SCANNING CALORIMETRY REVEALS A NEW PHASE TRANSITION IN L-α-DIPALMITOYLPHOSPHATIDYLCHOLINE

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ABSTRACT We report a new phase transition in fully hydrated dispersions of dipalmitoylphosphatidylcholine (DPPC). This new transition, called the sub-subtransition, exhibits a transition enthalpy of 0.25 kcal/mol with a T_m at 6.8°C. Unlike the subtransition, no extended low temperature incubation is required to observe the sub-subtransition. This new sub-subgel (SGII) phase may be a precursor to the subgel (SGI) phase, and this discovery is discussed in relation to the current knowledge regarding the polymorphic gel phases of both ester- and ether-linked lipids with identical acyl chains.

INTRODUCTION

L-α-Dipalmitoylphosphatidylcholine (DPPC) is probably the most well characterized phospholipid used in model lipid bilayer studies. Fully hydrated DPPC dispersions exhibit multiple endothermic phase transitions revealed by various physical techniques, both as the system temperature (Chapman et al., 1967; Tardieu et al., 1973; Tilcock, 1986) and pressure (Chong and Weber, 1983; Wong and Mantsch, 1983; Wong, 1984, 1986) is varied. Additional novel phases with acyl chain interdigitation occur when small amphiphilic molecules or thiocyanate ions are added to dispersions of saturated diacylphosphatidylcholines with identical acyl chains (McIntosh et al., 1983; Cunningham and Lis, 1986). Similarly, acyl chain interdigitation may be induced under conditions of high hydrostatic pressure (Braganza and Worcester, 1986).

DPPC, a phosphatidylcholine with two identical saturated acyl chains, undergoes three phase transitions above 0°C at ambient pressure (Chen et al., 1980; Füldner, 1981). The lowest of these three previously reported thermal transitions, the subtransition, involves a conversion from the subgel phase (SGI) to the metastable lamellar gel (GII) state. This subtransition may appear as two broad overlapping transitions, but these represent a single process (Wu et al., 1985; Yang et al., 1987). The SGI phase requires an extended incubation of the GII phase at low temperatures for its formation (Chen et al., 1980; Fuldner, 1981; Ruocco and Shipley, 1982; Finegold and Singer, 1984).

The nature of the structural changes involved in forming the SGI phase from the GII phase has been investigated at the molecular level by a number of physical studies (Ruocco and Shipley, 1982; Wong and Mantsch, 1983; Mushayakarara et al., 1986). The results are consistant with a conversion of the acyl chain packing from hexagonally disordered packing in the GII phase to a more ordered orthorhombic (Ruocco and Shipley, 1982) or triclinic packing (Mushyakarara et al., 1986) in the SGI phase. Low angle x-ray diffraction suggests that these changes are also accompanied by a dehydration in the polar headgroup region (Ruocco and Shipley, 1982).

Binary mixtures of phosphatidylcholines differing in chain length by a single methylene unit exhibit a much slower rate of SGI phase formation than what is observed in the pure components (Finegold and Singer, 1984), even though ideal mixing is expected in both the gel and liquid crystalline phase. Similarly, binary mixtures containing a small amount of the opposite headgroup stereoisomer (5% D-DPPC) or cholesterol are unable to form the SGI phase (Boyanov et al., 1986). Clearly, the behavior of the SGI phase in mixtures emphasizes that its formation is sensitive to the ability of the system to attain long range order in both the acyl chain region and the polar headgroup/interface region.

Our new transition, in contrast to the subtransition, appears immediately upon cooling the GII phase, is reversible, and appears at temperatures below all reported

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occurrences of the subtransition. This new SGII phase precedes the appearance of the SGI phase; furthermore, we believe that this phase is metastable with respect to the SGI phase, and acts as an intermediate state during the formation of the SGI phase.

METHODS

L-α-Dipalmitoylphosphatidylcholine (DPPC, 99+%) was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL) and Sigma Chemical Co. (St. Louis, MO). The dry lipid power was dissolved in benzene and lyophilized. Multilamellar dispersions were prepared by dispersing the dried, weighed lipid (15 mg/ml) into solution, heating the sample to 50°C for at least 30 min to ensure hydration, and intermittently vortexing the solution. This was followed by several cycles of heating, vortexing, and cooling to 0°C to ensure complete hydration of the lipid dispersion before use. High resolution differential scanning calorimetry was performed using a Microcal MC-2 equipped with the DA-2 digital interface and data acquisition utility (Microcal, Inc., Amherst, MA). Because the reproducibility in high resolution differential scanning calorimetry (DSC) may be limited by the accuracy of sample filling (Krishnan and Brandts, 1978), the low temperature incubation time dependence of SGII and SGI phase formation was determined by repetitive runs on a single sample, and also rechecked by observations on independent samples. In all experiments, samples were cooled in the calorimeter cell from a common temperature (50°C) in order to eliminate systematic differences in incubation times resulting from the finite thermal equilibration time of the calorimeter. Equilibration was complete in 75 min.

The thermotropic phase behavior of high purity phosphatidylethanolamines differ depending on the manufacturer (Finegold et al., 1985). We therefore compared DPPC from two independent sources and observed no difference. The transition enthalpy and transition midpoints were determined as previously described (Xu and Huang, 1987).

RESULTS

Calorimetric scans for samples incubated at 0.5 ± 0.1°C are shown Fig. 1 as a function of the variable low temperature incubation period. The magnitude of the transition enthalpy determined as a function of this low temperature incubation period reveals the rate of interconversion between two slowly equilibrating phases.

All samples exhibit the expected pretransition and main-phase transition (data not shown). Baseline scans performed on water and buffer reveal no transitions at much higher magnification than the scale shown in Fig. 1, indicating that the observed sub-subtransition is not an instrument baseline artifact.

The sub-subtransition at 6.8°C appears immediately upon cooling, exhibiting a transition enthalpy of 0.25 kcal/mol (Fig. 1 *A*). Initially, there is no evidence for a subtransition at higher temperatures. Increasing the low temperature incubation time results in the appearance of a distinct subtransition peak in addition to this sub-subtransition (Fig. 1, *B–D*). Although the sub-subtransition enthalpy and transition midpoint both appear to increase slightly at longer incubation times, this results from a small additive contribution by the broad subtransition. At ~8.5 h incubation time, the sub-subtransition appears to merge with the subtransition (Fig. 1 *D*). Our results suggest that the formation of the SGI phase is preceded by an immediately reversible SGII phase which undergoes a thermal transition at temperatures below the subtransition.

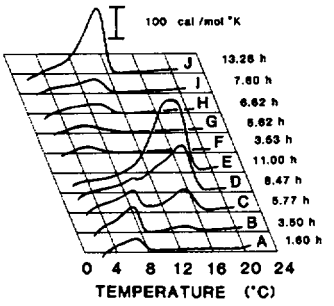

FIGURE 1 Differential scanning calorimetry of the sub-subtransition and subtransition in DPPC. A series of thermograms are shown as a function of the incubation time at low temperature (0.5 ± 0.1°C) before starting the scan. Thermograms *A–E* represent samples of DPPC in water. Thermograms *F–J* represent DPPC samples in buffer containing 25 mM phosphate, 50 mM KCl, 100 mg/ml ethanol. Incubation times are as follows: (*A*) 1.60 h, (*B*) 3.50 h, (*C*) 5.77 h, (*D*) 8.47 h, (*E*) 11.00 h, (*F*) 3.53 h, (*G*) 5.62 h, (*H*) 6.62 h, (*I*) 7.60 h, (*J*) 13.28 h. These represent the elapsed time from the start of cooling until the initiation of the scan, and include a thermal equilibration time of ~75 min.

The thermotropic behavior which we observe is independent of whether the sample is prepared in water, phosphate buffer (25 mM potassium phosphate, pH 7.4), or phosphate buffer with monovalent salt (25 mM potassium phosphate, 200 mM KCl, pH 7.4). In addition, both the sub-subtransition and the subtransition occur in the presence of 100 mg/ml ethanol (Fig. 1, *F–J*). Ethanol decreases both transition temperatures, and appears to decrease the sub-subtransition enthalpy. However, ethanol does not appear to change the limiting value of the subtransition measured for long (>2 wk) incubation periods.

DISCUSSION

The SGII phase that we report may have previously escaped notice for at least two reasons. The small transition enthalpy is unresolved from the baseline if the thermogram is examined on the same scale with which the other transitions are observed, and could easily be dismissed as erratic baseline behavior. Furthermore, the SGI phase is usually detected only after a low temperature incubation period, while the SGII phase can be observed calorimetrically within a limited time window. Perhaps a combination of these factors precluded the observation of the SGII phase in other studies of the SGI phase. As noted above, the SGII phase appears immediately upon cooling the GII phase. This SGII phase may then undergo further structural changes upon extended low temperature incubation, giving rise to the SGI phase. A phenomenological description models the formation of the SGI phase in terms of nucleation followed by two-dimensional propagation (Nagle and Wilkinson, 1982). The interpretation of the experimentally observed temperature dependence of the rate of SGI phase formation by this model requires that below a certain temperature, the growth rate is exceedingly slow; above a certain temperature, nucleation is rate limiting. In either situation, the SGI phase does not

appear. It is conceivable that either the nucleation phenomena involves the formation of the SGII phase, or else nucleation occurs from within the SGII phase.

Provided, however, that the SGII phase is the precursor to SGI phase, the temperature dependence of the rate of SGI phase formation may be reinterpreted to be a function of the relative ratio of the SGII phase in coexistence with the GII phase at any given incubation temperature. The SGII phase is absent above 8°C; similarly, the SGI phase is absent in samples incubated at or above this temperature (Chen et al., 1980; Wong, 1986).

Additionally, the fully reversible, low enthalpy subtransition reported for ether-linked phosphatidylcholines may be analogous to the sub-subtransition which we report for ester-linked phosphatidylcholines. Recent studies comparing the phase behavior of ether- and ester-linked phosphatidylcholines report a subtransition occuring in 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (DHPC), an ether-linked analogue of DPPC (Ruocco et al., 1985). Low angle x-ray diffraction reveals that this DHPC subtransition involves a conversion from an interdigitated gel phase with hexagonal packing to an interdigitated gel phase with orthorhombic packing upon cooling below the subtransition temperature. Analogous to the new sub-subtransition we report for DPPC, this DHPC subtransition is also immediately reversible, with an order of magnitude smaller transition enthalpy compared with the corresponding DPPC subtransition. We believe the subtransition in ether-linked phosphatidylcholines is related to the sub-subtransition in ester-linked phosphatidylcholines, in light of the similar transition enthalpies and reversibility. Can the sub-subtransition occur if the acyl chains of DPPC are fully interdigitated?

At an ethanol concentration greater than that required to abolish the pretransition, GII phase DPPC bilayers exist with fully interdigitated, hexagonally packed acyl chains (Simon and McIntosh; 1984, Rowe, 1985). Although our results indicate that ethanol decreases both the sub-subtransition and the subtransition temperatures, the newly discovered SGII phase nevertheless appears in the presence of 100 mg/ml ethanol. Therefore, it appears possible for both the SGII and SGI phases to form in fully interdigitated gel phase DPPC bilayers.

Although our calorimetry results do not offer a molecular interpretation of the structural changes involved in SGII phase formation, we can offer a plausible explanation consistent with much of the available data concerning the SGI phase. A likely possibility is that the structural change upon cooling the GII phase involves a reorientation of the acyl chains from hexagonally disordered packing to orthorhombic packing. Such a sequential change in acyl chain packing has previously been suggested in order to explain the complex temperature dependent phase behavior of the various phases of DPPC (Wong, 1986). Furthermore, previous x-ray studies (Ruocco and Shipley, 1982) characterizing the formation of the SGI phase note that there are significant changes in the positions of the wide-angle reflections which occur before the onset of the more gradual changes in bilayer periodicity accompanying the formation of the SGI phase. These rapid changes in hydrocarbon chain packing appear to occur on a timescale consistent with our calorimetric evidence for the existence of this additional SGII phase.

In summary, we believe that the SGII phase forms immediately upon cooling the GII phase. The sub-subtransition represents the conversion of the SGII phase back into the GII phase. Upon low temperature incubation, the SGII phase spontaneously undergoes further transformation to form the SGI phase. The immediate formation of the SGII phase upon cooling the GII phase may occur regardless of the state of acyl chain interdigitation.

This investigation was supported by U.S. Public Health Service grant GM-17452 from the National Institutes of General Medical Sciences, National Institutes of Health, Department of Health, and Human Services.

Received for publication 7 April 1987 and in final form 26 June 1987.

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