IR Spectroscopic Determination of Gel State Miscibility in Long-Chain Phosphatidylcholine Mixtures

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ABSTRACT We report on the gel-state microaggregation in binary mixtures of diacylphosphatidylcholines over temperatures ranging from −19°C to near the gel-to-liquid crystal transition. Microaggregates with lateral dimensions in the range 1–100 chains were detected and measured with an isotope infrared method that relates the splitting or the shape of the methylene scissors band to aggregate size. Measurements were made on fully hydrated dispersions of $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$, $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$, and $diC_{18}{}^{D}PC/diC_{24}{}^{H}PC$ at molar ratios of 4:1. Low levels of aggregation were determined with reference to the spectrum of the random mixture $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$. For $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$ at −19°C, which previous calorimetric measurements have indicated is a nearly ideal, we found about 4% of the minority component chains to be involved in aggregates. For $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$, the value increased to about 11%. $DiC_{18}{}^{D}PC/diC_{24}{}^{H}PC$ was found to be highly fractionated, in agreement with the earlier studies. The unit subcell, which defines the type of acyl-chain packing, was determined for the components of the mixtures. The temperature behavior of the phases and the temperatures at which the minority component domains undergo dissolution were determined.

INTRODUCTION

Biologically important properties of cell membranes are affected in many ways by lipid aggregation. For example, nonrandom lipid mixing affects water permeability (Carruthers and Melchior, 1983), the tendency to form nonbilayer phases (Tate et al., 1991), lateral diffusion of membrane proteins (Metcalf et al., 1986), and the function of membrane-bound enzymes (Watts and DePont, 1985, 1986). The detection and measurement of phase separation and microdomain formation in phospholipid membranes is an important and challenging problem, made especially difficult by the wide range of time and distance scales involved. A plethora of experimental and theoretical methods have been designed to determine and account for the extent and nature of aggregation. Calorimetry, a pioneering method (Mabrey and Sturtevant, 1976), measures the degree of nonideal behavior in binary phospholipid mixtures, although it provides relatively little specific information about the structure of the aggregates. Fluorescence microscopy is used to study the simultaneous coexistence of ordered and disordered phospholipid phases in monolayers (Nag et al., 1991) and in bulk phases (Parasassi et al., 1994). Its limiting resolution is about 0.5 μm. Fluorescence recovery after photobleaching (Almeida et al., 1992) provides information about domain connectivity (percolation) in gel-liquid crystal coexistence regions, and electron spin resonance (Sankaram et al., 1992) provides information about nanoscale structure. Electron microscopy (Hui and Yu, 1993) can be used to characterize ordered domains of dimensions larger than about 0.1 μm. Compositional fluctuations over distances greater than about 100 Å can be measured with neutron-scattering techniques (Knoll et al., 1981). Thus, it still remains difficult to characterize aggregates whose dimensions are smaller than 100 Å, although indirect approaches have been used in which the effects of microaggregation on the bulk properties (such as specific heat) of mixed-lipid membranes are exploited (Biltonen, 1990).

Infrared (Desormeaux et al., 1992) and Raman (Mendelsohn and Maisano, 1978; Koaouci et al., 1985) spectroscopies have been used to detect phase separation in binary mixtures by capitalizing on the differences in the thermal behavior of the components. This method takes the form of monitoring the temperature response of spectral markers, such as CH_2 and CD_2 stretching band frequencies, for mixtures in which one component of the mixture is proteated and the other deuterated.

We describe here another spectroscopic approach that enables the detection and measurement of microdomains of sizes ranging from 1 to 100 chains. This method, also based on H-D isotopes and initially used to study phase separation in crystalline orthorhombic binary *n*-alkane mixtures (Snyder et al., 1992, 1993, 1994), employs the splitting or the width of the infrared methylene-scissors band to determine domain size. It is necessary that the polymethylene chains of the component of interest have an isotopic composition (perproteated or perdeuterated) different from that of the other components. Then, because the scissors frequencies of proteated and deuterated chains greatly differ, the components of the mixture can be differentiated and, for binary mixtures, monitored simultaneously. The magnitude of the splitting depends upon coupling between the scissors vibrations of nearest neighbor chains that are isotopically alike. Because the vibrational coupling is short range and occurs only between like chains, it is confined within each

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domain. Under these conditions, the magnitude of the splitting is a function of the domain size, increasing with domain size. For domains larger than about 100 chains, the scissors-band splitting asymptotically approaches its maximum value (about 11 cm^{-1} for CH_2 and about 8 cm^{-1} for CD_2). For domains consisting of single chains, the splitting is zero, so that a single, symmetric band results.

Recently we demonstrated (Snyder et al., 1995) the feasibility of extending this method to lipids, in particular to binary mixtures of alkyl methyl esters in the crystalline state at room temperature and to binary mixtures of phosphatidylcholines in the gel state at −19°. In the case of the phospholipids, we measured aggregation in the three mixtures $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$, $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$, and $diC_{18}{}^{D}PC/diC_{24}{}^{H}PC$ at molar ratios of 4:1. The major component, $diC_{18}{}^{D}PC$, which is common to all of the mixtures, has deuterated acyl chains, and the minor components consist of homologs with proteated acyl chains whose lengths are greater than the majority component. The chain-length differences between the two components are thus 2, 4, and 6 carbons. These mixtures show a wide range of component miscibility that is generally in keeping with earlier calorimetric measurements on binary diacylphosphatidylcholine mixtures consisting of components with somewhat shorter acyl chains—in the range of 12 to 18 carbons (Van Dijck et al., 1977).

In the present study we have extended our earlier measurements (Snyder et al., 1995) to include the temperature dependence of the aggregation. In addition, we have quantitatively measured aggregation for those mixtures whose components are highly miscible and have established the type of chain packing in the mixtures.

In a typical experiment, a binary mixture is first heated above T_m (the gel-to-liquid crystal transition) to ensure mixing and then cooled to near −19°C over a period extending from 1 to 1.5 h. The temperature is then increased incrementally, and the infrared spectrum of the mixture is measured at each step to monitor the phase separation and chain packing.

The degree of aggregation was estimated from the splitting or width of the methylene scissors band using procedures developed earlier for *n*-alkane mixtures (Snyder et al., 1992, 1994). The type of chain packing was determined by using structure/spectra relations established in recent infrared studies of pure long-chain phosphocholines in the gel state (Snyder et al., manuscript in preparation). The latter studies complement concomitant x-ray studies on the gel state of these same lipids (Nagle et al., manuscript in preparation).

EXPERIMENTAL PROCEDURES

Materials

The phospholipids making up the mixtures were obtained from Avanti Polar Lipids (Alabaster, AL). These were (1,2-distearoylphosphatidylcholine ($diC_{18}{}^{H}PC$), acyl chain perdeuterated distearoylphosphatidylcholine ($diC_{18}{}^{D}PC$), 1,2-diarachidoylphosphatidylcholine ($diC_{20}{}^{H}PC$), 1,2-dibehenoylphosphatidylcholine ($diC_{22}{}^{H}PC$), and 1,2-dilignoceroylphosphatidylcholine ($diC_{24}{}^{H}PC$). Their purity was greater than 99%, a value consistent with that estimated from infrared spectra and differential scanning calorimetry transition widths measured for these samples. Therefore, the samples were used without further purification.

Mixtures

Binary lipid mixtures were prepared by dissolving separately a known weight of each of the individual components in $CHCl_3$/MeOH (4:1 or 9:1, v/v) and mixing appropriate volumes of the solutions. The solvent was removed under a gentle stream of N_2 gas. Complete drying was ensured by placing the sample under vacuum (<1 torr) for more than 2 h. The sample was then hydrated by adding the appropriate amount of highly purified H_2O to achieve a water/lipid weight ratio of about 2:1 and then sealed. Complete dispersal was achieved by vigorous, repetitive, vortex action (induced using a touch-activated mixer) at a temperature above T_m. That this procedure led to complete hydration was verified by the agreement of the literature values of T_m for the dispersions of the pure components (Cevc and Marsh, 1987) with T_m determined from infrared and differential scanning calorimetry measurements on dispersions prepared by the method described.

We have used a molar concentration ratio of 4:1 for all of the mixtures. With our technique, unbalanced molar ratios have certain advantages. If aggregation occurs, the minority domains tend to be well defined, and consequently the scissors band splitting also tends to be well defined. If the level of aggregation is low, the scissors band of the minority component appears as a singlet, facilitating detection. At a 4:1 concentration ratio, the scissors band of the minority component is still sufficiently intense to provide data suitable for reduction.

Infrared spectroscopy

Samples for infrared measurements were prepared by sandwiching aqueous phospholipid dispersions between CaF_2 windows separated by a Teflon spacer of an appropriate thickness (6, 12, or 25 μM). This sandwich was fixed in a brass block, which was in turn placed in a thermostatted chamber. Temperatures were controlled to 0.1°C with a Neslab refrigerated circulation bath (water or methanol). The lowest temperature attainable was about −19°C.

The infrared spectra were measured with a Nicolet Magna 550 interferometer equipped with a cooled MCT/InSb detector. The spectra were derived from 128 sample interferograms, collected at 1 cm^{-1} resolution, co-added, Fourier transformed with one level of zero filling, and ratioed against a background interferogram.

Highly accurate band frequencies were determined by using second-derivative spectra. Band separations as small as 3 cm^{-1} could be measured for overlapping bands with half-widths (full width at half-maximum, FWHM) severalfold greater than the separation. Because each derivative operation degrades the signal-to-noise ratio by a factor of 3 to 5, it was followed by 7-point smoothing before further analysis. For this reason, each smoothing operation results in a small decrease in the apparent resolution. The smoothing and second derivative operations were carried out with software (OMNIC) supplied by the instrument manufacturer.

To determine accurate widths for the CH_2 scissors bands, it was necessary to isolate these bands from a spectral background consisting primarily of choline methyl bands. The methyl bands (near 1480 and 1490 cm^{-1}) were eliminated by subtracting from spectrum of the mixture a scaled temperature-matched $diC_{18}{}^{D}PC$ spectrum, which shows the methyl bands but not the CH_2 scissors band.

BACKGROUND STUDIES

Spectra and structure of pure phospholipid gels

The binary mixtures of interest have a common majority component, $diC_{18}{}^{D}PC$. Therefore, for reference, the spec-

trum of pure di$C_{18}{}^{D}$PC in its gel state was measured as a function of temperature. The spectra in the CD_2 scissors region and the frequencies of the scissors bands are displayed as a function of increasing temperature in Figs. 1 and 2, respectively.

Similar measurements were made for the minority component di$C_{24}{}^{H}$PC. The data for pure di$C_{24}{}^{H}$PC are displayed in Figs. 3 and 4. Measurements on pure di$C_{20}{}^{H}$PC and di$C_{22}{}^{H}$PC (not shown here) indicate a temperature behavior for the scissors band similar to that for di$C_{24}{}^{H}$PC.

There are two kinds of orthorhombic chain packing for the diacylphosphatidylcholines in their gel state at −19°C and for their binary mixtures as well. These are referred to as the distorted orthorhombic (G_d) phase and the ordered orthorhombic (G_o) phases. The G_d phase is usually associated with lipids of even-numbered acyl chains with lengths in the range of 12–16 carbons. The G_o phase, only recently observed for diacylphosphatidylcholines, has been characterized by x-ray diffraction (Nagle, manuscript in preparation) and infrared spectroscopy (Snyder, manuscript in preparation). It occurs for homologues with even-numbered acyl chains of lengths in the range of 18–24 carbons. Some phosphocholine lipids, such as diC_{18}PC, can exist in either the G_d or G_o phase at −19°C.

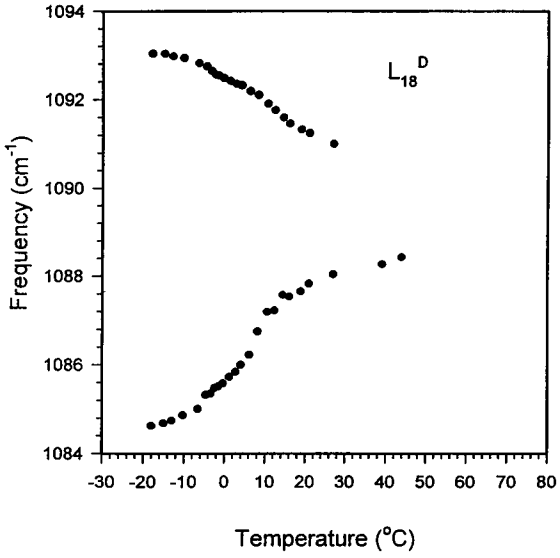

FIGURE 2 Frequencies of the CD_2 scissors bands for di$C_{18}{}^{D}$PC as a function of increasing temperature.

For a pure PC in its gel state at −19°C, the G_d and G_o phases are distinguishable on the basis of the frequency and shape of their scissors bands. For G_d, the components of the scissors doublet are separated by about 9.2 cm^{-1} for pro-

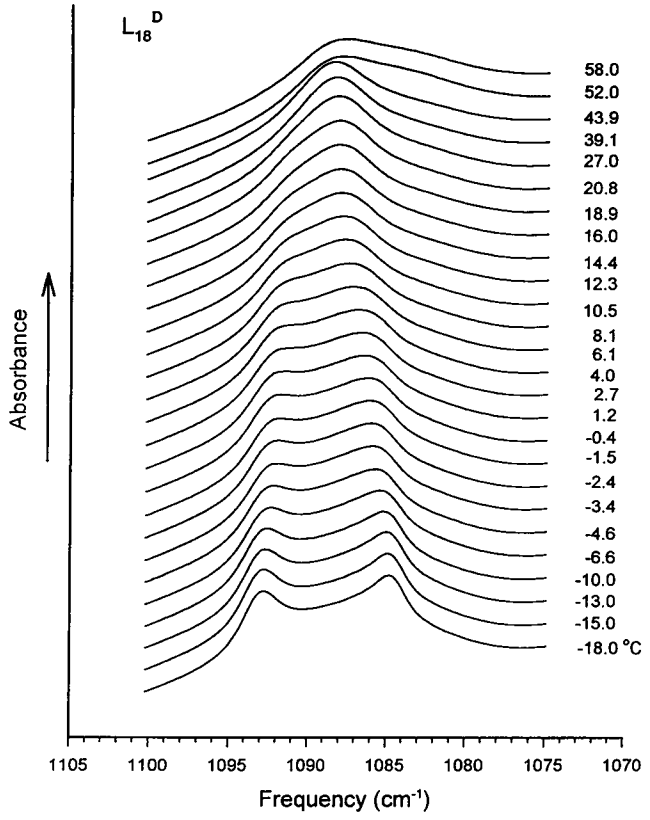

FIGURE 1 Stacked infrared absorbance spectra of the CD_2 scissors region for di$C_{18}{}^{D}$PC ($L_{18}{}^{D}$), at increasing temperatures from −18°C to 58°C. The temperature is indicated for each spectrum. (On this figure and on those that follow, the phospholipid diC_nPC will be designated as L_n.)

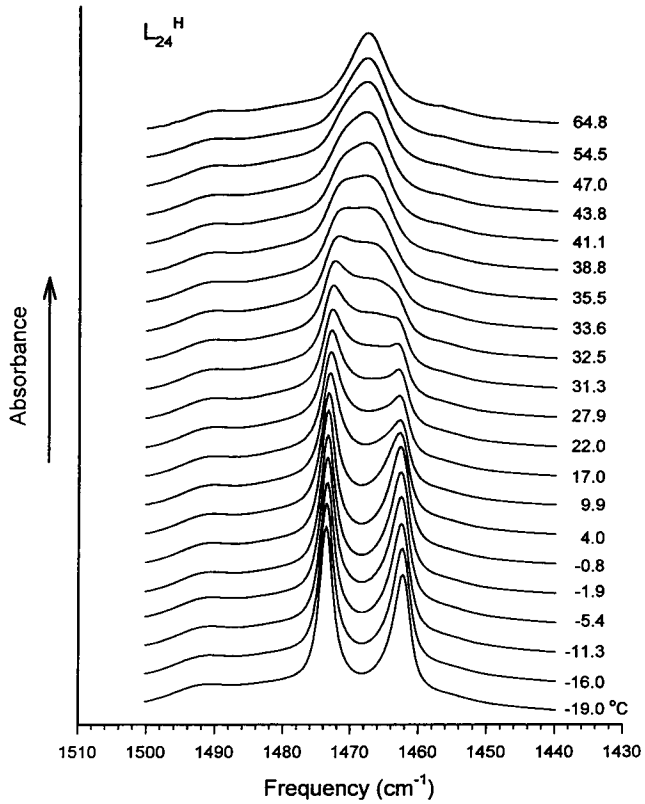

FIGURE 3 Stacked infrared absorbance spectra of the CH_2 scissors region for di$C_{24}{}^{H}$PC, at increasing temperatures from −19°C to 64.8°C. The temperature is indicated for each spectrum.

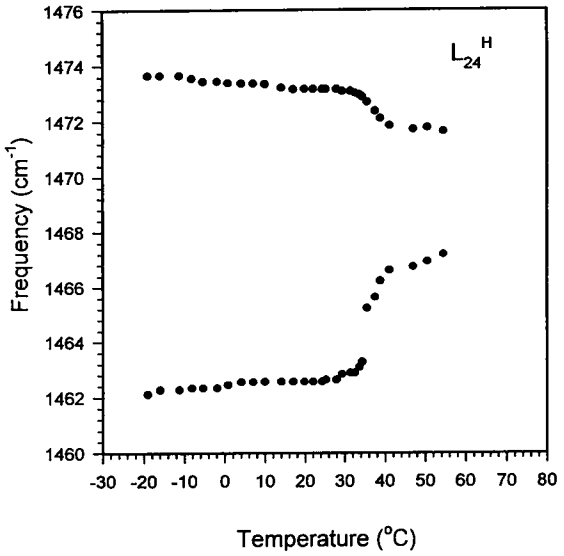

FIGURE 4 Frequencies of the CH_2 scissors bands for $diC_{24}{}^{H}PC$ as a function of increasing temperature.

teated acyl chains (6.6 cm^{-1} for deuterated chains). In addition, the shapes of the component bands differ, the higher-frequency one being much narrower. For the G_o form, the band separation is 11.6 cm^{-1} for proteated chains (8.4 cm^{-1} for deuterated chains), both components being equally narrow. The spectral differences between phases may be seen in Fig. 5, which shows the scissors bands for the ordered orthorhombic (G_o) and the distorted orthorhombic (G_d) phases for the proteated and deuterated phosphocholine lipids at −19°C.

It is important to know the type of chain packing, because the splitting depends on whether the gel is G_d or G_o and the average domain size is determined from the magnitude of the splitting. The chain packing for mixtures can be determined in the same way as for the pure lipids, that is, from the shapes of the components of the split scissors band.

The temperature behavior of the gel, as reflected by the scissors band, is complex for the pure PCs and their mixtures. The temperature behavior of the pure lipids is similar, regardless of their phase (G_d or G_o), acyl chain length, or isotope substitution. With increasing temperature, the two scissors-band components shift toward each other (Fig. 4). Beginning at −19°C (our lowest accessible temperature), there is a temperature region of slow convergence, followed, more or less discontinuously, by a region of faster convergence. The discontinuity is more pronounced for the G_o gel than for the G_d. The convergence culminates in an apparent merging of the bands. These events occur at higher temperatures as the acyl chain length is increased, suggesting a close relation between phase/structural changes and bilayer lateral expansion.

The temperature behavior of the binary mixtures tends to be similar to that for pure lipids. There are, however, additional complexities for the mixtures. These stem from aggregation, isotopic composition, and chain-length differences.

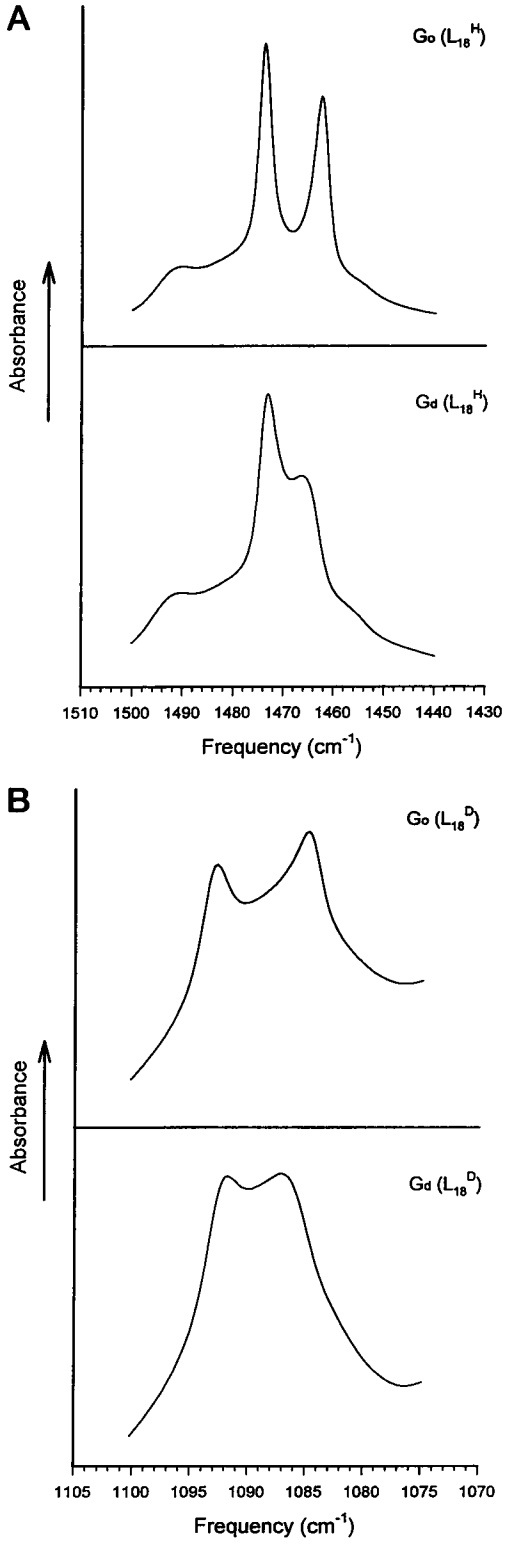

FIGURE 5 Characteristic shapes of the methylene scissors bands for the ordered orthorhombic (G_o) and the distorted orthorhombic (G_d) phases of phosphocholine lipid dispersions at −19°C. The phospholipids measured are indicated on the figure. The spectrum of $diC_{18}{}^{D}PC$ in its G_d form is not that for the pure lipid, but is that observed in its 4:1 mixture with $diC_{24}{}^{H}PC$. However, the spectrum of the undiluted lipid would be expected to be quite similar. The scissors bands for the deuterated lipids rest upon a phosphate band and this tends to distort their shapes, especially that of the lower frequency component.

$DiC_{18}DPC/DiC_{18}HPC$: a random mixture

The infrared method measures total clustering, regardless of origin. When we speak of "aggregation" in a mixture, we refer to the difference between this mixture and a random mixture in the average size or compactness of the domains. The spectrum of an equal chain-length mixture, such as $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$, therefore plays an important role as a reference because this mixture can be assumed to be randomly mixed.

We will focus first on the 4:1 $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$ random mixture at −19°C. The scissors band for the majority component ($diC_{18}{}^{D}PC$) appears as a doublet whose separation is only slightly less than that observed for pure $diC_{18}{}^{D}PC$ at the same temperature. This is understandable because the majority component is in effect a single, large domain, whether the mixture is a solid solution or is phase separated. Therefore, the magnitude of the splitting is near its maximum value and is insensitive to the degree of aggregation of the minority component.

The scissors band of the minority component for randomly mixed 4:1 $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$ at −19°C appears as a single, nearly symmetric band (Fig. 6). This band is much more sensitive to aggregation than the majority component. Its sensitivity increases as the domain size decreases. The width of this band increases rapidly with aggregation, reflecting an increase in the unresolved or nearly unresolved splitting. This is apparent in Fig. 6, where the minority bands for the 4:1 mixtures $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$ and $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$ are shown together with the same band for the random mixture. The bands appear broader as the chain-length difference increases. The dependency of the width on aggregation will be exploited below when these mixtures are considered.

We will touch briefly upon the more general problem of determining aggregation in mixtures having concentration ratios other than 4:1. For such measurements, we need the width and the splitting of the methylene scissors band of a random mixture as a function of concentration. The width is markedly concentration dependent. For example, for 4:1 $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$, its value is 5.8 cm^{-1}. In going to the 19:1 mixture, it decreases to 5.1 cm^{-1} as a result of an increase in the isolation of the proteated chains.

The concentration dependence of the scissors band splitting is indicated in Fig. 7 for the components of the $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$ mixture at −19°C. The magnitude of the splitting is dependent on phase, so we have drawn separate curves for G_o and G_d. (The mixtures studied here are mostly in the G_d phase.) In drawing the G_o curve, we were guided by the analogous curve determined earlier for the room-temperature binary *n*-alkane mixture $C_{36}{}^{D}/C_{36}{}^{H}$ (Snyder et al., 1992), which is shown in Fig. 7. The phospholipid curve for the G_o phase should resemble the *n*-alkane curve, because the chain packing in $C_{36}{}^{D}/C_{36}{}^{H}$ is orthorhombic perpendicular.

To make quantitative estimates of domain size, we need the relation between domain size and splitting. We have

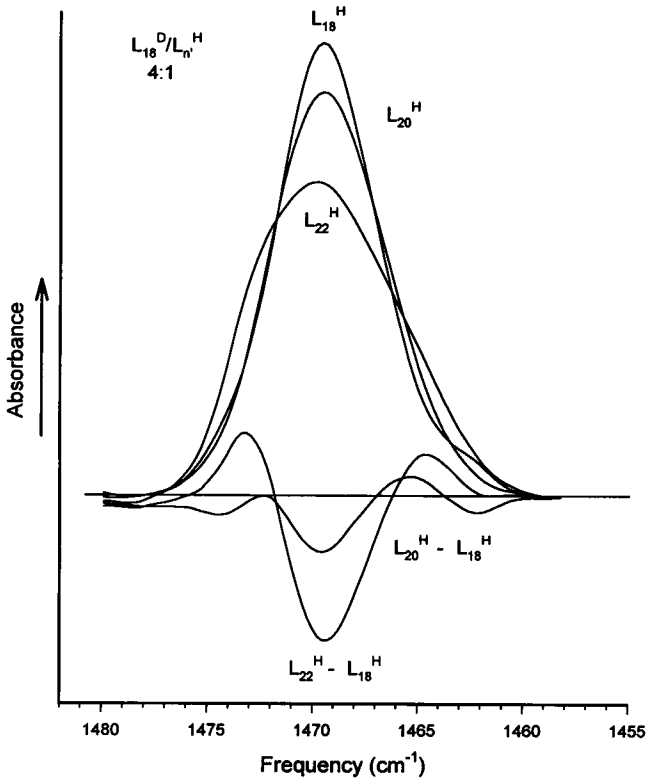

FIGURE 6 The CH_2 scissors band for the minority components in the mixtures $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$, $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$, and $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$, all at −19°C and at a concentration ratio of 4:1. (The scissors bands were isolated by subtracting the background contribution from the methyl groups.) The increase in widths indicates increasing aggregation of the minority phospholipids. The differences in the band shapes were obtained by subtracting the scissors band of the minority component $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$ (which is randomly mixed) from the corresponding band for the $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$ and $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$. Before the subtraction, the bands were normalized to the same integrated intensity. The difference spectra indicate changes in the size and in the concentration of domains that result from increasing the chain-length difference between the components.

argued that the "calibration curve" expressing this relation for *n*-alkane mixtures should also be appropriate for the phospholipid mixtures, except at very small domain sizes, in which case a (generally small) correction may be required (Snyder et al., 1995).

Distinguishing two similar singlet scissors bands

There are two different scissors bands, both singlets and both having frequencies within the narrow interval 1468–1470 cm^{-1}, that represent chains in quite different environments. Distinguishing them is important for establishing the temperature behavior of the mixtures. The bands are only marginally distinguishable on the basis of the small difference in their frequency, but clearly distinguishable from the temperature dependence of their frequency. One of the bands is associated with proteated chains that are isolated in an environment of deuterated chains. In this situation, the proteated chain is vibrationally uncoupled and therefore

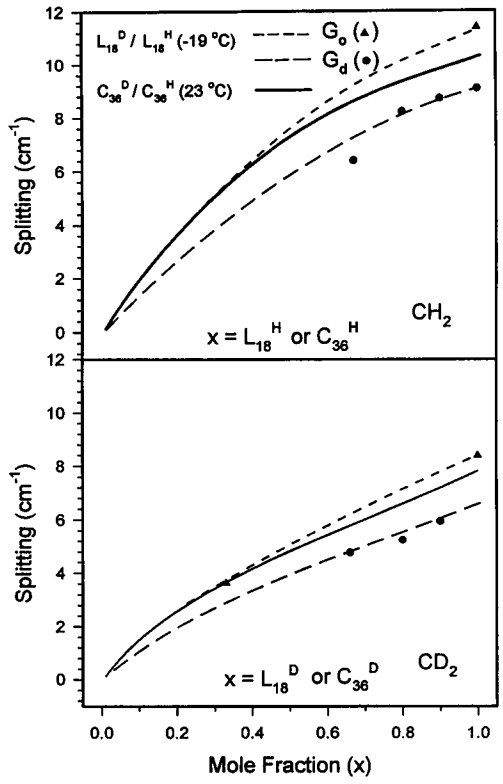

FIGURE 7 Approximate composition dependence of the magnitude of the frequency separation (splitting) between the two components of the scissors band for the phospholipid mixture $diC_{18}^{D}PC/diC_{18}^{H}PC$ at −19°C. For reference, the binary crystalline n-alkane mixture C_{36}^{D}/C_{36}^{H} at 23°C is included. The splitting for the phospholipid mixture is estimated both for distorted orthorhombic (G_d, ●) and ordered orthorhombic (G_o, ▲) chain packing. The splitting for the orthorhombic *n*-alkanes is that reported earlier (Snyder et al., 1992). (*Top*) The splitting for the proteated components, in which case the mole fraction is for the proteated component. (*Bottom*) The splitting for the deuterated component.

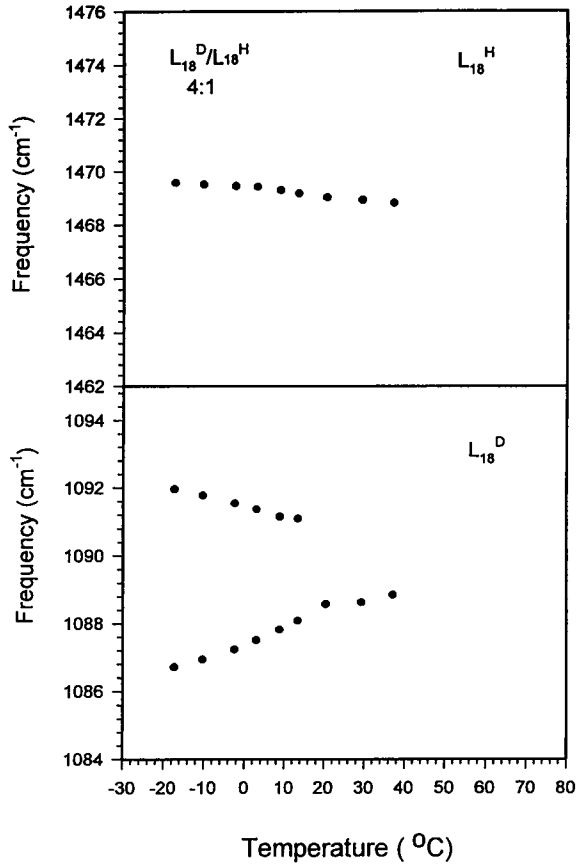

FIGURE 8 Temperature dependence of the methylene scissors frequencies for the components in the 4:1 mixture of $diC_{18}^{D}PC/diC_{18}^{H}PC$. (*Top*) CH_2 band for $diC_{18}^{H}PC$. (*Bottom*) CD_2 band for $diC_{18}^{D}PC$.

appears as a single band. For the 4:1 $diC_{18}^{D}PC/diC_{18}^{H}PC$ mixture at −19°C, the frequency of this band is near 1469.6 cm^{-1}, but at 40°C it has decreased to 1468.8 cm^{-1} (Fig. 8). This band is often found in the spectra of mixtures in which the proteated chains make up the minority component.

The other band, which looks similar, is also associated with proteated chains that are vibrationally uncoupled from their neighbors. In this case, however, the proteated chains are in an environment of other proteated chains. These chains are uncoupled, not through isotopic isolation, but rather as a result of high-amplitude libration-rotation about the long axis of the chains that sets in with increasing temperature by virtue of the lateral expansion of the lattice. Above 40°C, at which temperature the gel is in the G_d phase, the corresponding band in the spectrum of the pure lipid has a frequency (1468.6 cm^{-1}) that is essentially the same as that of the band isotopic isolation in a mixture at the same temperature. However, the frequency of this band, unlike that of the band associated with isotopic isolation, decreases only very slightly with increasing temperature. At temperatures above T_m, it has a nearly constant value of 1468.3 cm^{-1}.

MEASUREMENT OF AGGREGATION IN THE 4:1 $DiC_{18}^{D}PC/DiC_{n'}^{H}PC$ (n' = 20, 22, 24) MIXTURES

4:1 $DiC_{18}^{D}PC/DiC_{20}^{H}PC$ mixture

The high miscibility expected for an acyl chain-length difference of only 2 carbons is reflected in the similarity between the scissors band spectrum of this mixture at −19°C and that for randomly mixed 4:1 $diC_{18}^{D}PC/diC_{18}^{H}PC$ at the same temperature. In each case, the minority component scissors bands are singlets and have nearly the same shape (Fig. 6).

As reported earlier (Snyder et al., 1995), some aggregation in the minority component of $diC_{18}^{D}PC/diC_{20}^{H}PC$ mixture at −19°C is indicated by a broadening of this band. The width (FWHM) of the $diC_{20}^{H}PC$ band is 6.8 cm^{-1}, significantly greater than the 5.8 cm^{-1} observed for $diC_{18}^{H}PC$ in the random $diC_{18}^{D}PC/diC_{18}^{H}PC$ mixture (Fig. 6). Earlier estimates based on band widths indicate an average domain size of about 8 chains for the $diC_{18}^{D}PC/diC_{20}^{H}PC$ mixture and about 6 for the random mixture (Snyder et al., 1995).

The width of this band, if augmented by aggregation, should decrease with increasing temperature as the aggre-

gates are dissipated. This is observed. In Fig. 9, the width of the minority band for 4:1 $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$ is plotted as a function of temperature, along with the width of the corresponding band for the random $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$ mixture. As the temperature is increased, the width of the $diC_{20}{}^{H}PC$ band decreases, finally leveling off in the temperature region 10–20°C, where its value is near that for the random mixture. The aggregates of $diC_{20}{}^{H}PC$ chains in the 4:1 $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$ mixture thus disappear at about 15 ± 5°C.

The fraction of chains involved in the aggregates can be estimated from the changes in the band shape in going from a random mixture to a nonrandom mixture. The changes were measured by subtracting the 4:1 $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$ spectrum from that of $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$. Before subtracting, the integrated intensities of the two bands were scaled to the same value. The distribution of intensity in the difference spectrum, displayed in Fig. 6, indicates that 4 ± 2% of the $diC_{20}{}^{H}PC$ chains are involved in the aggregates.

To interpret the splitting of the majority component band for the 4:1 $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$ mixture at −19°C, it is necessary to know the acyl-chain packing subcell. On the basis of the width of the low-frequency component of the scissors band, the packing was identified as G_d, the same as for $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$. Band frequencies confirmed G_d packing (Fig. 10).

The majority component ($diC_{18}{}^{D}PC$) splitting for $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$ (now established to be in the G_d phase) is 6.1 cm^{-1} at −19°C (Fig. 11). As expected, this value is less than the 6.6 cm^{-1} for pure $diC_{18}{}^{D}PC$ in the G_d phase. On the other hand, it is significantly larger than the value of 5.3 cm^{-1} observed for 4:1 $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$ (also G_d) at the same temperature—in keeping with the expected trend to larger values as the majority component becomes purer as a result of demixing. The increase seems too large to be explained entirely on this basis, however.

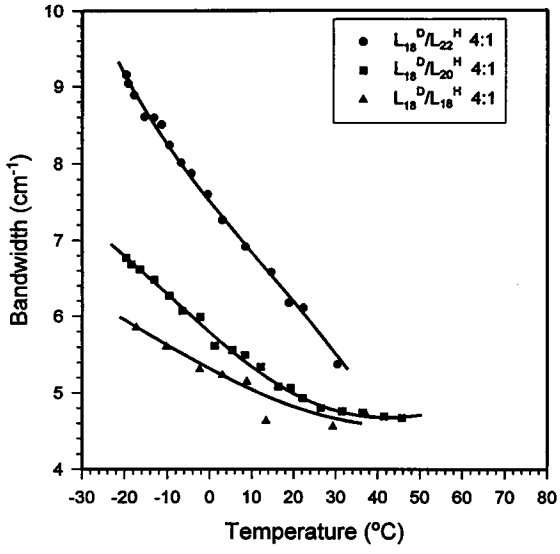

FIGURE 9 Temperature dependence of aggregation as indicated by the width (FWHM) of the CH_2 scissors band for the minority component in 4:1 binary mixtures of $diC_{18}{}^{D}PC$ paired with $diC_{18}{}^{H}PC$ (▲), $diC_{20}{}^{H}PC$ (■), or $diC_{22}{}^{H}PC$ (●).

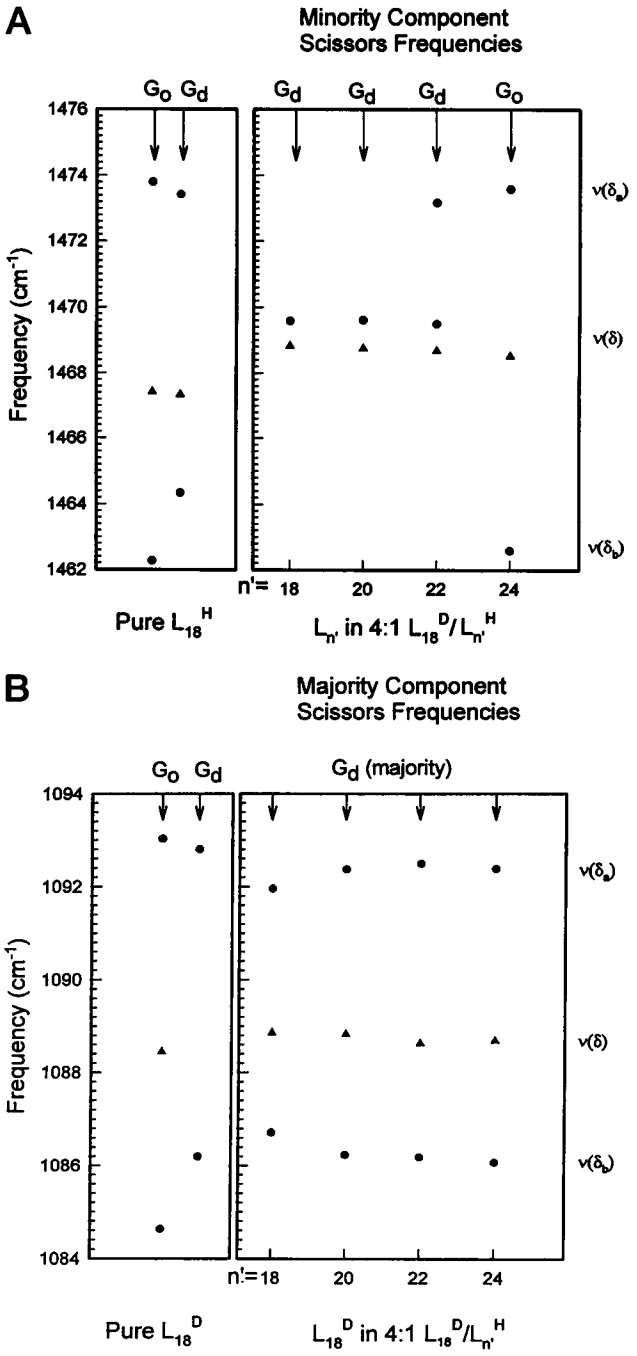

FIGURE 10 Summary of the scissors band frequencies for pure phospholipids and the 4:1 binary mixtures at −19°C (·). The packing subcell, which is G_o or G_d, is indicated. For samples at temperatures around 50°C, the packing is hexagonal-like and only a single band appears at frequencies indicated by ▲. (*a*) is for proteated phospholipids, pure and as minority components in the 4:1 mixtures. (*b*) is for deuterated phospholipids, pure and as the majority components. It is notable that the mixtures are all in the G_d phase except for the minority component, $diC_{24}{}^{H}PC$, in 4:1 $diC_{18}{}^{D}PC/diC_{24}{}^{H}PC$, which is G_o.

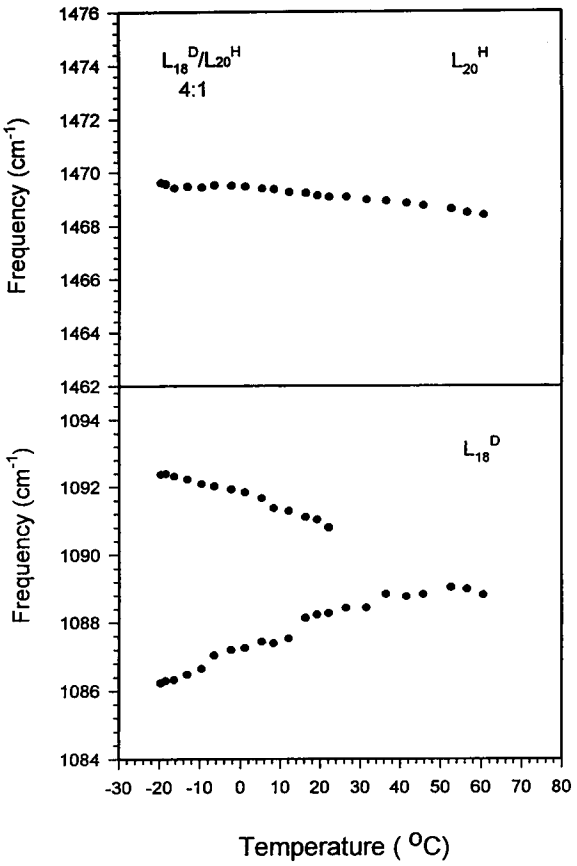

FIGURE 11 Temperature dependence of the methylene scissors frequencies for the components in the 4:1 mixture of $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$. (*Top*) CH_2 band for $diC_{20}{}^{H}PC$. (*Bottom*) CD_2 band for $diC_{18}{}^{D}PC$.

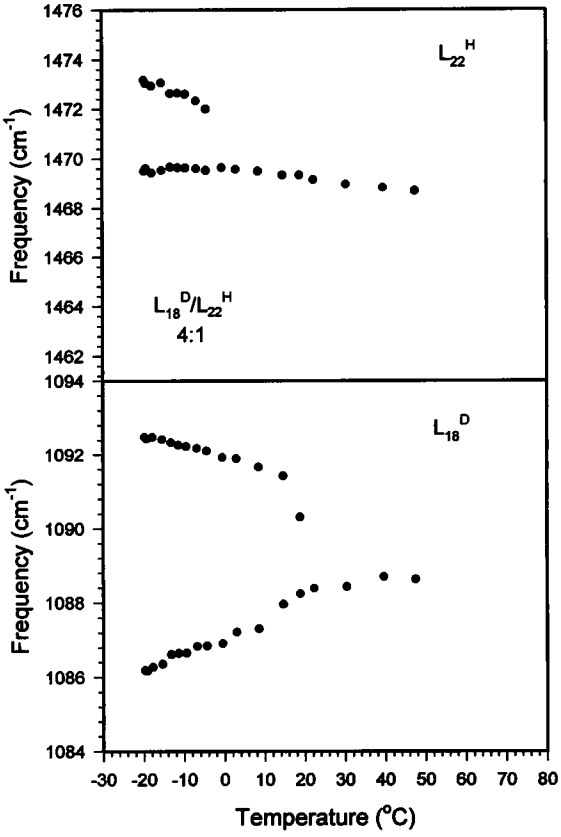

FIGURE 12 Temperature dependence of the methylene scissors frequencies of the components in the 4:1 mixture $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$. (*Top*) CH_2 band of $diC_{22}{}^{H}PC$. (*Bottom*) CD_2 bands of $diC_{18}{}^{D}PC$.

The 4:1 $DiC_{18}{}^{D}PC/DiC_{22}{}^{H}PC$ Mixture

Aggregation of the minority component ($diC_{22}{}^{H}PC$) is observed in this mixture at −19°C. Although still relatively small, it is significantly greater than that of the minority component in $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$. The average size of the $diC_{22}{}^{H}PC$ domains in $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$ has been estimated to be approximately 14 chains (Snyder et al., 1995). That the aggregation is more pronounced than in $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$ is evident from Fig. 6. The scissors band for $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$ is broader than that for $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$ and shows significant asymmetry due to a high-frequency shoulder. The frequency of the band responsible for the shoulder, which has the value 1473.1 cm^{-1} (determined from the second-derivative spectrum), strongly suggests that this band is the high-frequency component of a doublet originally from $diC_{22}{}^{H}PC$ domains. The lower-frequency member of the doublet could not be detected, presumably because of its greater breadth. The temperature behavior of the frequency of the "shoulder band," indicated in Fig. 12, also supports our assignment.

The dissolution of minority component aggregates in the 4:1 $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$ mixture as its temperature is increased from −19°C was monitored from the width of the minority component band (Fig. 9). The width decreases with increasing temperature roughly linearly to about 40°C, at which point its value is near that associated with random mixing, indicating complete dissolution.

The relative number of chains involved in the minority component aggregates in $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$ was estimated by using the spectral subtraction procedure described above for 4:1 $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$. The value found, 11 ± 2%, is nearly 3 times larger than that determined for $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$.

Although some of the $diC_{22}{}^{H}PC$ molecules in the 4:1 $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$ mixture are involved in aggregates, most exist in near isolation in a matrix of $diC_{18}{}^{D}PC$. This is confirmed by the observation that the frequency of the $diC_{22}{}^{H}PC$ scissors band (1469.6 cm^{-1} at −19°C) has the same value as the corresponding band (that of $diC_{18}{}^{H}PC$) in the 4:1 $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$ mixture, in which case the $diC_{18}{}^{H}PC$ chains are randomly dispersed.

The splitting of the majority component band in $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$ is 6.3 cm^{-1}, slightly larger than the 6.1 cm^{-1} found for $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$. The $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$ and $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$ mixtures both exist in the G_d phase at −19°C, so their splittings can be compared. Their values are consistent with the trend toward larger splitting with increasing phase separation, as is evident in Fig. 10. The temperature dependence of the frequencies and

shapes of the scissors bands of the majority component in the $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$, $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$, and $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$ mixtures are all similar, reflecting the fact that the two components making up each mixture are highly miscible.

The 4:1 $DiC_{18}{}^{D}PC/DiC_{24}{}^{H}PC$ mixture

This mixture at −19°C is extensively demixed, as evidenced by the large splitting for *both* the minority and majority component scissors bands (Figs. 13 and 14). It is notable that the chain packing is different for the two components—the $diC_{24}{}^{H}PC$ domains are in the G_o phase, whereas those of $diC_{18}{}^{D}PC$ are G_d. Pure $diC_{24}{}^{H}PC$ is normally G_o, whereas pure $diC_{18}{}^{D}PC$ can be either G_o or G_d.

Convincing evidence for nearly complete separation lies in the magnitude of the splitting observed for the minority component $diC_{24}{}^{H}PC$. The splitting is 10.9 cm^{-1}, only a little less than the full splitting of 11.6 cm^{-1} observed for pure $diC_{24}{}^{H}PC$ at −19°C. Extensive fractionation is also indicated by the absence of a third band between the components of the doublet. Such a band, if present, would indicate a significant concentration of $diC_{24}{}^{H}PC$ molecules in the majority phase domains.

As the 4:1 $diC_{18}{}^{D}PC/diC_{24}{}^{H}PC$ mixture is warmed, the scissors bands undergo changes related to structural transitions. The spectral changes observed for the minority component $diC_{24}{}^{H}PC$ are similar to those observed for pure $diC_{24}{}^{H}PC$ (Fig. 14). Specifically, increasing the temperature of the mixture from −19°C initially results in a slow, continuous collapse of the $diC_{24}{}^{H}PC$ scissors band doublet, similar to that observed upon warming pure $diC_{24}{}^{H}PC$, except that the change occurs over a shorter temperature interval. At higher temperatures, the scissors band is transformed into a singlet associated with the high-temperature hexagonal-like G_d phase, a behavior also similar to that of pure $diC_{24}{}^{H}PC$.

The onset of the dissolution of $diC_{24}{}^{H}PC$ domains is signaled by the appearance of a band at 1468.8 cm^{-1} that is associated with isotopically isolated acyl chains of $diC_{24}{}^{H}PC$. (The assignment of this band is based on the temperature dependence of its frequency, as indicated under Distinguishing Two Similar Singlet Scissors Bands, above.) This band appears at about 24°C, at which temperature chains from the $diC_{24}{}^{H}PC$ domains begin to dissolve into the majority component ($diC_{18}{}^{D}PC$) domains. Complete dissolution of the $diC_{24}{}^{H}PC$ domains occurs at a temperature somewhat above 47°C.

Finally, we note that the temperature behavior of the majority component ($diC_{18}{}^{D}PC$) of the 4:1 $diC_{18}{}^{D}PC/diC_{24}{}^{H}PC$ mixture (Fig. 14) resembles that for pure $diC_{18}{}^{D}PC$ (Fig. 2). The difference in behavior is mainly due to the different phase of the majority component (G_d and G_o, respectively) in the two cases.

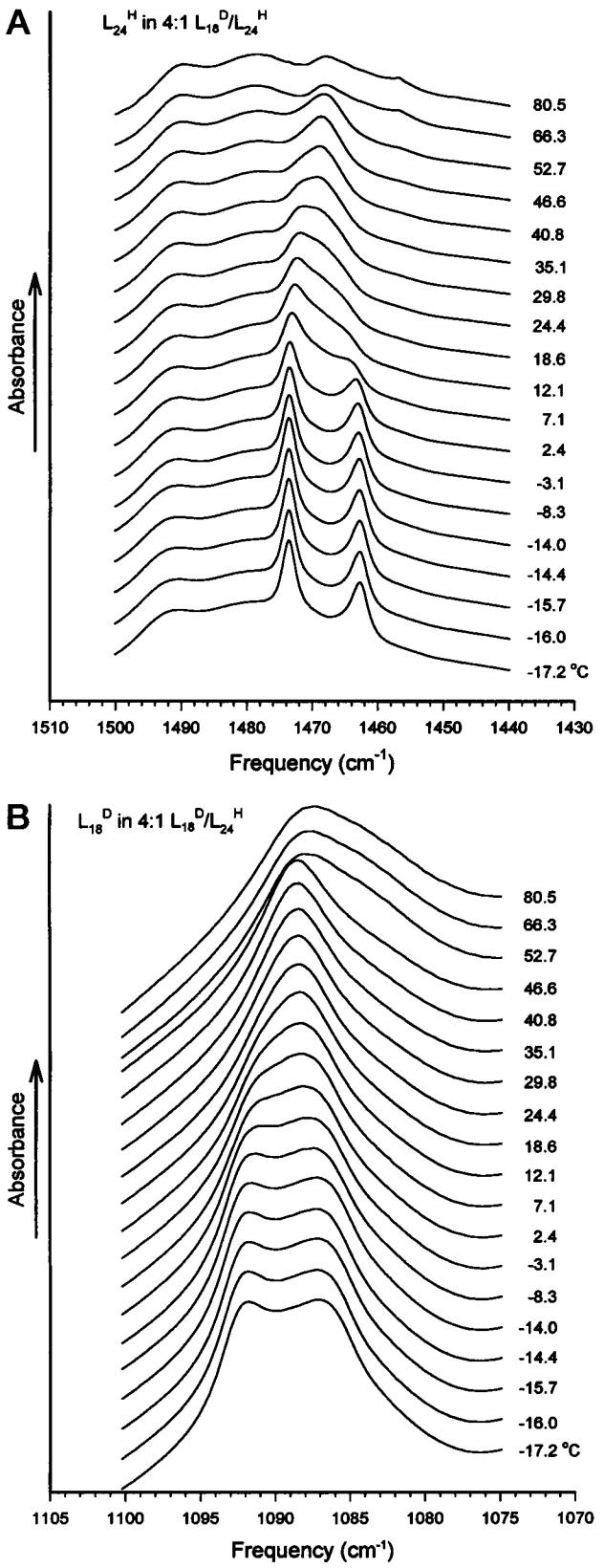

FIGURE 13 (*a*) Stacked infrared absorbance spectra in the CH_2 scissors region for the $diC_{24}{}^{H}PC$ component in a 4:1 $diC_{18}{}^{D}PC/diC_{24}{}^{H}PC$ mixture over the temperature range −17.2°C to 80.5°C. The temperature is indicated for each spectrum. (*b*) As in (*a*), but for the CD_2 scissors region of the $diC_{18}{}^{D}PC$ component.

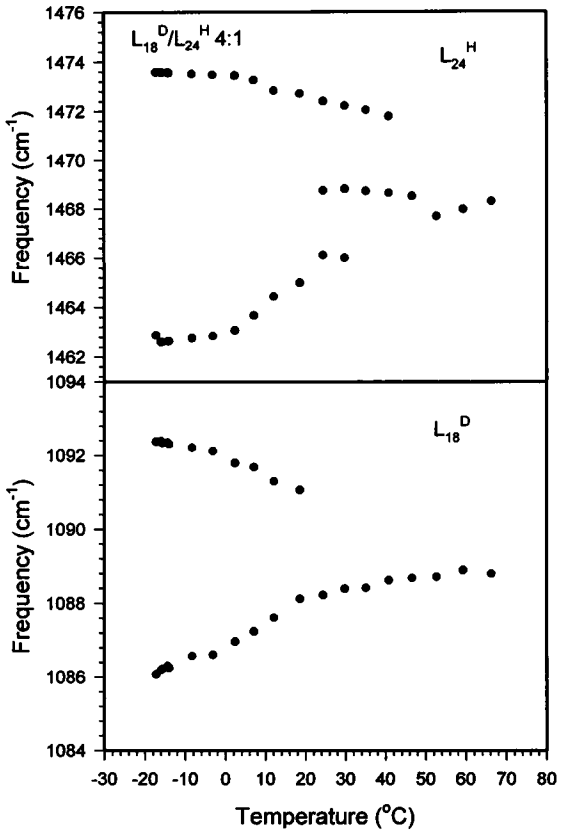

FIGURE 14 Temperature dependence of the methylene scissors frequencies of the components in the 4:1 mixture $diC_{18}{}^{D}PC/diC_{24}{}^{H}PC$. (*Top*) CH_2 band of $diC_{24}{}^{H}PC$. (*Bottom*) CD_2 bands of $diC_{18}{}^{D}PC$.

DISCUSSION AND SUMMARY

The mixed-isotope (hydrogen/deuterium) infrared method provides a quantitative measure of the degree of aggregation in phospholipid mixtures. Among its virtues are the ability to detect and measure aggregates in the size range of 1–100 molecules (for both components, in the case of a binary mixture) and, concomitantly, to monitor structure and crystal phase. It is required that the acyl chain packing be orthorhombic perpendicular and that one component be proteated and all the others deuterated (or the other way around). The aggregation is measured by the splitting (or broadening, if the degree of aggregation is low) of the methylene scissors band associated with the acyl chains. The splitting, which occurs only for orthorhombic packing, originates from vibrational interaction between chains that are isotopically alike.

We have applied the method to a series of binary mixtures of diacylphosphatidylcholines. The mixtures ($diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$, $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$, $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$, and $diC_{18}{}^{D}PC/diC_{24}{}^{H}PC$ at molar concentration ratios of 4:1) are aqueous dispersions in the gel state. The mixtures have acyl chain-length differences of 0, 2, 4, and 6 carbons between the components.

General relations between component miscibility and chain-length difference have been established from earlier calorimetric studies on binary phospholipid mixtures that are analogous to those studied here, but with acyl chain lengths in the range of 12–18 carbons. It was found that a chain-length difference of 2 carbons leads to near-ideal mixing (implying no aggregation), whereas a difference of 6 carbons leads to nearly complete phase separation. For the intermediate case of a 4 carbon length difference, a mixture generally considered highly nonideal, the nature and degree of the phase separation, in particular for the $diC_{14}{}^{H}PC/diC_{18}{}^{H}PC$ mixture, have been debated (Chapman et al., 1974; Knoll et al., 1981; Schmidt and Knoll, 1986; Cevc and Marsh, 1987; Biltonen, 1990).

Our results for the 4:1 $diC_{18}{}^{D}PC/diC_{n'}{}^{H}PC$ ($n' = 20, 22, 24$) mixtures are generally consistent with the qualitative estimates of miscibility derived from calorimetry. However, the determination of aggregation by the infrared method provides a more sensitive, quantitative, and structurally oriented characterization. Its sensitivity is particularly well displayed in application to the 4:1 $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$ mixture at −19°C. Although this mixture, with its chain-length difference of 2 carbons, would be classified by calorimetry as an essentially ideal solid solution, infrared measurements indicate significant aggregation, albeit at a low level. The aggregation is apparent in the greater width of the scissors band of the minority component ($diC_{20}{}^{H}PC$) relative to the width associated with the corresponding component ($diC_{18}{}^{H}PC$) of the randomly mixed 4:1 $diC_{18}{}^{D}PC/diC_{18}{}^{H}PC$. The shapes of the scissors bands for these two mixtures (Fig. 6) show differences that can be utilized to estimate the fraction of minority component chains involved in the aggregates. We find that about 4% of the $diC_{20}{}^{H}PC$ molecules in 4:1 $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$ at −19°C are so involved. For 4:1 $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$ at −19°C, a mixture with a 4-carbon chain-length difference, the situation is similar in that the minority component ($diC_{22}{}^{H}PC$) also undergoes a small degree of aggregation. The amount, however, is significantly greater than in 4:1 $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$. This is evident from the shape of the $diC_{22}{}^{H}PC$ scissors band, which is much broader than that of $diC_{20}{}^{H}PC$ in the 4:1 $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$ mixture (Fig. 6). Difference spectra indicate that about 11% of the minority $diC_{22}{}^{H}PC$ molecules in the $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$ mixture at −19°C are involved in aggregates. The 4:1 $diC_{18}{}^{D}PC/diC_{24}{}^{H}PC$ mixture at −19°C belongs in a separate category. Its components are nearly immiscible, in keeping with the results of calorimetric studies on other phospholipid mixtures having this chain-length difference (Cevc and Marsh, 1987).

The packing structure of the acyl chains in the various mixtures at −19°C was determined from the shape of the scissors band. The two possible orthorhombic arrangements are G_o or G_d, which refer, respectively, to ordered or distorted unit subcells. For 4:1 mixtures in which there is a low degree of aggregation, the packing may be assumed to be that of the majority component. In this case, it is usually possible to determine the packing from the shape of the majority component band, because the splitting is large and therefore the component bands are well defined. The 4:1

$diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$ and $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$ mixtures at $-19°C$ are in the G_d phase. If the mixture is highly demixed, the packing can be different for the two phases and can be determined because the splittings for both components will tend to be large. The 4:1 $diC_{18}{}^{D}PC/diC_{24}{}^{H}PC$ mixture represents such a case. The packing for the majority component is G_d, but for the minority component it is G_o.

The temperature dependence of aggregation and phase, which are likely to be more or less coupled, was measured as the mixture was warmed. The temperature at which the dissolution of the minority aggregates is complete was measured for the 4:1 $diC_{18}{}^{D}PC/diC_{20}{}^{H}PC$, $diC_{18}{}^{D}PC/diC_{22}{}^{H}PC$, and $diC_{18}{}^{D}PC/diC_{24}{}^{H}PC$ mixtures and found to be $15 \pm 5°C$, $40 \pm 3°C$, and $>47°C$, respectively. The onset temperature for dissolution was also determined for the $diC_{18}{}^{D}PC/diC_{24}{}^{H}PC$ mixture and estimated to be $24 \pm 3°C$. Phase transitions and structural changes are observed as the mixtures are warmed. The majority component generally behaves in a manner similar to that component in its pure state. However, for the mixtures, the changes occur at lower temperatures and over a shorter temperature interval.

We conclude with some comments concerning the two requirements inherent to the infrared method. The requirements are that, for a binary mixture, the acyl chains of one of the components must have an isotopic composition (proteated or deuterated) different from that of the other components and that the acyl chains must pack in a perpendicular orthorhombic unit subcell. The presence of both proteated and deuterated components in the same mixture makes it necessary to consider the effect of isotope composition on the tendency of a mixture to aggregate. For binary *n*-alkane mixtures, such effects can be surprisingly large (Snyder et al., 1994). In summary, more extensive microphase separation (or equivalently, larger average domain sizes is anticipated for a D/H mixture than for the corresponding H/D mixture. The tendency to separate is nearly the same for the H/H and D/D combinations, lying between that of D/H and H/D. (The first isotope in these combinations represents the shorter chain.) The isotope effect on demixing is associated with the slightly lower melting points for perdeuterated chains and the slightly smaller molar volume of the CD_2 group relative to the CH_2 group (Snyder et al., 1994; Dorset et al., 1991).

The necessity for orthorhombic chain packing would seem to render the sampling of disordered phases a difficult proposition, limiting applications, given that most biological membranes exist predominantly in more or less disordered phases. Such systems might be measured by first quenching them at a rate rapid enough and to a temperature low enough to preserve the high-temperature structure. By controlled annealing, it may be possible to induce conformational ordering and orthorhombic packing without introducing significant lateral diffusion. This process has been observed during the annealing of amorphous films of *n*-alkanes prepared by deposition from the vapor (Hagemann et al., 1987).

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