-subtransition is the only low-temperature event for incubation times at 0°C for up to 3 h. Slater and Huang (1987) observe the first patterns of the subtransition, together with the sub-subtransition, in their thermogram taken after 3.5 h at 0.5°C. Thus, the lifetime of the SGII phase at 0°C could be estimated at ~3 h before the beginning of its conversion into the L_c phase can be detected.

According to the calorimetric observations of Slater and Huang (1987), the subtransition appears as a distinct peak, in addition to the sub-subtransition, upon increasing the low-temperature storage time. After its first appearance in a thermogram recorded after 3.50 h at 0.5°C, it grows in enthalpy and temperature while the sub-subtransition decreases. On a thermogram recorded after 11-h incubation, only the subtransition persists (Slater and Huang, 1987). This behavior is indicative for a formation of the L_c phase from within the SGII phase. Data reported recently by Tristram-Nagle et al. (1994) do not seem to contradict this assumption. In their study, the equilibrium L_c phase is observed to nucleate from a phase with a major (20) wide-angle band at 0.425 nm identified by the authors as the gel phase. The value of 0.425 nm is higher than most of the reported wide-angle reflections of the $L_{\beta'}$ phase (cf. 0.418 nm in Ruocco and Shipley (1982b) and Akiyama et al. (1987), 0.417 nm in Ruocco and Shipley (1982a), and 0.419 nm in the present study). The figures in Tristram-Nagle et al. (1994) show wide-angle diffraction patterns limited to scattering angles below 21.3° (Bragg spacing 0.41 nm) and, thus, the positions of (11) peaks cannot be compared. Because the lamellar repeat period of the SGII coincides with that of $L_{\beta'}$ phase, and considering the unambiguous demonstration of a fast SGII phase formation upon cooling below 7°C provided in the present work, it is reasonable to assume that these authors observe formation of the L_c phase from within the SGII phase, at least in their protocol at 2.3°C.

Earlier data of Ruocco and Shipley (1982b) also provide support for the assumption that the formation of the L_c phase takes place from within the SGII phase. According to their report, the $L_{\beta'} \rightarrow L_c$ structural transformation proceeds in two time domains: a rapid first step involving no change in the lamellar repeat period and change of the characteristic $L_{\beta'}$ wide-angle reflections into two well separated reflections at 0.43 and 0.40 nm; and further slower alterations to the final L_c phase starting after 1.5–2 h at −2°C. The result of the first step has been observed in their x-ray pattern collected after 40-min incubation at −2°C. It is close to the pattern of the SGII phase found to form readily upon cooling in the present work. These authors, however, could not detect a calorimetric transition upon immediate reheating.

In the present study, the formation of a metastable subgel intermediate — the SGII phase — has been found to take place at ~7°C upon cooling. According to our earlier experience, the subtransition does not appear in DPPC samples cooled to 8–10°C and stored at this temperature for more than two months. This observation is in support of the analysis of the L_c phase formation kinetics of Yang and Nagle (1988), where a conclusion has been drawn that nucleation does not occur above 7°C, i.e., the L_c phase does not form above this temperature without preceding cooling to lower temperature. Thus, the formation of SGII phase appears to be an intermediate event preceding the formation of L_c phase in a sequence $L_{\beta'} \rightarrow$ SGII $\rightarrow L_c$ (Fig. 5). This hypothesis is supported also by the lack of sub-subtransition in DL-DPPC, i.e., in the absence of an apparent SGII phase in the racemic lipid, the formation of L_c phase is suppressed.

The main argument supporting the distinction of SGII and $L_{\beta'}$ as different phases stems from the cooperativity of the interconversion between them. As illustrated in Fig. 3, the changes in structural parameters involved in this conversion take place in the temperature range 5–12°C upon heating and 10–3°C upon cooling. No significant changes in the phase structures are observed out of these temperature ranges for either SGII or $L_{\beta'}$ phase. The peaks in the heat capacity curves observed calorimetrically provide independent proof for a cooperative SGII $\rightarrow L_{\beta'}$ transition. Finally, the existence of a limiting temperature for the nucleation of the L_c phase as concluded by Yang and Nagle (1988) also documents the existence of a sharp boundary at ~7°C between the two different phase structures — $L_{\beta'}$ and SGII.

There are at least two substantial differences between the metastable subgel SGII phase, identified in this report, and other reported metastable subgel phases in DPPC, obtained using various temperature protocols (Silvius et al., 1985; Tristram-Nagle et al., 1987; Lewis and McElhaney, 1990). First, although the latter metastable subgel phases have been found to form with low-temperature incubation times of the order of days, the rapid SGII-$L_{\beta'}$ conversion proceeds reversibly, with a relaxation time of 15 s. Second, the formation of these metastable phases is strongly pathway-dependent, i.e., they could be eliminated using proper temperature protocols, as recently demonstrated by Tristram-Nagle et al. (1994), whereas the SGII phase appears to be obligatory and to persist for ~3 h after cooling to temperatures below 7°C.

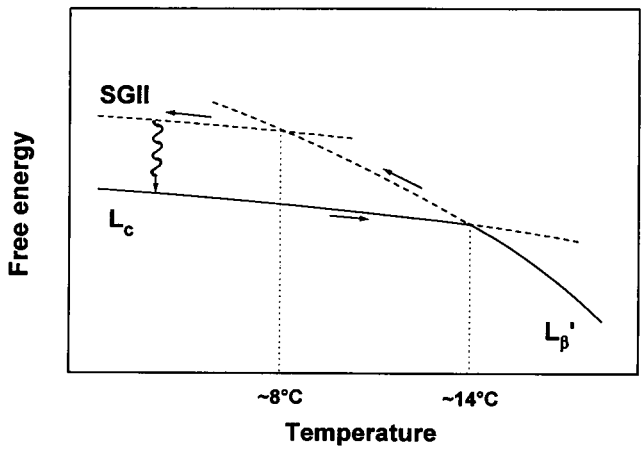

FIGURE 5 Schematic free energy versus temperature plot for hydrated DPPC at low temperatures illustrating the different transition pathways for the L_c-$L_{\beta'}$ transformation in cooling ($L_{\beta'}$-SGII-L_c) and heating (L_c-$L_{\beta'}$) directions. Solid lines indicate stable phases, and dashed lines indicate metastable phases.

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