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RAMAN TEMPERATURE STUDY OF CONFORMATIONAL CHANGES IN ANHYDROUS DIPALMITOYLPHOSPHATIDYLCHOLINE

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The gradual randomization of anhydrous dipalmitoylphosphatidylcholine below the main transition is monitored by Raman spectroscopy at the headgroup, the acyl linkages, and the hydrocarbon chains. The thermodynamic properties of the conformation changes occurring in the different regions of the phospholipid molecule are investigated using the symmetric C_4N^+ stretch, C=O stretch, and the CH and C–C stretch spectral regions. The first Raman spectra of intermediate stages in the temperature-induced change of the C=O doublet (arising from inequivalent acyl rotamers) to a singlet are presented. A fractional order parameter for the acyl region, $\theta_{C=O}$, is defined. Temperature plots of the fractional order parameters for the CH stretch (θ_{CH}) and the chain C–C stretch (θ_{C-C}) are nearly co-linear, while that of $\theta_{C=O}$ is parallel to but below the θ_{CH} and θ_{CC} plots. We observed that the onset of hydrocarbon chain randomization proceeds first and that conformation change in the acyl region begins after the *trans-gauche* isomerization of 4 to 5 C–C bonds per molecule in the chains. Thereafter, the rate of change in the hydrocarbon chain and acyl regions are approximately equal. Van't Hoff plots for the hydrocarbon chain randomization and acyl region conformational changes from 50 to 90°C give ΔH values of 3.2 ± 0.4 kcal/mol and 4.4 ± 0.5 kcal/mol, respectively. The electrostatic interactions of the headgroups are not broken until the transition. It appears that ionic interactions at the headgroup are responsible for the exceptionally high ΔH values for acyl C_2-C_1 rotamerization.

Introduction

In a number of biological membrane processes, strong interactions at the phospholipid headgroups which dehydrate the lipid surface are likely to occur. For example, multi-bilayer formation (as in myelin), membrane fusion, and bilayer-to-hexagonal H_{II} transitions may require close contact between adjacent bilayers. Strong attractive ionic forces between headgroups facilitate close approach of lipid headgroups and extrusion of water between bilayers. Also, ionic binding at the headgroup, which may occur with metallic cation binding or protein binding, may displace water molecules.

Studies of conformation changes in anhydrous phospholipids will provide the background necessary for understanding mechanisms involving strong ionic interactions at the headgroup. Also, studies of both anhydrous phospholipids and phospholipids in aqueous dispersion will, when compared, reveal the effect of bound water on lipid conformation and thermodynamics. Raman

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Abbreviations: DPPE, dipalmitoylphosphatidylethaneolamine; DPPC, dipalmitoylphosphatidylcholine; DLPE, dilauroylphosphatidylethaneolamine.

spectroscopy can be used to study molecular conformation in a variety of states and is used in the present study to investigate temperature-induced conformational change in anhydrous dipalmitoylphosphatidylcholine (DPPC) at temperatures well below that of the main anisotropic melting.

X-ray diffraction studies of phospholipids [1–3] have provided detailed structural information about the phospholipid ordered state. The hydrocarbon chains are shown to be in an extended all-*trans* configuration below the ester linkage region. The two side chains differ at the ester linkage, with the *sn*-2 chain undergoing a *gauche* rotation at the C-C bond adjacent to the carbonyl group, while the *sn*-1 chain C_2-C_1 is in the *trans* configuration. There are slight differences in the polar head group conformation depending upon the makeup of the headgroup. A model structure is shown in Fig. 1.

The hydrocarbon side chains undergo randomization as the result of the introduction of *gauche* conformers about the C-C bonds [6]. The *trans-gauche* rotation has been shown to begin at the methyl end of the hydrated phospholipid by NMR [8], ESR [9], and fluorescence spectroscopy [10–12]. In addition it has been shown that there is a great deal of rearrangement of the side chains prior to the main melting transition [13] in hydrated lipids.

The conformational difference at the ester linkage produces two carbonyl groups which are spectroscopically distinct. The existence of two sharp C=O stretch bands in the Raman spectrum of crystalline DLPE [4], anhydrous DPPC [4,5] and DPPE [4] has been demonstrated. Dispersing these phospholipids in water causes the two sharp carbonyl stretch bands to be replaced by one broad band of double intensity.

In this paper, Raman spectroscopy is used to probe the structural changes that occur prior to the main hydrocarbon chain disruption in anhydrous DPPC, which provide information about the hydrocarbon chains. We have also followed the carbonyl stretching region and the C-N^+ stretch band of the polar headgroup. The structural changes that occur in these three distinct phospholipid structural regions are compared. The results for anhydrous DPPC are compared to studies of hydrated DPPC and the effect of hydration is discussed.

Experimental

Synthetic dipalmitoylphosphatidylcholine (DPPC) was obtained from Sigma. The stated purity was 99%, and thin-layer chromatography revealed one band only.

A DPPC sample was dried by heating under vacuum for 24 h and was then placed in a capillary tube. The Raman spectrum was then measured by placing the sealed tube in a Harney-Miller cell, which regulated sample temperature by a stream of warmed or cooled N_2 gas. A thermocouple was attached to the sample capillary close to the site at which the laser beam was incident. Temperature fluctuation during each Raman measurement was less than 1°C. 45 min equilibration time was allowed between each temperature increment and the Raman measurement.

The 514.5 nm laser line of an argon laser was used for sample excitation. Scattered light was collected at 90° to the incident beam and analyzed by a Spex 14018 double monochromator equipped with a spatial filter to reduce stray light. The photon counting system included a cooled RCA-C31034 photomultiplier tube. Spectral bandpass was set at 5 cm^{-1}.

Results

Phospholipid molecules may be divided into three molecular regions, as shown in Fig. 1, which correspond to three regions of lipid bilayer which are distinct in physical properties and function. Using Raman spectroscopy, we were able to investigate all regions of the phospholipid molecule (and hence the bilayer) simultaneously, without requiring synthetic probes. In order to compare the pre-melt properties of the interior, interface and headgroup regions of the anhydrous dipalmitoylphosphatidylcholine molecule, we use Raman CH stretch bands for monitoring hydrocarbon chain conformation and lateral interchain interactions, the hydrocarbon chain C-C stretch bands for monitoring *trans-gauche* isomerization, the C=O stretch bands for monitoring acyl linkage conformation and motional freedom at the interface, and the choline symmetric C_4N^+ stretch for monitoring headgroup electrostatic interactions and motional freedom. These three Raman spec-

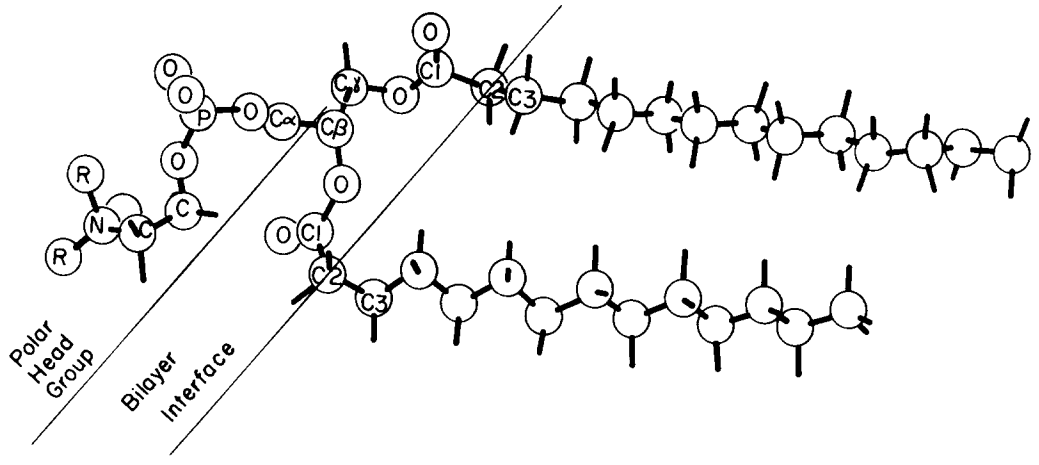

Fig. 1. The interior, acyl interface and headgroup regions of the phospholipid molecule. Conformation is taken from the X-ray diffraction study of dilauroylphosphatidylethanolamine of Ref. 1.

tral regions are shown in Fig. 2 for two temperatures below the main hydrocarbon chain transition, 20 and 70°C, and one temperature, 110°C, just above the transition.

Hydrocarbon chain randomization

The Raman CH stretch band at 2880 cm^{-1} is sensitive to both intrachain conformation and interchain interactions [14]. Both *trans-gauche* isomerization in the chain and changes in chain packing can alter the shape of this band. Its peak height, ratioed to the relatively constant peak height of the 2850 cm^{-1} symmetric CH_2 stretch band, decreases with hydrocarbon chain randomization [13–17]. We observed that the peak height ratio I_{2880}/I_{2850} decreases sharply as expected, at the main transition for crystalline DPPC at approx. 100°C. However, a gradual decrease in the 2880 cm^{-1} band begins well below the transition, indicating that some randomization of the hydrocarbon chains develops gradually with temperature before the cooperative melting takes place. This pre-melt temperature range was studied, between 50°C and 90°, in greater detail in order to investigate changes leading up to the transition. The difference between r_t, the value for I_{2880}/I_{2850} for the 'fully' ordered hydrocarbon chains and r, the value observed at any given temperature, is ratioed to the difference $(r_t - r_g)$ for the values for the fully ordered and fully disordered chains. The ratio is used to express a fractional order parameter, θ_{CH}, for the hydrocarbon chains.

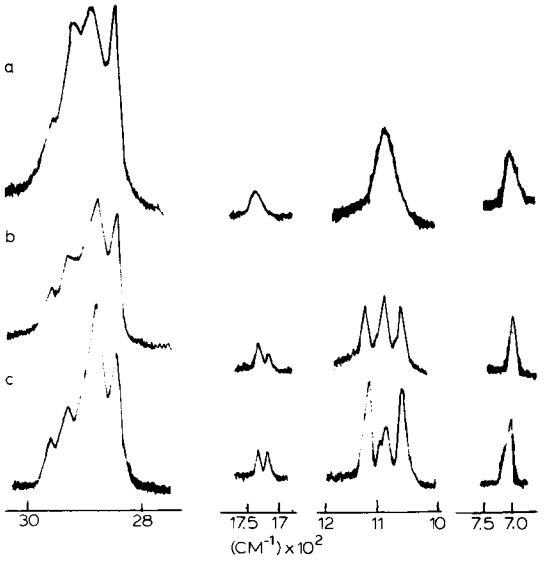

Fig. 2. Raman spectra of the CH stretch, C=O stretch and C-C stretch regions of DPPC at (a) 20°, (b) 70°, and (c) 110°C.

$$\theta_{CH} = \frac{r_t - r}{r_t - r_g}$$

The values for r_t and r_g were approximated by values obtained from spectra of anhydrous DPPC measured at 18 and 110°C, respectively. θ_{CH} is plotted in Fig. 3 as a function of temperature. The

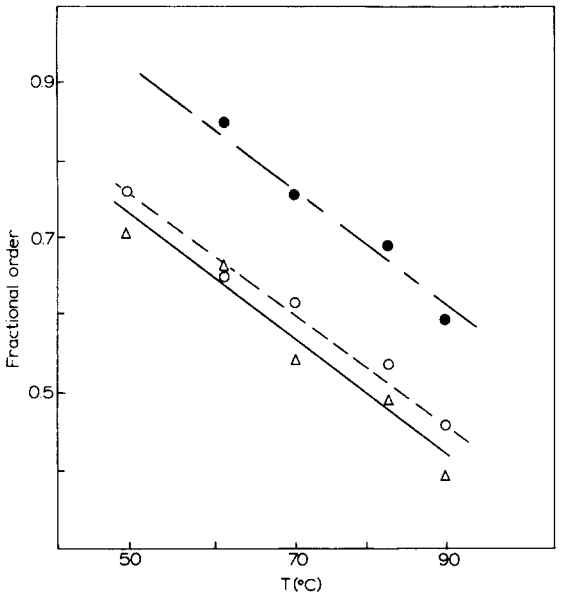

Fig. 3. Fractional order determined from the C-C stretch (○), the C-H stretch (△), and C=O stretch (●) spectral regions.

change in θ_{CH} with temperature is approximately linear below the main transition with a slope of -7.8 ± 1.0 obtained by least-squares analysis.

The carbon-carbon stretching modes for the hydrocarbon chains are observed at 1060 to 1130 cm^{-1}, Two bands attributed to segments of all-*trans* carbon-carbon bond conformers appear at about 1060 and 1130 cm^{-1}. The *gauche* carbon-carbon bond conformers produce a very broad band at about 1090 cm^{-1}. Therefore, the relative intensities of the 1130 cm^{-1} and 1090 cm^{-1} bands may be used to estimate relative populations of *trans* rotamers in all-*trans* segments and rotamers in disordered segments. The symmetric C_4N^+ choline stretch at 710 cm^{-1} is used as an internal standard below the main transition. The fractional order measured by the hydrocarbon chain C-C stretch modes is then taken as the ratio of (I_{1130}/I_{720}) to (I^t_{1130}/I^t_{720}), where I^t_{1130} and I^t_{720} are peak heights measured at a low temperature (18°C in the present study) where the side chains are considered to be all-*trans*. θ_{CC} is also plotted against temperature in Fig. 3 and also appears to be linear between 50 and 90°C. A least-squares analysis produced a value of -7.0 ± 0.9 for the slope.

Though θ_{CH} is sensitive to both interchain interactions and chain conformation while θ_{CC} is sensitive to chain conformation only, the plots for θ_{CH} and θ_{CC} are nearly parallel and differ at any given temperature by a small amount, about 0.03. This result suggests that, for the pre-melt region, either the changes in lateral chain interactions are insignificant or that the changes in interchain interactions are proportional to changes in chain conformation.

Acyl region conformation changes

For anhydrous DPPC at 20°C (Fig. 2a), the ester C=O stretch region contains two bands of equal intensity, appearing at 1741 and 1724 cm^{-1}. In the melted state at 110°C (Fig. 2c), a single band of double intensity appears at 1739 cm^{-1}. The present study shows for the first time intermediate stages in melting in the acyl region (Fig. 2b, for example), which begin below the transition temperature. The intensity in the C=O stretch doublet begins to gradually shift from the lower frequency band to the higher frequency band in the pre-melt region. This change does not begin to appear, however, until the temperature is raised about 50°C, which is well above the temperature at which initiation of hydrocarbon-chain randomization was observed by the CH and CC stretch modes.

The frequencies observed for the C=O stretch bands and the ratio of intensities of the lower and higher frequency bands are presented in Table I. The frequency of the lower band shifts upward slightly with temperature, from 1724 cm^{-1} at 18°C to 1729 cm^{-1} at 81°C. However, the most notable

TABLE I

CHANGES IN THE ACYL CARBONYL STRETCH MODES WITH TEMPERATURE

Temperature of measurement (°C)	Frequencies (cm^{-1})		Ratio of intensities I_1/I_2
	ν_1	ν_2	
18	1724	1741	1.0
62	1726	1741	0.83
72	1727	1742	0.74
81	1729	1742	0.64
110	–	1739	0.0

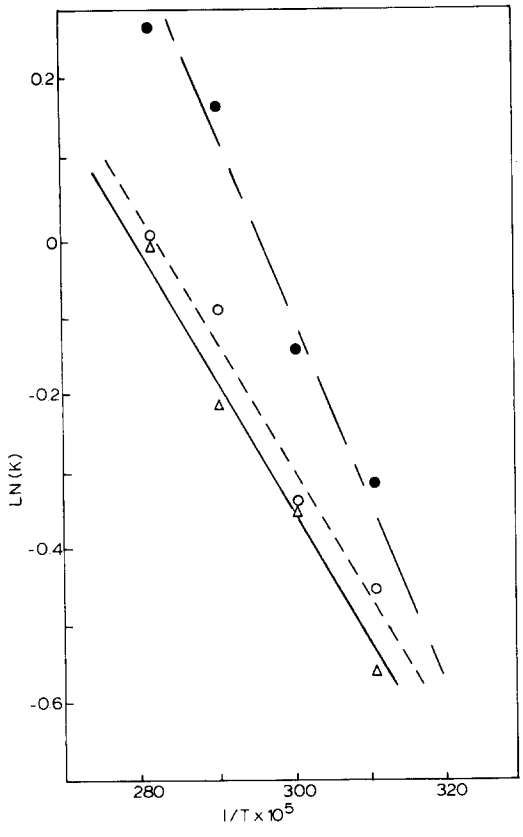

Fig. 4. Van't Hoff plots for the C-C stretch (○), the C-H stretch (△), and C=O stretch (●) spectral regions.

change observed in the lower frequency band is a decrease in its intensity which occurs concomitantly with an increase in intensity of the higher frequency band. Thus two well-resolved C=O stretch bands separated by 17 to 13 cm^{-1} co-exist between 18 and 81°C. The ratio of intensity of the lower frequency band to the higher frequency band is 1.0 between 18 and 50°C, and changes gradually from 1.0 at 18°C to 0.64 at 81°C. After anisotropic melting at the transition, the intensity ratio is approximately zero at 110°C.

We have previously assigned the doublet observed in anhydrous DPPC to a molecular conformation in which the two ester linkages differ in configuration in such a way as to produce different C=O stretch frequencies [4]. We believe the single strong C=O stretch band is observed for DPPC in aqueous dispersion because the specific configurational inequivalence of the ester linkages which gives rise to the doublet has been removed. We have provided evidence [4] that the C=O stretch frequency is sensitive specifically to rotation about the C_2-C_1 bond. In anhydrous DPPC, we assume the *sn*-1 chain contains the C_2-C_1 *trans* conformer and the *sn*-2 chain contains the C_2-C_1 *gauche* conformer, as is the case for crystalline DLPE [1]. Hence, we observe a doublet in the Raman spectrum. Hydration causes the intensity of the lower frequency band to shift upwards and merge with that of the higher frequency band [4]. Although the hydrocarbon chain conformations for the hydrated gel state and the melted state are not similar, the spectroscopic evidence suggests that the *sn*-1 and *sn*-2 chains both contain nearly equivalent (probably *gauche*) C_2-C_1 bond rotamers for both states.

Therefore, it appears that two principle conformations exist for the acyl linkage region, and that change from one conformation to the other occurs by a discrete transition. We propose that a rotation about the C_2-C_1 bond occurs in one of the two acyl linkages of the molecule from the rotamer which gives rise to the lower frequency C=O stretch at about 1724 cm^{-1} to the rotamer which gives rise to the higher frequency band at about 1740 cm^{-1}. The relative populations of the two acyl rotamers change with temperature in the pre-melt region, causing the relative intensities of the two Raman C=O stretch bands to change dramatically.

We now define a fractional order parameter for the acyl region using the relative intensities of the C=O stretch doublet. We define conformer I as that of the low temperature crystalline configuration of anhydrous DPPC with inequivalent C_2-C_1 rotamers in the *sn*-1 and *sn*-2 chains. Thus, conformer I is assigned to a C=O doublet having equal intensities in both bands. Conformer II is assigned to conformations which have equivalent C_2-C_1 rotamers in the *sn*-1 and *sn*-2 chains, or those which give rise to a single higher frequency C=O stretch band. If we refer to conformer I as the ordered conformer, then $\theta_{C=O}$ = conformer I population/(conformer I population + conformer II population). Thus,

$$\theta_{C=O} = 2I_{1724}/(I_{1724} + I_{1740})$$

$\theta_{C=O}$ is plotted against temperature below T_m in

Fig. 3. $\theta_{C=O}$ also varies linearly with temperature in the pre-melt region and is nearly parallel to the fractional order plots for the hydrocarbon chain CH stretch and C-C stretch regions, but is about 0.15 greater over the 50–90°C temperature range than θ_{CH} and $\theta_{C=O}$. Thus, hydrocarbon chain randomization begins at lower temperatures than does conformational change in the acyl region. However, above 50°C, each changes at the same rate.

Headgroup

The shift in frequency of the symmetric C_4N^+ choline stretch from 710 cm^{-1} to 720 cm^{-1} upon hydration is believed to be due to the reduction of electrostatic interactions involving the choline group [5]. We agree with this interpretation and believe that the shift from 710 to 716 cm^{-1} observed with the melting of anhydrous DPPC indicates melting of the headgroup, reducing average ionic interactions between choline and phosphate groups. The symmetric C_4N^+ stretch frequency is observed at 716 cm^{-1} in the 110°C. The frequency shift occurs only after the hydrocarbon chains have completed melting.

Discussion

The Raman temperature study of anhydrous DPPC shows that conformational and packing changes associated with melting proceed sequentially, beginning with the hydrocarbon chains, for which the onset of chain randomization occurs at about room temperature. After the fractional order for the hydrocarbon chains has decreased linearly with temperature to about 0.75, the acyl region packing begins to change above 50°C. The acyl region order decreases above 50°C in the pre-melt temperature region at the same rate with temperature as the hydrocarbon chain order. Apparently, strong ionic interactions hold the headgroup region rigid until the transition occurs, since no change in the choline C_4N^+ stretch frequency (which is sensitive to ionic interactions) occurs in the pre-melt region.

The fractional order determined from the relative intensity of the chain C-C stretch mode at 1130 cm^{-1} is sensitive to the number and length of segments of all-*trans* rotamers. If we assume that *gauche* rotamers are introduced first at the methyl terminal end and then are added sequentially from the methyl end towards the acyl end, we can estimate the average number of *gauche* rotamers per hydrocarbon chain from θ_{CC}. Using the information given in Fig. 3, we estimate that three or four *gauche* rotamers per chain must be introduced at the methyl end before conformational changes can occur in the acyl region. That is, randomization must extend from the methyl end through four or five carbon atoms before sufficient energy is obtained for disrupting packing in the acyl region.

Enthalpy requirements for rotamer isomerization in the hydrocarbon chain region and the acyl region are determined from the slopes of Van't Hoff plots of $\ln \theta/(1-\theta)$ vs. $1/T$ (K) (see Fig. 4). Both the plot using θ_{CH} and the plot using θ_{cc} give an enthalpy value of 3.2 ± 0.4 kcal/mol for rotamer isomerization in the hydrocarbon chain. The Van't Hoff plot for $\theta_{C=O}$ gives a value of 4.4 ± 0.5 kcal/mol for rotamer isomerization, presumably about the C_2-C_1 bond in the acyl region. This value is large compared to the enthalpy change of 0.5 kcal/mol measured for rotation about the C_2-C_1 bond of small ester molecules [18]. The additional energy requirement is likely due to restraints imposed upon rotation by tight packing in the interface region. The rotation is facilitated by hydration, since one C=O stretch band of double intensity is observed for aqueous dispersions of DPPC at 25°C [4]. Yet packing in the interface region is very ordered and rigid for DPPC in aqueous dispersion at 25°C, as measured by the Raman acyl C_2-C_1 stretch bands [19]. Two possible conclusions follow. The first is that hydration of the ester carbonyl group may stabilize the conformation achieved after rotation, though the acyl interface is still rigid for the gel phase at 25°C. The second is that headgroup interactions and headgroup-acyl group interactions in addition to acyl packing restraints are likely to contribute to the high enthalpy requirement for rotamer isomerization in the acyl region for anhydrous DPPC.

The mechanisms and thermodynamics of randomization in anhydrous DPPC are applicable to biological membrane processes in which strong ionic interactions may displace hydrated water. Examples of such phenomena may include changes

to non-bilayer aggregates, membrane fusion, multi-bilayer formation (as in myelin), complex formation with metallic cations and possibly sòme types of phospholipid-protein binding. For these systems also, both strong headgroup interactions and strong constraints on C_2-C_1 rotamer conformation and isomerization in the acyl region may be present and may contribute to the mechanisms involved in these processes. In Raman temperature studies in progress, we have already observed evidence for this in an intermediate step in the bilayer to H_{II} transition of phosphatidylethanolamine.

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