Subgel Phases of *n*-Saturated Diacylphosphatidylcholines: A Fourier-Transform Infrared Spectroscopic Study†

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ABSTRACT: The subgel phases of a homologous series of saturated straight-chain diacylphosphatidylcholines with hydrocarbon chains consisting of 10–18 carbon atoms were studied by Fourier-transform infrared spectroscopy. All of these lipids initially form a subgel phase which is spectroscopically similar to that obtained when fully hydrated multilamellar dispersions of dipalmitoylphosphatidylcholine are incubated at 0–4 °C for 2–4 days. However, further low-temperature incubation of those phosphatidylcholines with acyl chains of 16 or fewer carbon atoms results in the sequential formation of 1 or more additional, spectroscopically distinct subgel phases, with the number of such phases increasing as hydrocarbon chain length decreases. Our data indicate that the formation of all of these subgel phases involves both reorientation of the acyl chains and major changes in hydration and/or hydrogen-bonding interactions at the polar/apolar interfacial region of the lipid bilayer. We suggest that the driving force behind the formation of these L_c phases is the formation of an extended hydrogen-bonding network in the interfacial region of the bilayer and that the optimization of this network probably requires some distortion of the optimal packing of the acyl chains. As a result, an increase in acyl chain length makes the formation of these L_c phases less favorable and eventually prevents optimization of the hydrogen-bonding network at the bilayer polar/apolar interface.

Aqueous dispersions of phosphatidylcholines (PCs)[1] are perhaps the most intensively characterized of all model lipid bilayers studied. Of these, the *n*-saturated diacyl-PCs, particularly DPPC, have been thoroughly studied by a wide range of physical techniques, and as a result, most of the structural and dynamic aspects of their thermotropic phase behavior (especially the pretransition and the main gel/liquid-crystalline phase transition) are relatively well understood. However, our understanding of the gel-state polymorphism exhibited by these compounds remains incomplete. In particular, the so-called subtransition first characterized by Chen et al. (1980) has recently been shown to be considerably more complex than was originally thought (Finegold & Singer, 1984, 1986; Silvius et al., 1985; Tristram-Nagle et al., 1987; Lewis et al., 1987b). DSC studies have shown that the thermodynamic properties of the subtransition of DPPC observed after incubation of fully hydrated multilamellar vesicles at 0–4 °C for 3–4 days differ significantly from those exhibited by samples that have been incubated under the same conditions for 6–8 weeks (Silvius et al., 1985; Tristram-Nagle et al., 1987; Lewis et al., 1987b). The change in properties is manifest by significant increases in the calorimetrically determined subtransition temperature (from 18 to 22 °C) and the associated enthalpy change (from 3–4 to 5–6 kcal/mol). This and similar observations of other *n*-saturated diacyl-PCs have led us and others to the conclusion that the formation of the stable L_c phases in this homologous series of PCs is a complex multistage process which proceeds via a number of intermediates which themselves are highly ordered L_c-like structures (Finegold & Singer, 1984, 1986; Lewis et al., 1987b). However, dilatometric studies of DPPC have shown that despite the increase in calorimetric enthalpy, the subtransition temperature determined under equilibrium or near-equilibrium conditions (13 °C) does not change when the samples are incubated for prolonged periods (Tristram-Nagle et al., 1987). This observation led these authors to suggest that the observed changes in the calorimetrically determined subtransition temperature which occur upon long-term low-temperature incubation of DPPC may be a kinetic artifact. They further suggest that the increase in the calorimetrically determined enthalpy may be the result of an increase in the size of the microdomains and not the result of any significant structural change. This point of view may be consistent with some NMR spectroscopic data which show that the calorimetric changes detected were not accompanied by any changes in the ^{31}P NMR powder patterns even after incubation at 0–4 °C for at least 2 months (Lewis et al., 1984). Despite these observations, however, the basis of the calorimetric behavior remains unclear, mainly because all of the structural information on the subgel phase of DPPC and other *n*-saturated diacyl-PCs that is currently available in the literature (Fuldner, 1981; Ruocco & Shipley, 1982a,b; Cameron & Mantsch, 1982; Church et al., 1986) was obtained from studies on multibilayers that have been incubated for relatively short or undefined periods of time. Thus, it is not clear whether the structural data obtained relate to the stable L_c phase formed by these compounds or to their metastable L_c-like phase intermediates.

The chain length dependence of the gel phase polymorphism exhibited by the *n*-saturated diacyl-PCs is another aspect of their thermotropic behavior that is poorly understood at present. Recent studies have shown that the phenomenological picture of the polymorphism exhibited by the *n*-saturated diacyl-PCs is markedly dependent on chain length (Finegold & Singer, 1986; Lewis et al., 1987b) and have even suggested that the gel phase polymorphism of one of the shorter chain lipids (DLPC) may in some ways be significantly different from its longer chain homologues (Morrow & Davis, 1987). To date, the majority of the structural studies on the subgel

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[1] Abbreviations: PC, phosphatidylcholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; FT-IR, Fourier-transform infrared; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; C=O, carbonyl; CH_2, methylene.

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phases of the *n*-saturated diacyl-PCs have been done on DPPC multibilayers, and given the results of recent studies on the shorter chain homologues [see Finegold and Singer (1986), Lewis et al. (1987b), and Morrow and Davis (1987)], one cannot be certain that the data obtained from those studies would give an accurate picture of the behavior of the entire homologous series. Indeed, recent studies on glycolipid bilayers (Mannock et al., 1988, 1990; Sen et al., 1990; Lewis et al., 1990) have shown that there can be marked structural and behavioral discontinuities between the shorter and longer chain members of a homologous series of lipids. Such observations and other studies demonstrating the behavioral differences between the odd- and even-numbered members of a homologous series of lipids (Lewis & McElhaney, 1985a,b; Lewis et al., 1987a,b, 1988, 1989; Mantsch et al., 1985, 1987, 1989b; Church et al., 1986; Yang et al., 1986) clearly underscore the pitfalls inherent in the widespread practice of using a single member of a homologous series lipids as a model for all other homologues. Given this, we have reexamined the gel phase polymorphism of the homologous series *n*-saturated diacyl-PCs (chain length ranging from 10 to 18 carbon atoms) using FT-IR spectroscopy as a noninvasive method of probing the structural basis of their gel phase polymorphism. In these studies, we have also examined the changes which occur upon long-term incubation of the L_c phase of these lipids, so as to obtain some insight into the basis for the calorimetric changes that are observed.

Materials and Methods

The PCs used in this study were synthesized in this laboratory and were from the batch of highly purified materials previously used for DSC studies [see Lewis et al. (1987b)]. To prepare samples for infrared spectroscopy, 3–4-mg samples of the dry lipid were hydrated by the addition of 50 mL of D_2O followed by vigorous vortexing at temperatures near 70 °C. The hydrated samples were then squeezed between the BaF_2 windows of a heatable liquid cell to form a 25-μm film and mounted in a cell holder attached to a circulating water bath that was used to regulate the temperature. Samples were treated in situ to form their respective subgel phases by following the protocol recommended by Lewis et al. (1987b). Infrared spectra were recorded on a Digilab FTS-40 Fourier-transform infrared spectrometer using data acquisition and data processing parameters that are essentially similar to those described by Mantsch et al. (1985).

In the spectral regions of interest, the observed absorption bands are usually the result of a summation of broad overlapping components. In such cases, Fourier deconvolution was used to accurately determine the frequencies of the component bands, and curve-fitting procedures were used to obtain some information about the widths and integrated intensities of the component bands. Both procedures were done with software supplied by Digilab, Inc. Deconvolution of the spectra, using parameters which lead to a bandwidth reduction by a factor of 2, was routinely done. Under our experimental conditions, the signal to noise ratio (minimally 200) and absorption due to residual water vapor were such that the deconvolution leading to a bandwidth reduction of 2.5–2.6 could easily be accomplished without introducing any significant distortion of the spectra.

Results

In these studies, infrared spectra of the *n*-saturated diacyl-PCs were recorded as a function of temperature and of storage time under conditions previously shown to induce the formation of their subgel phases [see Lewis et al. (1987b)]. The infrared spectroscopic changes coincident with the pre-

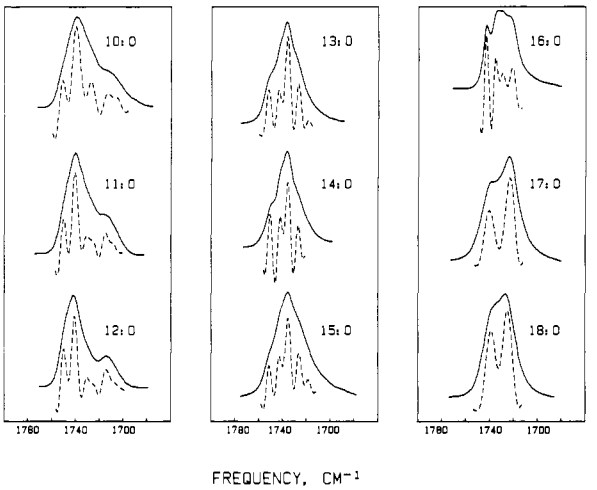

FIGURE 1: Chain length dependent changes in the C=O stretching region of the infrared spectra of the stable L_c phases of the *n*-saturated diacyl-PCs. The data are presented as absorbance spectra with the solid lines representing the normal spectra and the dashed lines representing the spectra obtained after resolution enhancement by Fourier self-deconvolution methods.

transitions and the gel/liquid phase transitions of all of these PCs are essentially similar to those previously reported for DPPC [see Mendelsohn and Mantsch (1986) and references cited therein] and will not be discussed here. Illustrated in Figure 1 is the region of the infrared spectrum which encompasses the C=O stretching vibration of the subgel phases of the diacyl-PCs used in this study. In a previous infrared spectroscopic characterization of DPPC (Cameron & Mantsch, 1982), it has been demonstrated that the structural changes coincident with the conversion of the L_β phase to the L_c phase can result in significant changes in the C=O stretching as well as the methylene deformation regions of the infrared spectrum. We also find that in addition to the changes in the C=O stretching bands, the formation of the L_c phases of these lipids is accompanied by distinctive changes in the CH_2 scissoring and the α-methylene bands. Our results also show that the spectroscopic properties of the stable L_c phases of these lipids vary significantly with increasing acyl chain length. Evidently, there is a gradation of spectroscopic properties which reflect chain length dependent changes both in acyl chain packing interactions and in the organization of the carbonyl ester interfacial regions of the respective lipid bilayers. In addition, we find that the chain length dependent structural changes in the stable L_c phases of these lipids are reflected in the spectroscopic properties of group frequencies attributable to the phosphate polar headgroup (data not shown). However, owing to the low signal to noise ratio in this region of the infrared spectrum and poor resolution from neighboring bands, it is not easy to properly characterize the latter, and those aspects will not be discussed here. With most of the lipids, there is evidence of major spectroscopic differences between the subgel phases formed and those previously reported for DPPC (Cameron & Mantsch, 1982). Indeed, the data also indicate that, with decreasing acyl chain length, the spectra become more complex and that the differences between these data and those previously reported for DPPC become more pronounced. From the data shown in Figure 1, it is also apparent that the stable L_c phases of these *n*-saturated diacyl-PCs can be conveniently assembled into four spectroscopically different groups, of different chain lengths (i.e., N = 10–12; N = 13–15; N = 16; N = 17–18). The details of the spectroscopic changes which typify the formation of the L_c phases of these groups are presented below.

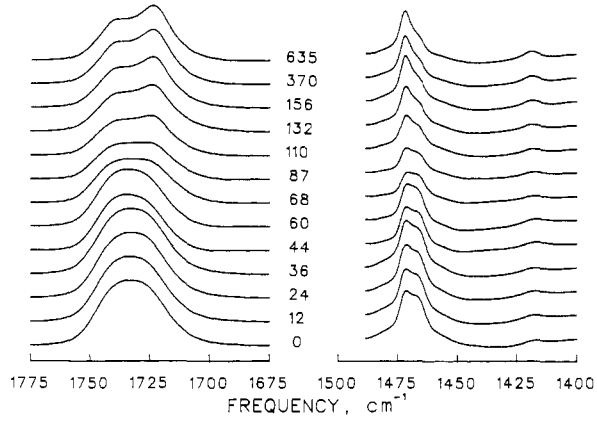

FIGURE 2: Time-dependent changes in the C=O stretching (left panel) and CH_2 deformation bands (right panel) of the infrared spectrum of diheptadecanoyl-PC as its L_β phase converts to the L_c phase at 0–4 °C. The data were acquired at the times indicated (hours) and are presented in the absorbance mode.

Longer Chain Phosphatidylcholines (N = 17, 18). Illustrated in Figure 2 are the time-dependent changes which typify the C=O stretching and methylene deformation regions of the infrared spectra of the longer chain lipids as the L_β phase converts to the L_c phase at 0–4 °C. The data indicate that the changes observed are generally similar to those previously observed with DPPC [see Cameron and Mantsch (1982)]. The L_β phase of these lipids exhibits a broad C=O stretching band contour centered at 1735 cm^{-1}, and it resolves into two components (at 1740 and 1727 cm^{-1}) after deconvolution to enhance band resolution. These bands were originally thought to arise from the stretching vibrations of the *sn*-1 and *sn*-2 ester carbonyls, respectively (Levin et al., 1982; Mushayakarara et al., 1982; Mushayakarara & Levin, 1982). However, a recent study (Blume et al., 1988) has demonstrated that the two bands each contain significant and comparable contributions from the stretching vibrations of both the *sn*-1 and *sn*-2 ester carbonyl groups. Blume et al. (1989) also provided strong evidence that with hydrated lipid bilayers such as the ones used in our studies, the two bands probably arise from populations of "free" and hydrogen-bonded ester carbonyl groups. With the formation of the L_c phase, the broad C=O ester band becomes more asymmetric, apparently as a result of an increase in the intensity of the low-frequency component. Upon resolution enhancement, it becomes clear that the observed changes are the result of a shift of the low-frequency band component from 1727 to 1724 cm^{-1}, as well as a decrease in its bandwidth. This shift to lower frequency is consistent with a strengthening of hydrogen bonding to an ester carbonyl group [see Blume et al. (1988) and Wong & Mantsch (1988) and references cited therein] and, at the very least, indicates that there is a marked change in the hydrogen-bonding interactions at the C=O ester interfacial region of the lipid bilayer. Since interfacial water is the sole source of the hydrogen donors for such hydrogen-bonding interactions, it seems logical to suggest that the change in hydrogen bonding to the C=O ester group(s) is itself a reflection of a change in interfacial hydration when the L_c phase forms. Upon further analysis of the C=O band using band-fitting procedures (not shown here), it soon became obvious that with the formation of the L_c phases of these longer chain lipids there was a considerable narrowing of both band components. This observation is consistent with a reduction of the mobility of the C=O ester groups, which in turn is compatible with the formation of the ordered crystallike structure which is generally assumed for the L_c phases of most lipid bilayers.

As was also observed with DPPC [see Cameron and Mantsch (1982)], the formation of the L_c phase of these longer chain PCs also involves significant changes in the CH_2 deformation region of the infrared spectrum. At temperatures near 0–4 °C, the CH_2 scissoring band of the L_β phase of these lipids exhibits a broad contour which resolves into two bands at 1472 and 1466 cm^{-1}, respectively. Initially, the two components are of comparable integrated intensities, but as the L_c phase forms, the integrated intensity of the low-frequency component decreases to about 25–30% of that of the high-frequency band. The two components of the CH_2 scissoring band are a reflection of the orientational inequivalence which occurs when the polymethylene chains are arranged with nonparallel zigzag planes. In infrared spectroscopic studies of solid hydrocarbons [see Casal et al. (1983) and references cited therein], this factor group splitting has been correlated with the assembly of the polymethylene chains into an orthorhombic subcell, and in such systems, the CH_2 scissoring band tends to split into components of comparable integrated intensity when the zigzag planes of the polymethylene chains are perpendicular to each other. Thus, the data suggest that, in the L_β phase from which the L_c phase is nucleated, the zigzag planes of the hydrocarbon chains are perpendicular and that the formation of the L_c phase probably involves a net reorientation of the all-trans polymethylene chains. The fact that the low-frequency band persists in the L_c phase of these longer chain PCs indicates that in the L_c phase the zigzag planes of hydrocarbon chains are not parallel.

The formation of the L_c phase of these lipids also coincided with a significant change in the contours of the α-methylene bending band near 1418 cm^{-1}. In the L_β phase, this band is weak and fairly broad. Upon formation of the L_c phase, there is a significant increase in the overall intensity of this band, and it appears as a single band at 1418 cm^{-1} even after resolution enhancement. Given the expected conformational differences between the α-methylene groups of the *sn*-1 and *sn*-2 acyl chains, it seems unlikely that the two groups would exhibit similar spectroscopic properties. Thus, we suggest that the appearance of a single α-methylene band in the L_c phase is probably the result of the sharpening of one of the α-methylene bands owing to a conformational change which results in a reduction in the mobility of one of the α-methylene groups. Thus, in addition to the hydration changes at the C=O ester interface and the reorientation of the polymethylene chains, our data also suggest that the formation of the L_c phase of these longer chain PCs also involves a conformational change in the region near the linkage between one of the acyl chains and the glycerol moiety.

Dipalmitoylphosphatidylcholine (N = 16). Figure 3 illustrates the changes which typify the C=O stretching the CH_2 deformation regions of the infrared spectrum DPPC during the transformation of its L_β phase to its stable L_c phase. It is clear that the early stages of this process are phenomenologically similar to those involved in the formation of the L_c phases of the di-17:0 and di-18:0 compounds and are essentially identical with the infrared spectroscopic studies reported previously (Cameron & Mantsch, 1982). Thus, it is logical to conclude that the L_c phase formed upon short-term incubation of DPPC multibilayers at 0–4 °C is structurally similar to those of the longer chain homologues. However, it is also clear that the initial events are eventually superseded by more drastic events which, in these studies, are manifest by changes in the C=O stretching bands, the principal methylene deformation modes of the main chain methylene bands near 1468 cm^{-1}, and the α-methylene bands at 1418

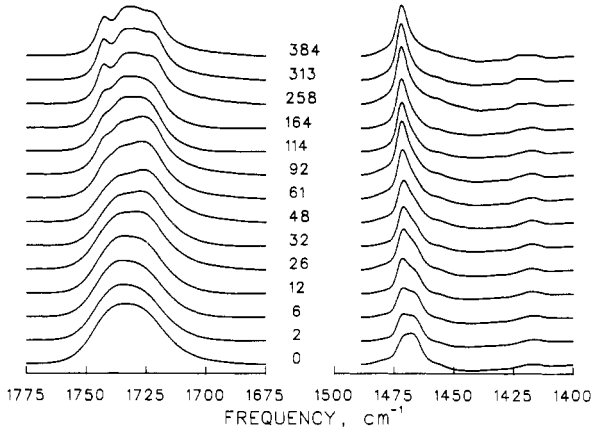

FIGURE 3: Time-dependent changes in the C=O stretching (left panel) and CH_2 deformation bands (right panel) of the infrared spectrum of DPPC as its L_β phase converts to the L_c phase at 0–4 °C. The data were acquired at the times indicated (hours) and are presented in the absorbance mode.

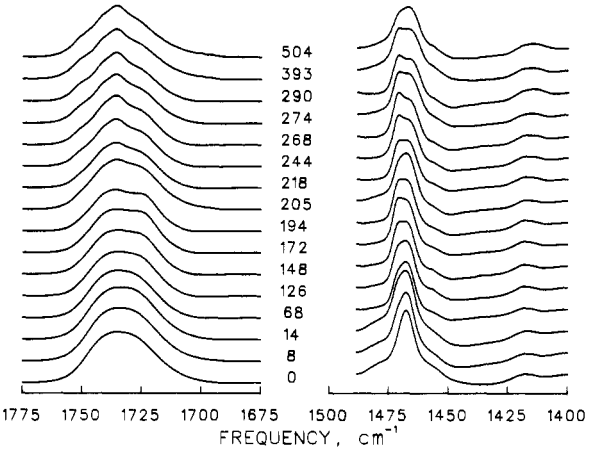

FIGURE 4: Time-dependent changes in the C=O stretching (left panel) and CH_2 deformation bands (right panel) of dipentadecanoyl-PC as its L_β phase converts to the L_c phase at −8 °C. The data were acquired at the times indicated (hours) and are presented in the absorbance mode.

cm^{-1}. These spectroscopic changes clearly indicate that the L_c phase formed after a few days incubation at 0–4 °C is a metastable phase, which is structurally different from the more stable L_c phase formed by long-term incubation under the same conditions. Figure 3 shows that upon long-term incubation at 0–4 °C, the low-frequency component of the CH_2 scissoring band (1466 cm^{-1}) gets progressively weaker with time and eventually disappears leaving a single band at 1472 cm^{-1}. This indicates that changes in the hydrocarbon chain packing continue during this phase eventually resulting in the reorganization of the hydrocarbon chains into a different subcell. The data indicate that the frequency of the CH_2 band is in the range expected of hydrocarbon chains packed in a triclinic subcell with the zigzag planes of the polymethylene chains parallel to each other (Snyder, 1961). Figure 3 also shows that significant changes in the region near the linkage between the acyl chains and the glycerol moiety also occur upon long-term incubation of DPPC under the given experimental conditions. We find that the single α-methylene band at 1417 cm^{-1}, which is evident after a few days incubation, is eventually replaced by two sharper bands near 1424 and 1416 cm^{-1}. These two bands could possibly arise as a result of the conformational inequivalence of the α-methylene groups on the *sn*-1 and *sn*-2 acyl chains of the lipid molecule. Their appearance as relatively sharp bands at this stage in the process suggests that a conformational and/or hydration change resulting in a reduction of the mobility of these groups occurs when the metastable L_c phase converts to the more stable form.

The conversion of the metastable L_c phase of DPPC to its final stable form is also accompanied by some interesting spectroscopic changes in the C=O stretching region of the infrared spectrum. In particular, the data indicate that the bands at 1738 and 1725 cm^{-1} which were resolved by deconvolution of the C=O band contour of the metastable L_c phase each appear to split to produce bands at 1743, 1735, 1728, and 1721 cm^{-1} (see Figure 3) when the stable L_c phase eventually forms. These four bands are relatively sharp, indicating that there is a marked reduction in the mobility of the C=O ester groups as would be expected when the lipid "crystallizes" into a stable form. We expect that the complex pattern of bands is a reflection of major changes in the hydrogen-bonding interactions between the solvent and the interfacial C=O ester groups of the lipid probably as a result of the formation of crystalline hydrates of this lipid. Interestingly, the frequencies of all of the bands resolved are lower than the 1748 cm^{-1} value recorded for neat anhydrous triacetin [see Mushayakarara et al. (1986)]. Given that C=O ester stretching frequencies near 1750 cm^{-1} have been correlated with non-hydrogen-bonded ester carbonyl groups (Mushayakarara et al., 1986), our observations may be indicating that in the stable L_c phases of DPPC, both the *sn*-1 and *sn*-2 ester carbonyls are involved in hydrogen-bonding interactions with the solvent phase.

Medium-Chain Phosphatidylcholines (*N* = *13*, *14*, *15*). The spectroscopic changes which characterize the formation of the L_c phases of these medium chain PCs are illustrated in Figure 4. The data clearly show that the process involves major structural reorganizations of both the hydrophobic and the polar/apolar interfacial domains of the these lipids. The initial stages of the process (see spectra $t < 190$ h in Figure 4) are evidently similar to those observed during the early phases of the formation of the L_c phases of DPPC and the longer chain homologues. As was the case with their longer chain homologues, there is factor group splitting of the CH_2 scissoring band into components near 1466 and 1472 cm^{-1}, and as the L_c phase begins to form, there is a progressive increase in the intensity of the high-frequency band relative to the low-frequency band. This similarity between these compounds and their longer chain homologues is more clearly seen in the C=O stretching region of the spectrum where, during the early of stages of L_c phase formation (Figure 4, spectra $t < 190$ h), there is a sharpening and growth in the relative intensity of the low-frequency component of the band contour and both bands shift to lower frequencies.

The data also show that the spectroscopic changes which occur during the later stages of the "crystallization" of these lipids are radically different from those which occur with DPPC and the longer chain homologues. During the early stages of L_c phase formation, there is a progressive decrease in the relative intensity of the low-frequency component (at 1466 cm^{-1}) of the CH_2 scissoring band contour. This trend is arrested and soon reversed as the stable L_c phase forms, and during the later phases, the CH_2 scissoring band contour resolves into components of comparable integrated intensity at 1466 and 1472 cm^{-1}. Thus, we expect that in the stable L_c phase of these lipids the polymethylene chains are oriented with their zigzag planes perpendicular (or nearly so) to each other. This is similar to that observed when the L_β phases of these lipids are cooled to temperatures well below those of their respective pretransitions. Subtle structural differences between the stable L_c phases of these medium-chain compounds and

their longer chain homologues are also apparent from the a-methylene bending bands near 1418 cm^{-1}. The formation of the stable L_c phase of these medium-chain lipids coincides with the appearance of two α-methylene bands at 1420 and 1413 cm^{-1}. Their appearance as sharper bands during the later stages of L_c phase formation is indicative of the reduction in the mobility of the linkages between the acyl chains and the glycerol moiety, as would be expected when the lipid forms a highly ordered crystalline structure. The fact that the frequencies of these bands clearly differ from those of the stable L_c phases of DPPC and the other longer chain homologues probably reflects subtle differences in the conformation of their respective α-methylene groups, possibly as a result of hydration or even conformational changes in the polar/apolar interfacial region of the respective lipid bilayers.

The spectroscopic changes which occur in the C=O stretching region of the infrared spectra of these medium-chain lipids provide further evidence that their stable L_c phases are structurally different from those of any of their longer chain homologues. During the early stages of L_c phase formation, the structural changes are spectroscopically similar to those which occur with the long-chain homologues (see Figure 4, spectra $t < 190$ h). We expect that these early events are phenomenologically similar to the formation of the metastable L_c phase formed by DPPC, since the spectroscopic changes observed initially coiincide with the occurrence of the metastable subgel phases that were observed in previous DSC studies of these lipids (Lewis et al., 1987b). As was the case with the longer chain homologues, their C=O stretching bands were initially resolvable into two components which narrowed and shifted toward lower frequencies as the metastable L_c phase formed. However, with the conversion to the stable L_c phase, there was a progressive buildup of spectral intensity between 1735 and 1755 cm^{-1} (Figure 4, spectra $t > 190$ h). The observed spectroscopic changes were shown by deconvolution (see Figure 1) to be the result of the growth of fairly narrow bands at 1750, 1742, and 1735 cm^{-1} coupled with an increase in the frequency (from 1725 to 1728 cm^{-1}) of the low-frequency component of the C=O stretching band contour. The narrowness of the bands is consistent with the formation of a crystallike structure in which the mobility of the ester carbonyls has been considerably reduced. The appearance of the band at 1750 cm^{-1} is also significant since its frequency is similar to that of neat anhydrous triacetin (Mushayakarara et al., 1986), for which the ester carbonyls are known not to be hydrogen bonded. Thus, our observations suggest that there exists a population of non-hydrogen-bonded ester carbonyls in the stable L_c phases of these medium-chain PCs. We further suggest that the latter may be the result of significant dehydration of the polar/apolar interfacial regions of the lipid bilayer.

Short-Chain Phosphatidylcholines ($N = 10, 11, 12$). With the short-chain members of this homologous series of PCs, the initial stages of the formation of their L_c phases occur too quickly for the time course to be characterized. Nevertheless, from the data shown in Figure 5, it is clear that these lipids readily form an L_c phase which is spectroscopically indistinguishable from the stable L_c phases of the medium-chain homologues. However, it is also clear that continued incubation of these short-chain lipids at low temperatures results in further spectroscopic changes in both the carbonyl stretching and the methylene deformation regions of the infrared spectrum. Evidently, for these short-chain homologues, the L_c phase which is analogous to the stable L_c phase formed by the medium-chain homologues is itself metastable, and with time

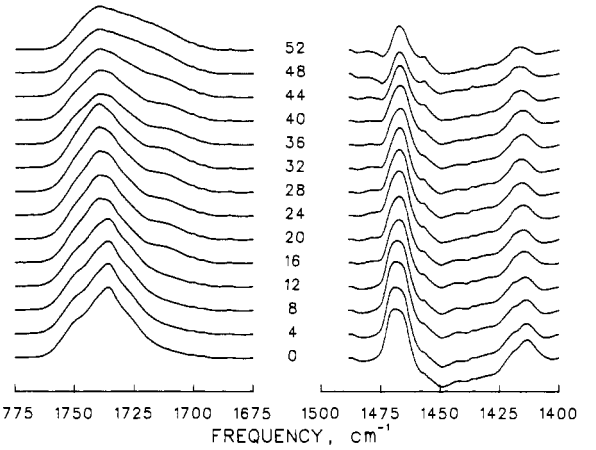

FIGURE 5: Time-dependent changes in the C=O stretching (left panel) and CH_2 deformation (right panel) bands of diundecanoyl-PC as its intermediate L_c phase converts to its stable L_c phase at −10 °C after initial nucleation at −20 °C. The data were acquired at the times indicated (minutes) and are presented in the absorbance mode.

converts to an even more stable L_c phase for which the hydrocarbon chain packing and the structure of the polar/apolar interface are radically different. In the C=O stretching region of the spectrum, the appearance of this stable phase coincides with the formation of a very broad C=O ester band at 1714 cm^{-1} and a slight decrease (from 1750 to 1748 cm^{-1}) in the frequency of the "non-hydrogen-bonded" ester carbonyls. These observations are consistent with further strengthening of hydrogen-bonding interactions involving the ester carbonyl groups of these lipids, which in turn suggests that a radical change in either hydration and/or hydrogen-bonding interactions involving the ester carbonyls has occurred. Most likely the changes in hydration and/or hydrogen-bonding interactions at the polar/apolar interface are closely linked to the spectroscopic changes in the α-methylene bending bands near 1418 cm^{-1}. In the metastable L_c phase of these short-chain PCs, the bending vibration of the α-methylene groups gives rise to a band contour which resolves into components which near 1420 and 1414 cm^{-1}. With the conversion to the stable L_c phase, a prominent band contour composed of narrow band components at 1417 and 1414 cm^{-1} is observed. Evidently, the formation of the stable L_c phase of these lipids involves further conformational changes in the linkages between the acyl chains and the glycerol backbone.

In the methylene scissoring region of the infrared spectrum, we also find that the formation of the stable L_c phase of these short-chain PCs result in the collapse of the factor group splitting of the CH_2 scissoring band originally present in the metastable L_c phase with the result that a single band at 1468 cm^{-1} is observed (Figure 5). Evidently, the conversion from the intermediate L_c phase of these short-chain lipids to their final stable states involves considerable distortion of acyl chain packing. Interestingly, this change in the properties of the CH_2 scissoring band is remarkably similar to that which occurs when the metastable L_c phase of short-chain β-D-glucosyldiacylglycerols converts to a very stable crystallike phase (Lewis et al., 1990). We expect that in a system containing all-trans polymethylene chains, a CH_2 scissoring frequency near 1468 cm^{-1} would typify orientationally disordered chains such as is found in the rotator phases of solid hydrocarbons (Casal et al., 1983), or the $L_{\beta'}$-type gel phases of lipid bilayers [see Mendelsohn and Mantsch (1986) and references cited therein]. With these short-chain PCs, it is unlikely that the acyl chains adopt such a loose packing in the stable L_c phase, since the bands in the methylene deformation and so-called fingerprint

Table I: Characteristic Infrared Absorption Bands (cm^{-1}) of the Gel and Subgel Phases of the Saturated Straight-Chain Diacylphosphatidylcholines

phase	N	C=O stretching	CH_2 scissoring	α-CH_2 bending
L_β	10–18	1735 (1740, 1728)[b]	1468[a]	1418
L_c1[c]	10–18	1738, 1723–1725	1471, 1466	1418
L_c1'[d]	16, 17–18	1738, 1723–1725	1471, 1466	1418
L_c2[e]	10–15	1750, 1742, 1735, 1726	1471, 1466	1420, 1413
L_c3	10–12	1748, 1740, 1724, 1714	1468	1417, 1414
L_c4	16	1743, 1735, 1728, 1721	1472	1424, 1417

[a] This band usually splits into components at 1471 and 1466 cm^{-1} when cooled to temperatures well below the gel/liquid-crystalline phase transition temperature. [b] The values in parentheses are the frequencies of the bands resolved after Fourier deconvolution. [c] The subgel phase that is initially formed by all of these lipids. [d] The stable phase formed by the longer chain homologues. This phase is spectroscopically similar to the L_c1 phase but is listed separately here because calorimetric studies have indicated that its thermodynamic properties differ from those of the L_c1 phase. [e] The stable phase formed by the medium-chain lipids. It is also transiently formed by the short-chain homologues en route to the formation of their stable gel phase.

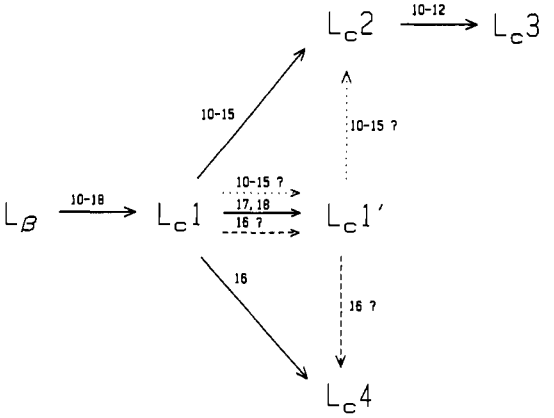

FIGURE 6: Block diagram outlining the processes involved in the formation of the stable L_c phases of the *n*-saturated diacyl-PCs. The solid arrows indicate the conversions observed, and the dashed and dotted arrows indicate possible alternative pathways. The acyl chain lengths of the PCs which follow the various paths are indicated by the numbers alongside the arrows.

region of the infrared spectrum are very sharp (not shown here). Thus, the acyl chains are probably in a rigid crystallike structure in which the acyl chains are orientationally disordered and either hexagonally packed or arranged in a complex hybrid subcell. These observations thus suggest that the formation of the stable L_c phases of these short chain PCs may even involve some weakening of strong interchain interactions which give rise to the factor group splitting of the CH_2 scissoring bands that is observed in the intermediate L_c phase.

Discussion

As was expected from previous studies, the incubation of the L_β gel phases of these saturated, straight-chain diacyl PCs at temperatures well below those of their respective gel/liquid-crystalline phase transitions results in the nucleation and growth of the so-called subgel (L_c) phase. In these studies, we have found that all members of this homologous series of lipids form a structurally similar type of L_c phase during the initial stages of that process. The type of L_c phase observed initially is exemplified by the subgel phase formed by short-term (2–4 days) incubation of DPPC at 0–4 °C and has been thoroughly characterized by a number of physical techniques [see Fuldner (1981), Ruocco and Shipley (1982a,b), Cameron and Mantsch (1982), Church et al. (1986), and Tristram-Nagle et al. (1987) and references cited therein]. This work and previous infrared spectroscopic studies (Cameron & Mantsch, 1982) suggest the type of L_c phase formed initially is nucleated from an L_β phase in which the acyl chains are arranged with their zigzag planes normal to each other. The type of packing seems to occur spontaneously when acyl chain motions are damped by cooling to temperatures well below that of the gel/liquid-crystalline phase transition. The data also suggest that during the formation of the initial L_c phase the acyl chain packing and the hydration of the C=O ester interfacial region of the lipid bilayer are altered. Form our results and previous work on DPPC (Cameron & Mantsch, 1982), it is clear that the acyl chains of the initial L_c phase are not parallel, and we can suggest that during the formation of this phase there may be either a reduction of the angle between the zigzag planes and/or changes in the orientation of the polymethylene chains within the unit cell. However, it is also clear that prolonged incubation of these lipids under conditions conducive to the nucleation and growth of the L_c phase results in further spectroscopic changes which are strongly chain length dependent. The major diagnostic features of the L_β phase and the four spectroscopically distinct subgel phases of these PCs are listed in Table I, and a block diagram outlining the probable course of events leading to their formation from the L_β phase is shown in Figure 6. With the longer chain compounds, we find that the subgel phases that they form initially (designated L_c1 in Table I and Figure 6) are spectroscopically similar to the most stable subgel phase we have observed so far. At this time, we are uncertain as to whether there are any structural differences between the "two subgel phases" of these longer chain homologues, but since calorimetric studies have indicated that their thermodynamic properties are different (Finegold & Singer, 1984, 1986; Silvius et al., 1985; Tristram-Nagle et al., 1987; Lewis et al., 1987b), we have designated the stable subgel phases of the long-chain homologues as L_c1' (see Table I and Figure 6) until this issue can be unambiguously settled.

The demonstration that changes in acyl chain length result in marked spectroscopic differences between the types of stable L_c phases formed by these lipids clearly indicates that all members of this homologous series do not spontaneously adopt structurally similar crystalline forms. At the present time, the only available single-crystal X-ray structure of a PC is that of DMPC reported by Pearson and Pascher (1979). This X-ray structure is often used to explain many aspects of the structure and properties of lipid bilayers. Our observations suggest that the crystal structure of DMPC may not be even typical of the saturated diacyl-PCs and as a result greater care should be exercised when that "model" is used in the interpretation of the phenomenology of lipid bilayers. These studies have also suggested that the subgel phase of DPPC may be structurally unique. Ironically, the bulk of the structural information currently available on the subgel phases of the saturated diacyl-PCs has come from studies on DPPC. This observation vividly underscores the pitfalls inherent in the widespread practice of using any single lipid as a representative model for other homologues.

The finding that the stable L_c phase of DPPC apparently has spectroscopic characteristics that are unlike that of any of the other members of this homologous series of PCs is surprising. This finding suggests that the stable L_c phase of DPPC may have unique structural features. However, at this time, this cannot be unambiguously established on account of the very slow rates with which the transformations involving the L_c phases of its longer chain homologues take place [see Lewis et al. (1987b)]. In this study, we found that the spectroscopic properties of most stable L_c phases formed by

the longer chain homologues are not too dissimilar from those exhibited by the L_c phases upon short-term incubation of these PCs. Thus, it is possible that the longer chain homologues may, with time, eventually transform to structures that are spectroscopically similar to that exhibited by DPPC. It should be noted, however, that we did not find any evidence that the longer chain homologues were undergoing structural transformations similar to those exhibited by DPPC (or indeed of any other homologue) on the time scale of 3–4 months, and it has been shown previously that the calorimetric characteristics of the subgel phases of diheptadecanoyl- and dioctadecanoyl-PCs change over that time scale (Finegold & Singer, 1984, 1986). Thus, it is also possible that the stable L_c phase of DPPC may indeed be structurally unique. Inevitably, this aspect of the chain length dependence of the gel phase polymorphism of these diacyl-PCs will remain uncertain until some method is found to accelerate the rate at which the longer chain homologues transform to their final stable states.

The demonstration that the L_c phase initially formed by DPPC is spectroscopically distinct from that obtained upon prolonged annealing has helped to clarify some of the uncertainties concerning the calorimetric properties of the subgel phases that it forms upon short- and long-term incubation at low temperatures. Our results clearly indicate that the L_c phase formed upon short-term low-temperature incubation of DPPC is a metastable structure, which upon prolonged incubation converts to a more stable L_c phase that is structurally different from that formed initially. Thus, the basis of the calorimetric data reported [see Silvius et al. (1985), Tristram-Nagle et al. (1987), and Lewis et al. (1987b)] is a bona fide structural change and not a kinetic artifact as suggested by Tristram-Nagle et al. (1987). At this time, details of the structure of the stable L_c phase are lacking mainly because the bulk of the data published so far has been obtained from samples which we presume have been annealed at low temperatures for relatively short periods. However, from our data, we can suggest that the stable L_c phase formed by this particular lipid may be a very highly ordered structure in which zigzag planes of the hydrocarbon chains are parallel, and in which the interfacial ester carbonyl groups are considerably more immobile and probably less hydrated that is the case with the initial L_c phase formed. Moreover, the appearance of four distinct carbonyl ester bands in the infrared spectrum may also indicate that the lipid has crystallized into a structure in which there are at least two inequivalent molecules in the unit cell. However, it is apparent that our understanding of the structural aspects of the stable L_c phase of DPPC is still, at best, rudimentary and that a careful reinvestigation of the physical aspects of the subgel phases of this molecule is in order. Moreover, it is also clear that the structural basis of the marked chain length dependence of the spectroscopic properties of the stable L_c phases of this entire homologous series of supposedly simple lipids is in need of further investigation.

The fact that changing the acyl chain length alters the structure and spectroscopic properties of the stable L_c phases formed by these lipids also provides some useful insights into the nature of the competing interactions driving the assembly of amphipathic lipid molecules into ordered crystallike forms. An important observation here is that there appears to be a marked decrease in the complexity of the hydrogen-bonding interactions around the C=O ester in the interfacial region of the lipid bilayer as the chain length increases. Furthermore, at shorter chain lengths, the formation of strong hydrogen-bonding interactions at the ester interface seems to coincide with a weakening of lateral interactions between the hydrocarbon chains. In spite of this, the calorimetric data on the L_c phases of the shorter chain compounds indicate that the energy required to disrupt these structures is quite large and can approach 18 kcal/mol [see Lewis et al. (1987b)]. However, with these short-chain lipids, only a small fraction of the total enthalpy change can be attributed to a pure chain-melting phenomena comparable to the L_β/L_α phase transition evident at longer chain lengths. Thus, it would appear that with the short-chain lipids it is the optimization of such polar interactions in the headgroup and interfacial regions of the lipid bilayer which make the major contribution to the free energy of the system and thus determine the type of crystalline structure which can be formed. The infrared spectroscopic data presented here even suggest that the gain in free energy from optimization of such interactions may be sufficiently great as to occur at the expense of optimal hydrocarbon chain packing if the acyl chains are sufficiently short. From the above arguments, it is also apparent that it is very unlikely that polar interactions in the headgroup and interfacial regions of the lipid bilayer can continue to dominate the physical properties of these (or any other) lipid bilayers as the length of the hydrocarbon chains increases. Since in any lipid bilayer, the optimization of such polar interactions is obviously opposed by the hydrophobic interactions, the increase in the energy of the interchain van der Waals interactions coincident with an increase in acyl chain length would inevitably shift the control of the physical properties away from the polar interactions at the bilayer surface and toward those which dominate the hydrophobic region. We suggest that it is this chain length dependent change in the balance between the polar and hydrophobic contributions to the free energy of the lipid bilayer which is responsible for the fact that the types of crystallike structures that are spontaneously formed by these lipids, under comparable conditions, differ so markedly with acyl chain length. Moreover, we also suggest that for all amphipathic lipids the formation of any subgellike phase may well have a minimal requirement for at least a partial optimization of polar interactions at the bilayer surface. Although to our knowledge this possibility has not been addressed in the literature, it may also help to explain why the formation of ordered crystallike phases in many of the phospho- and glycolipid lipid bilayers studied so far [see Lewis and McElhaney (1985a), Lewis et al. (1987a,b, 1990), and Mannock et al. (1988, 1990)] gets progressively less favorable as acyl chain length increases. We suggest that such chain length dependent phenomena arise naturally from the amphipathic properties of phospholipid molecules such as these and are probably responsible for most of the chain length dependent properties of these and other lipid bilayers.

Registry No. Didecanoyl-PC, 13699-47-3; diundecanoyl-PC, 24675-16-9; DLPC, 18285-71-7; ditridecanoyl-PC, 75340-69-1; DMPC, 13699-48-4; dipentadecanoyl-PC, 67896-63-3; DPPC, 2644-64-6; diheptadecanoyl-PC, 67896-64-4; dioctadecanoyl-PC, 4539-70-2.

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Fidelity in the Aminoacylation of $tRNA^{Val}$ with Hydroxy Analogues of Valine, Leucine, and Isoleucine by Valyl-tRNA Synthetases from *Saccharomyces cerevisiae* and *Escherichia coli*

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ABSTRACT: Several analogues of valine, leucine, and isoleucine carrying hydroxyl groups in the γ- or δ-position have been tested in the aminoacylation of tRNA by valyl-tRNA synthetases from *Saccharomyces cerevisiae* and *Escherichia coli*. Results of the ATP/PP_i exchange and of the aminoacylation reactions indicate that the amino acid analogues not only can form the aminoacyl adenylate intermediate but are also transferred to tRNA. However, the fact that the reaction consumes an excess of ATP indicates that the misactivated amino acid analogue is hydrolytically removed. Thus, valyl-tRNA synthetase from *S. cerevisiae* shows a high fidelity in forming valyl-tRNA. Although the much bulkier amino acid analogues allo- and iso-γ-hydroxyvaline and allo- and iso-γ-hydroxyisoleucine are initially charged to tRNA, the misaminoacylated $tRNA^{Val}$ is enzymatically deacylated. This cleavage reaction is mediated by the hydroxyl groups of the amino acid analogues which are converted into the corresponding lactones.

Protein biosynthesis is remarkably error free; about one mistake is made in 3000 amino acids incorporated (Loftfield, 1963). A crucial step in this process is the aminoacylation of transfer RNA (tRNA) with the cognate amino acid, a reaction catalyzed by the aminoacyl-tRNA synthetases. This reaction can be divided into two separable steps: (i) the activation of the amino acid by ATP to an enzyme-bound aminoacyl adenylate (activation step) and (ii) the transfer of the aminoacyl residue to the cognate tRNA (transfer step).

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