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Kinetics of subgel formation in DPPC: X-ray diffraction proves nucleation-growth hypothesis

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Abstract

Wide-angle and low-angle X-ray diffraction data were obtained during the time course of the gel to subgel phase transformation in fully hydrated DPPC. When the system was kept close to equilibrium by following a *T*-jump protocol, the X-ray data unequivocally demonstrate the coexistence of growing subgel and shrinking gel domains. When the system was supercooled and held further from equilibrium as in previous studies, the kinetic behavior was more complicated. These data prove that the basic mechanism for the gel to subgel phase transformation is one of nucleation of subgel domains followed by growth of the domains.

Key words: Lipid bilayer; Subgel transition; Phase transformation kinetics; X-ray diffraction; Phosphatidylcholine

1. Introduction

The subgel phase in phosphatidylcholines has been much studied [1–5] since its discovery in 1980 by Chen et al. [6]. This phase of fully hydrated multilamellar bilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), also called the C phase, has a smaller volume [7], lower enthalpy [6], and more X-ray diffraction reflections [4,8–11] than the gel phase. The subgel phase in DPPC forms from fully hydrated gel phase bilayers by lowering the temperature below approximately 7°C and waiting for periods of days or weeks [7]. Upon melting the subgel to the gel phase, conventional differential scanning calorimetry obtains a transition temperature (T_S) in excess of 17° [1–3,6,7] and transition temperatures as high as 24°C have been cited in the literature [12]. However, as was shown by dilatometry performed very close to equilibrium [1,7] and by adiabatic calorimetry [13], the true equilibrium transition temperature is in the range 13.5–14.0°C. Furthermore, after the subgel phase begins to form at temperatures less than 7°C and the temperature is then jumped to between 7 and 13°C, the transformation from gel to subgel continues [7,14], thereby proving that the gel phase is merely metastable below T_S and is not the thermodynamically stable phase. The failure to observe subgel formation when the gel phase is first cooled to between 7°C and 13°C is consistent with the classical theory of homogeneous nucleation [15]. According to this theory an excessively long time is required near T_S for a free energy fluctuation large enough to nucleate a critical domain with large enough radius so that it does not spontaneously collapse due to the boundary tension of the domain wall. After nucleation below 7°C, however, a supercritical subgel domain continues to grow at the expense of the surrounding gel phase. This suggests that the gel to subgel phase transformation can be thought of according to the classical picture consisting of the two steps, (i) nucleation of subgel domains followed by (ii) growth of the domains. Such a picture demands that there be transient coexistence of gel and subgel phases while the kinetic phase transformation takes place.

The classical nucleation-growth picture of the subgel phase transformation described above is flatly contradicted by all the reported X-ray data for subgel formation in DPPC [10,16,17]. Instead of a superposition of

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Abbreviation: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine.

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gel phase reflections and subgel phase reflections, with the former gradually disappearing with time as the latter become stronger, the Bragg spacing of each gel phase reflection shifted continuously towards the Bragg spacing of a subgel phase reflection, while new subgel reflections also appeared. In particular, the low-angle reflections were consistent with a continuous decrease in the lamellar repeat spacing and the wide-angle interchain peak at 4.25 Å appeared to shift continuously into the subgel peak at 4.44 Å. It has been suggested that a new kind of phase transformation takes place and the names 'sequential relaxation' [18] and 'lenient cooperativity' [17] have been proposed, although no very clear idea of a possible mechanism has emerged.

In this paper, we report X-ray diffraction data that are strikingly different from those reported previously. The basic change in these experiments was to employ a more appropriate thermal protocol for forming the subgel phase. Since the subgel forms rather slowly, it has often been considered convenient to lower the temperature to near 0°C because the initial transformation then proceeds more rapidly than in the range 4–7°C. However, we earlier showed that after 300 h the volume decrease when incubated at 4.9°C actually catches up with and surpasses the volume decrease when incubated at 0.5°C [1]. Also, subgel formed at the higher incubation temperatures gave cleaner, single peaked DSC endotherms upon melting [1]. Finally, when incubated at temperatures near 0°C, the kinetics of the volume versus time data, although monotonic, are reproducibly very complicated, suggesting a complex sequence of dominant kinetic regimes, that one has little hope of understanding [1,14]. In contrast, when the subgel phase was first initiated by cooling to 4°C for a few hours and then jumped to temperatures above 7°C, the volume versus time data were much simpler and could be fitted to the classical Kolmogorov-Avrami theory over the whole range [14], with the only unconventional aspect being the need for a small effective dimensionality. The interpretation we advanced for those results [1,14] was that the deep supercooling produces many nuclei which can only grow a little before impacting upon other domains. Such impacts would form defective domain walls because the individual domains are mutually misoriented. This would be an unfavorable protocol for forming the best subgel phase because the domain walls would be regions of disorder relative to a well formed subgel phase. Furthermore, they would anneal out only very slowly and would give rise to an extra, and complicated, kinetic process.

All the preceding kinetic X-ray diffraction data were obtained while incubating between −4°C and 4°C [4,8,9,10,11,16,17]. According to the analysis in the preceding paragraph, it is not surprising that the diffraction kinetics are not consistent with the conventional nucleation-growth hypothesis. The crucial experiment to test this hypothesis is to follow a thermal protocol that initiates only a small number of nuclei by incubating for a short time at a temperature below 7°C followed by a long incubation above 7°C. Under these circumstances, the classical nucleation-growth hypothesis requires coexistence of large domains of subgel in the gel phase during the phase transformation. Nonobservance of phase coexistence would invalidate the nucleation-growth hypothesis. Observance of phase coexistence would confirm the basic nucleation-growth hypothesis when the system is closest to equilibrium.

2. Materials and methods

Sample preparation

DPPC was purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. The high purity of this lipid was confirmed by thin-layer chromatography using chloroform/methanol/water (60:30:5, v/v) as the solvent system. Multilamellar vesicles (MLVs) were prepared by adding 50 mg of lyophilized DPPC (Lot No. 160PC-183) to 143 mg of deionized distilled water, and cycling the dispersion three times between 70°C and 5°C with 5 min of vortexing at each temperature. Thin-walled 1.0 mm glass capillaries (Charles Supper) were cleaned by sequentially washing with a chromic acid bath, deionized water, acetone and finally copious amounts of deionized water. After drying with nitrogen the capillaries were flame sealed at one end. The MLV dispersion was then loaded into the capillaries using a 1.0 ml Hamilton syringe. In order to remove air bubbles the capillaries were centrifuged for 10 min at $1100 \times g$ at room temperature. After centrifugation the capillaries were flame-sealed above the water layer, and this seal was dipped in Duco cement. Upon completion of the diffraction experiments, it was confirmed that the samples had remained fully hydrated by observing the presence of the excess water layer above the opaque lipid dispersion.

X-ray diffraction

The X-ray source was an Elliot rotating anode Type GX21, typically operated at 5.3 kW. After each X-ray exposure the sample was vertically translated by 1 mm relative to the beam. This ensured that X-ray data were obtained from sample that had not been previously exposed to radiation.

Film data. The X-ray beam was passed through a nickel filter to select CuK_{α} radiation and was pinhole collimated (two 0.3 mm holes 6.5 cm apart), and the patterns were recorded on Kodak DEF5 X-ray film (Charles Supper, Natick, MA) at a distance of 73.0 mm from the sample. The temperature was controlled to

±1°C using a YSI Model 72 Proportional Temperature Controller (Yellow Springs, OH) connected to a quartz heater. This heater and a finned coil cooled by a Model KR60A compressor (Polysciences, Niles, IL) were located within the insulated 2 × 2 × 2 ft radiation shield surrounding the sample and camera. Four small fans circulated air inside the chamber. Three insulated TFD probes (Omega Engineering, Stamford, CT) which had been calibrated to an NIST thermometer (Taylor Instrument, Rochester, NY) monitored the temperature at different sites within the chamber. The sample was mounted in an aluminum holder with mylar windows and cooled through the air. Temperature gradients across the sample were estimated to be negligible since the entire radiation chamber was cooled. Each X-ray exposure took 2–3 h.

PSD data. The samples were mounted in an Air Products Model DE-201 Cryostat connected to an Air Products Model HC-2 Compressor (Allentown, PA). The temperature was controlled to ±0.02°C with a Lake Shore DRC-91C Controller using a platinum resistance thermometer. An air-filled beryllium chamber thermally isolated the sample from the room. A graphite monochromator selected CuK_α radiation (λ = 1.5418 Å) and defined a beam with angular resolution of half-width at half-maximum $\delta(2\theta)$ (HWHM) = 0.05° in the horizontal direction. The slits before the sample were set to ±0.25 mm in the horizontal direction and ±0.5 mm in the vertical direction. A Braun (Munich, Germany) position sensitive detector (PSD) with an effective linear resolution of 0.2 mm was placed horizontally 57.35 cm from the sample. Evacuated flight paths contained the main beam and the scattered X-rays. Each set of exposures at low and wide-angle took 4 h; then the sample was translated as above.

Temperature-jump protocol

Samples were first cooled to a temperature T_J between 7°C and 13°C and the X-ray pattern of the typical gel phase was obtained. At time $t = 0$ the samples were then quenched to a temperature T_Q below 7°C (typically about 4°C) and held at T_Q for 7–10 h. Then, the temperature was jumped back up to T_J and held there for subsequent X-ray exposure.

3. Results

Fig. 1 shows the X-ray patterns recorded on film. Fig. 1A shows the typical pattern for $L_{\beta'}$ gel phase lipid. The low-angle reflections correspond to orders h = 3–5 of lamellar repeat and the wide-angle reflections at Bragg spacings of d_{20} = 4.25 Å and d_{11} = 4.06 Å are more separated at 10°C than they are at room temperature. After quenching to T_Q = 4°C for 7 h and T-jumping to T_J = 10°C, the phase transformation into

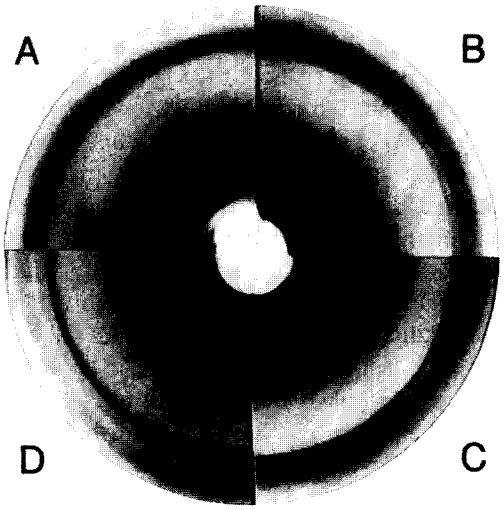

Fig. 1. Film data. $L_{\beta'}$ gel phase DPPC at 10°C (A), followed by T-jump data with T_Q = 4°C for 7 h and T_J = 10°C with time elapsed after quenching: 25 h (B), 48 h (C), 5 weeks (D).

the subgel proceeded as shown in the other quadrants of Fig. 1. By 25 h after quenching, the main subgel reflection at 4.46 Å is already visible in Fig. 1B. After 48 h, Fig. 1C shows that the main subgel reflection at 4.46 Å is nearly equal in intensity to the gel phase (20) reflection and two new subgel rings are visible at 6.81 Å and 10.2 Å. Although it is not so apparent in Fig. 1C, the lamellar h = 3 and 4 orders are broader, consistent with two sets of low-angle reflections, as will be seen more clearly when we turn to our higher resolution PSD data. Fig. 1D was obtained 5 weeks after quenching and is typical of a well-formed subgel phase with many sharp reflections.

Fig. 2 shows T-jump data taken with the PSD in the wide-angle region. Curve A shows the sharp (20) gel phase peak at scattering angle 2θ = 20.92° corresponding to a Bragg d-spacing of 4.25 Å. The [11] peak is lower and broader and appears at higher angles than shown. Subsequent to quenching and T-jumping, the intensity of the (20) peak gradually decreases with negligible shift in peak position. Simultaneously, a subgel peak proceeds to grow at scattering angle 19.9° corresponding to a d-spacing of 4.46 Å and there is negligible shift in the position of this peak with time.

Fig. 3 shows selected low-angle data for the same sample as in Fig. 2. The range for the scattering angle was chosen to contain the h = 4 and h = 5 lamellar reflections because the h = 5 subgel reflection is nearly extinct. Curve A shows the gel phase h = 4 order at 2θ = 5.59° and the h = 5 order at 2θ = 6.98°, both corresponding to a lamellar repeat distance of 63.3 ± 0.1 Å. Subsequent to quenching and T-jumping, the intensity of the h = 5 order gradually decreases with negligible shift in peak position. Simultaneously, the

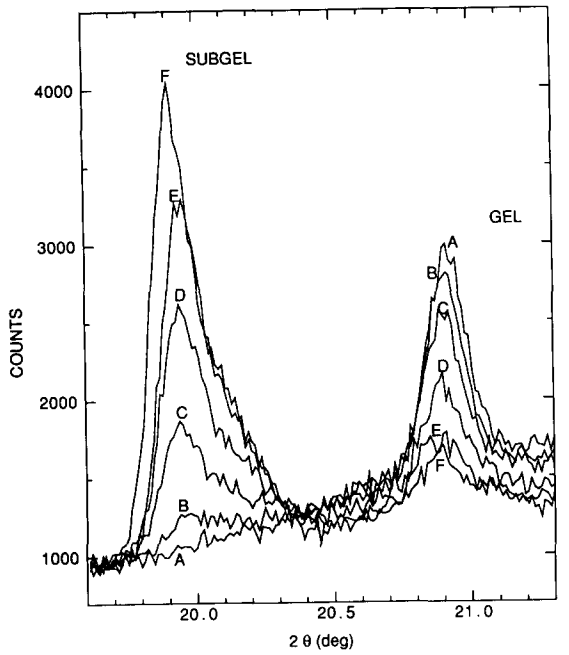

Fig. 2. Wide-angle X-ray diffraction peaks during subgel formation at $T_J = 10.3$°C following nucleation at $T_Q = 4.3$°C for 10 h. Time elapsed was 0 h (A), 20 h (B), 43 h (C), 93 h (D), 141 h (E), 189 h (F). Between E and F the sample was quenched to 2.3°C for an additional 19 h to nucleate any liposomes that might not have had any nuclei form during the first quench.

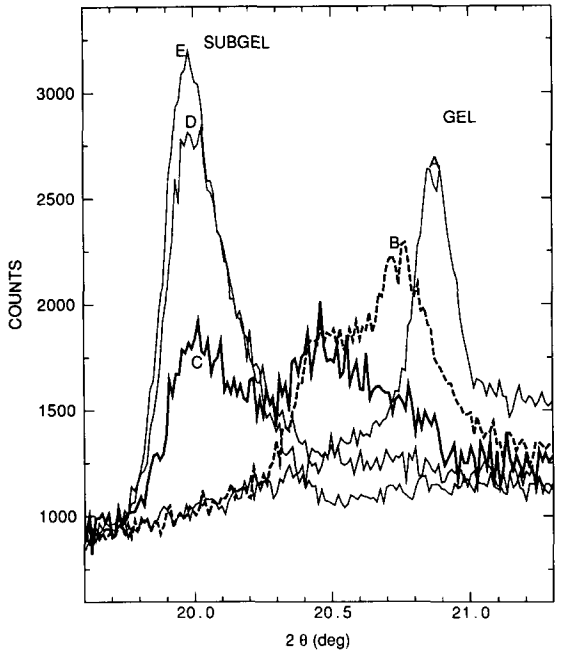

Fig. 4. Wide-angle X-ray diffraction data during subgel formation in DPPC at $T_Q = 2.3$°C with no subsequent T-jump. Time elapsed after quenching was 0 h (A), 2 h (B), 24 h (C), 46 h (D), 72 h (E). Curve B is shown with dashed lines and curve C with a bold solid line.

$h = 4$ subgel peak proceeds to grow in at scattering angle 5.95° corresponding to a d-spacing of 59.4 ± 0.1 Å while the $h = 4$ gel phase peak decreases. Even when the $h = 4$ gel and subgel peaks have nearly the same intensity, they are distinguishable as shown by curve C in Fig. 3.

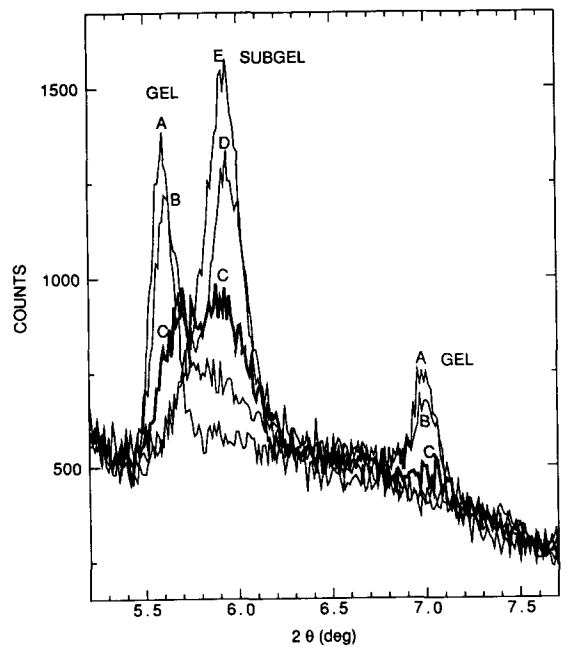

Fig. 3. Low-angle ($h = 4$ and 5) diffraction peaks for the sample in Fig. 2. Time elaspsed after quenching was 0 h (A), 23 h (B), 95 h (C), 143 h (D), 191 h (E). Scan C is shown with a bold line for clarity. Between D and E the sample was requenched as in Fig. 2.

For contrast, we also studied the subgel phase transformation following an incubation protocol closer to previous studies [10,16,17]. The temperature was simply decreased to 2.3°C and held there. Fig. 4 shows wide-angle data that are more consistent with previous data in that the (20) peak in the gel phase shown in curve A appears to shift in curve B. Unlike previous data, however, there also appears to be a new peak near scattering angle 20.46°. This new peak persists to later times as shown in curve C which also shows the subgel peak at 20.0°. Fig. 5 shows the corresponding low-angle data for the same sample. These data are consistent with previous data [10,16,17] in that the positions of the $h = 4$ and 5 orders of the lamellar repeat appear to move continuously with incubation time.

In our first film experiment using the T-jump protocol (data not shown) the sample was not translated with respect to the beam for subsequent X-ray exposures. It was observed that the subgel phase transformation proceeded much more slowly compared to our volume measurements taken under the same T-jump protocol [14] and not all the subgel reflections were visible even after 9 days. The capillary was then translated to expose previously unirradiated sample with strikingly different results. The subgel transformation

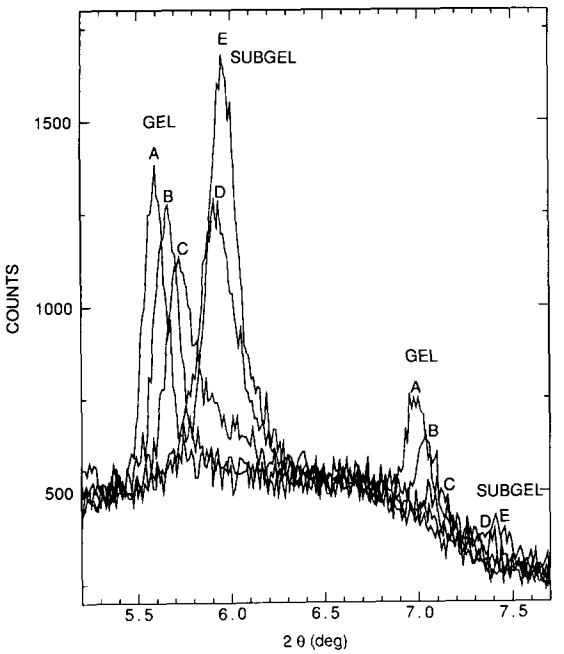

Fig. 5. Low-angle ($h = 4$ and 5) diffraction peaks for the sample in Fig. 4. Time elapsed after quenching was 0 h (A), 4 h (B), 25 h (C), 49 h (D), 74 h (E).

was virtually complete with many subgel reflections (as in Fig. 1D) in the unexposed part of the same sample. All subsequent X-ray patterns were consequently taken by translating the sample to expose fresh sample after 2–4 h irradiation.

4. Discussion

The X-ray data in Figs. 1–3 clearly demonstrate two-phase coexistence of the gel and subgel phases during the gel to subgel phase transformation using the *T*-jump protocol described herein. This definitively proves that the classical nucleation-growth hypothesis is valid. It also demonstrates that a kinetic regime can be found where the steps of nucleation and growth can be well separated.

It may be of interest to compare this result with results for other lipid phase transformations. Of course, by the Gibbs phase rule there can be no *equilibrium* coexistence of phases when there is excess water and pure one-component lipids except at the unique temperature of the equilibrium phase *transition*, so the issue is whether there is transient coexistence of initial and final phases during the kinetic phase *transformation*. For the $P_{\beta'}$ to L_{α} main phase transformation in DPPC Tenchov et al. [18] presented the case that there is no coexistence. For the $L_{\beta'}$ to $P_{\beta'}$ transformation in DPPC, Yang and Nagle [14] suggested that calorimetric data were consistent with coexistence for the gel to ripple phase transformation but not with the reverse ripple to gel phase transformation. This proposed irreversibility in the kinetic mechanism could be related to the formation of a metastable ripple phase upon cooling found by Tenchov et al. [18]. Our present data showing coexistence in the gel to subgel phase transformation in DPPC does not prove that such coexistence necessarily occurs in the reverse subgel to gel phase transformation. Yang and Nagle [14] have shown that calorimetric data were consistent with such coexistence, but Tenchov et al. [18] did not observe coexistence in diffraction studies. Both experiments, however, used constant heating rates that drive the system far enough from equilibrium that the apparent subgel transition temperature is raised by at least 3°C to over 17°C. Fuldner [8] observed coexistence at one temperature in the middle of the subgel to gel transition, but this could be due to equilibrium two-phase coexistence caused by impurities since no kinetics were reported. In a different lipid, phosphatidylethanolamine, coexistence has been shown using X-ray diffraction for the L_{α} to H_{II} phase transformation [19] and also for the L_{β} to L_{α} phase transformation [20].

The kinetics of the subgel phase transformation were exquisitely sensitive to radiation damage. When the sample was exposed to X-rays for 3 h per day for one week at the same position, only a rather poor subgel phase is formed as evidenced by the number and breadth of the diffraction reflections. This was initially surprising since the total irradiation dose was comparable to doses used in our gel phase studies [21] where it was verified that radiation damage did not significantly change the data. This can be understood, however, as a kinetic slowing down of the phase transformation due to the necessity to continually eject impure material from the newly formed subgel domains. This observation suggests that one might even consider using the kinetics of the subgel phase transformation as an assay for minute amounts of impurity. Nevertheless, the possibility of radiation damage was easily circumvented in this study by using a previously unexposed portion of the sample for each new measurement.

We have also confirmed in this paper that the kinetics of the gel to subgel phase transformation behave differently when further from equilibrium. When the DPPC sample was supercooled to 2.3°C the lamellar repeat distance moves continuously, in agreement with all previous experiments [10,16,17]. This latter behavior is understandable if it is assumed that there are many small domains. Then, both the gel and the subgel domains are small so most of the interlamellar space is close to both a gel domain and a subgel domain. Under these circumstances, the forces that establish the interlamellar distance would be a compromise between the gel and subgel forces, leading to a

compromise lamellar spacing throughout the entire MLV. Only when the domains are large enough to establish separate lamellar spacings would one expect to see phase coexistence in the low-angle region. These results when the sample was excessively supercooled are consistent with our colliding domain picture [1,14] as well as with the 'small-scale' coexistence discussed by Tenchov et al. [18]. However, our result contradicts the assertion [18] that failure to observe large-scale coexistence far from equilibrium precludes large-scale coexistence near equilibrium.

Calorimetric studies [1,22,23] and infrared studies [24] of DPPC incubated below 4°C yield complex kinetics that have been interpreted as initial formation of a metastable subgel intermediate C* followed by formation of the stable subgel phase C. For $T_{incubation} = 0.1$°C we [1] obtained $\tau_{G \rightarrow C^*} \approx 0.5$ days and $\tau_{C^* \rightarrow C} \approx 18$ days and these times are comparable to those that can be inferred from IR data [24] with $T_{incubation}$ in the range 0–4°C. We believe, however, that the data in Figs. 1–3, obtained using the T-jump protocol, do not involve the C* intermediate, for the following reasons. First, there is no discernable shift in the subgel X-ray spacings in Fig. 1 which include incubation times from 1 day to 5 weeks. This indicates that the T-jump protocol yields only a single subgel structure. This result is consistent with our earlier calorimetric and dilatometric data [1] that showed no metastable intermediates when higher $T_{incubation}$ was employed. Second, the X-ray spacings obtained using the T-jump protocol are the same as for a sample incubated for 14 months at 2–4°C (data not shown). These incubation conditions are generally agreed to yield the stable subgel phase. Therefore, our T-jump protocol appears to yield the stable subgel phase, at least in so far as our present X-ray data can determine.

When DPPC was supercooled to $T_{incubation} = 2.3$°C in the present study, the wide-angle X-ray data in Fig. 4 show the transient appearance of a new peak near 20.46°, suggesting that this might be the X-ray signature of the metastable C* phase. We are not certain of this interpretation, however, because this peak disappears in less than 2 days, which is rather rapid compared to $\tau_{C^* \rightarrow C}$ of about 18 days at 0.5°C [1], although $T_{incubation}$ is slightly different for the two studies. In addition, subgel formation has been shown to be faster at higher lipid concentration in an isobranched phosphatidylethanolamine [22], so direct comparison between previous calorimetry and X-ray kinetics may not be possible. Compared to our T-jump data in Fig. 2, our wide-angle data in Fig. 4 seem to be more consistent with previous X-ray data [10,16,17], although detailed comparison cannot be made because previous studies had an order of magnitude poorer resolution.

Finally, it is appropriate to compare the different protocols for forming subgel. We believe that our T-jump protocol is a better choice than the previous deep supercooling protocols, for two reasons. The first reason is that it forms more perfect subgel structure, as evidenced by the smaller volume found [1]. The second reason is that it allows for a simpler and more fundamental interpretation of the kinetics of the phase transformation, as evidenced by the ability to fit the Kolmogorov-Avrami theory to the volumetric data [14] and now by the ability to detect clean large-scale phase coexistence using X-ray diffraction. These conclusions are not surprising since the T-jump protocol keeps the system closer to equilibrium where theory is better developed and more understandable and where ordered structures grow more perfectly. Establishment of this fundamental picture provides a basis for discussing the effects of complications, such as colliding domains, that are likely to occur when further from equilibrium.

5. Acknowledgments

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