INFLUENCES OF MEMBRANE CURVATURE IN LIPID HEXAGONAL PHASES STUDIED BY DEUTERIUM NMR SPECTROSCOPY

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The presence of reversed hexagonal phase, H$_n$, favoring lipids in membranes has been proposed to be significant in various biological processes. Therefore an understanding of the H$_n$ phase and the transition from the lamellar to hexagonal phase is of importance. We have applied deuterium NMR spectroscopy to study the bilayer and reversed hexagonal phases of 1-perdeuteriopalmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine. The difference in packing between the H$_n$ and L$_a$ phases leads to smaller segmental order parameters in the former case. Since the order profiles are sensitive to the geometry of the aggregates, they can be used to extract structural information about the phases. We present a new means of calculating the radius of curvature, R$_1$, for the H$_n$ phase from $^2$H NMR data. This method gives a value of R$_1$ = 18.1 Å, which is in agreement with current understanding of the structure of the H$_n$ phase and with x-ray diffraction data.

Phospholipids, like most amphiphiles, can form a variety of different structures in aqueous environments. These include lamellar or bilayer aggregates, which comprise the basis for lipid organization in biological membranes (1). Yet a significant portion of membrane lipids tend to form nonbilayer phases such as the reversed hexagonal, H$_n$, and cubic phases (2,3). It has been suggested that the presence of these nonbilayer favoring lipids and the proximity to the bilayer to non-bilayer phase transition (3,4) are implicated in various biological functions.

Abbreviations: DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DPPC-d$_{62}$, 1,2-diperdeuteriopalmitoyl-sn-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; $^2$H NMR, deuterium nuclear magnetic resonance; H$_n$, reversed hexagonal phase; L$_a$, liquid crystalline phase; MOPS, morpholinopropane sulfonic acid; PLPC-d$_{31}$, 1-perdeuteriopalmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine; PLPE-d$_{31}$, 1-perdeuteriopalmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPE-d$_{31}$, 1-perdeuteriopalmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; R$_1$, radius of curvature; S$_{CD}$, carbon-deuterium bond order parameter; TLC, thin layer chromatography; $\Delta v_Q$, quadrupolar splitting.

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mediated by membrane proteins (3-7). Given that these factors may be necessary for function in natural systems, it is important to understand the structural parameters of the \( \text{H}_n \) phase and how they differ from the \( \text{L}_\alpha \) phase. The most successful technique in accomplishing this to date has been x-ray diffraction, which has provided the first evidence of these non-bilayer phases, as well as some of the most current measurements of structural properties of the \( \text{H}_n \) phase (2,8,9). This knowledge has led to an improved understanding of some of the forces leading to formation of the \( \text{H}_n \) phase (10). However, low-angle x-ray diffraction studies give little or no information at the molecular level, nor any dynamic information.

Deuterium NMR spectroscopy, \(^2\text{H}\) NMR, has been utilized to extract such knowledge for lipids in the \( \text{L}_\alpha \) phase (11-14), including phosphatidylcholines, phosphatidylethanolamines, glycolipids, as well as other membrane lipids (11-16). \(^2\text{H}\) NMR has also been used to characterize certain geometric properties of the \( \text{L}_\alpha \) phase, including the average bilayer thickness and the cross-sectional area per molecule (16-20). Lafleur \textit{et al.} (21) have recently shown that the orientational order in the \( \text{H}_n \) phase is less than in the \( \text{L}_\alpha \) phase for the case of 1-perdeuteriopalmitoyl-2-oleoyl-\( \text{sn-glycero-3-phosphoethanolamine} \) (POPE-d\(_{31}\)) (21). We have carried out similar studies, and in this paper describe the use of \(^2\text{H}\) NMR to compare the \( \text{L}_\alpha \) and \( \text{H}_n \) phases of 1-perdeuteriopalmitoyl-2-linoleoyl-\( \text{sn-glycero-3-phosphoethanolamine} \) (PLPE-d\(_{31}\)). These results clearly indicate that the orientational order is less for the \( \text{H}_n \) phase compared to the \( \text{L}_\alpha \) phase. The differences in the order parameters between the phases arise from changes in the acyl chain packing, which we relate to the radius of curvature in the \( \text{H}_n \) phase. The information obtained is in agreement with and extends current knowledge of the structure of hexagonal phases (7,8).

**Materials and Methods**

Palmitic acid (Sigma grade) was obtained from Sigma (St. Louis, MO) and was perdeuterated by passage of deuterium gas over the sample in the presence of a 10% palladium on charcoal catalyst (Aldrich, Milwaukee, WI) as described (16). 1-perdeuteriopalmitoyl-2-linoleoyl-\( \text{sn-glycero-3-phosphoethanolamine} \) (PLPE-d\(_{31}\)) was synthesized as follows: First, 1,2-diperdeuteriopalmitoyl-\( \text{sn-glycero-3-phosphocholine} \) (DPPC-d\(_{62}\)) was prepared by acylating the cadmium chloride adduct of \( \text{sn-glycero-3-phosphocholine} \) with the anhydride of palmitic acid-d\(_{31}\) (22,23). Next, 1-perdeuteriopalmitoyl-2-linoleoyl-\( \text{sn-glycero-3-phosphocholine} \) (PLPC-d\(_{31}\)) was prepared by treating DPPC-d\(_{62}\) with snake venom phospholipase A\(_2\) from \textit{Crotalus adamanteus} (E.C. 3.1.1.4) (Sigma, MO), followed by acylation of the \( \text{sn-2} \) position with linoleic acid obtained from Nu Chek Prep, Inc. (Elysian, MN). Finally transphosphatidylation of the PLPC-d\(_{31}\) was carried out using phospholipase D (from locally obtained Savoy cabbage) in the presence of ethanolamine to yield the desired PLPE-d\(_{31}\) product (24,25). The PLPE-d\(_{31}\) was purified using silica gel chromatography and gave a single spot upon TLC analysis in CHCl\(_3\)-MeOH:H\(_2\)O (65/35/5). The product was dried under high vacuum, transferred to a 8 mm test tube, and mixed with 50 wt % buffer comprising 20 mM MOPS and 1 mM EDTA in deuterium depleted...
water, pH = 7.1. It was then vortexed lightly (< 1 min) and freeze-thawed five times to ensure homogeneity.

The $^2$H NMR spectra were acquired with a General Electric GN-300 spectrometer (Freemont, CA) operating at a magnetic field strength of 7.05 tesla ($^2$H frequency of 46.1 MHz), using a home-built, high-power probe with a horizontal solenoid inductor. A phase-cycled quadrupolar echo pulse sequence was employed, with a 1.8 μs 90° pulse and a recycle time of 0.5 s (26,27). For the liquid-crystalline phase 2000 transients were acquired with a digitization dwell time of 2 μs, whereas 10000 acquisitions were used for the reversed hexagonal phase with a dwell time of 12 μs. Both quadrature channels were employed, taking care to initiate Fourier transformation at the maximum of the quadrupolar echo. The data were then transferred to a Digital Equipment Corporation Microvax II computer for off-line processing. All spectra were numerically deconvoluted, that is de-Paked, to obtain subspectra corresponding to the $\theta$=0° orientation of either the bilayer normal for the Lα phase or the cylinder axis for the HII phase, relative to the main magnetic field (28,29). In the case of the Lα phase, the C-2H bond segmental order parameters, $S_{CD}^{(i)}$, of the de-Paked $^2$H NMR spectra were calculated from the quadrupolar splittings using the relation

$$\Delta v_Q^{(i)} = \frac{3}{2} \frac{e^2qQ}{h} \left( \frac{3 \cos^2 \theta - 1}{2} \right) |S_{CD}^{(i)}|, \quad (1)$$

where $(e^2qQ/h) = 170$ kHz. For hexagonal phases there is an additional axis of symmetry, about which motions are averaged, which corresponds to the cylinder axis (30). Therefore an additional rotation of coordinates must be made to determine the segmental order parameters, and equation (1) becomes

$$\Delta v_Q^{(i)} = \frac{3}{2} \frac{e^2qQ}{h} \left( \frac{3 \cos^2 \zeta - 1}{2} \right) \left( \frac{3 \cos^2 \theta - 1}{2} \right) |S_{CD}^{(i)}|. \quad (2)$$

Here $\zeta$ is the angle between the average orientation of the molecular long axis (identical to the bilayer normal in the Lα phase) and the cylinder axis. This angle is assumed to be $\zeta$=90°, and thus in the absence of other effects the quadrupolar splittings, $\Delta v_Q^{(i)}$, in the HII phase are reduced by a factor of one-half relative to the values observed for the Lα phase.

In equations (1) and (2) the C-2H bond segmental order parameters are defined by

$$S_{CD}^{(i)} = \frac{1}{2} <3\cos^2 \beta_i - 1>, \quad (3)$$

where $\beta_i$ is the angle between the C-2H bond direction at any instant and the local axis of motional symmetry, i.e. the lamellar normal for the Lα phase or the local normal to the cylinder axis for the HII phase. The brackets indicate an average over all conformations sampled on the $^2$H NMR time scale. The segmental order parameters can then be used to estimate the average length of the acyl chain $<L>$ and average chain cross-sectional area $<A>$, using a simple statistical model developed by Schindler and Seelig (17) and later modified by Salmon et al. (16). See Thurmond et al. (20) for complete details on the method of calculation.

Results and Discussion

Figure 1 shows de-Paked $^2$H NMR spectra, representing the $\theta$ = 0° orientation of the motional symmetry axis relative to the magnetic field, for an aqueous dispersion containing 50 wt% PLPE-d31 in the Lα and HII phases; excess water is present in both cases. Compared to the
Figure 1. Representative de-Paked $^2$H NMR spectra of aqueous dispersions of 50 wt% PLPE-d$_{31}$ containing 20mM MOPS buffer (pH=7.1) in (a) the L$_a$ phase at 50°C and (b) the H$_II$ phase at 60°C. The de-Paked spectra correspond to the $\theta=0^\circ$ orientation of the motional symmetry axis with respect to the magnetic field. In the L$_a$ phase the symmetry axis is the bilayer normal, whereas in the H$_II$ phase it is the cylinder axis. Note that the frequency axis for the L$_a$ phase (a) is twice that of the H$_II$ phase (b).

L$_a$ phase (part a of Figure 1), the quadrupolar splittings in the H$_II$ phase (part b) are reduced as is expected; cf. equations (1) and (2). However the reduction is greater than the factor of one-half anticipated from geometrical considerations alone. We have also carried out preliminary $^2$H NMR transverse relaxation rate, $R_2$, studies of PLPE-d$_{31}$ in both phases, and have found that the $R_2$ rates are higher in the H$_II$ phase than in the L$_a$ phase (31). Use of a simple model for the motions (30-32) indicates that the change in relaxation rate reflects an additional relaxation mechanism due to diffusion of the lipids around the water cylinders.

Using equations (1) and (2), the segmental order parameters, $S_{CD}^{(l)}$, can be extracted from the splittings of the de-Paked $^2$H NMR spectra. Figure 2 shows that the values of the segmental order parameters for PLPE-d$_{31}$ in the H$_II$ phase (60°C) are substantially less than in the L$_a$ phase (50°C). This decrease is more than expected if the change were due only to the increased temperature. It is also apparent that the shapes of the order profiles are different, and that the segmental order parameters, $S_{CD}^{(l)}$, decrease more gradually down the chain for the H$_II$ phase than...
Figure 2. Segmental order parameters, $S_{\text{CD}}^{(i)}$, as a function of acyl chain position ($i$) for an aqueous dispersion of 50 wt% PLPE-d$_{31}$ containing 20 mM MOPS buffer (pH=7.1) in the L$_a$ (●) and H$_n$ (○) phases at 50°C and 60°C, respectively. The values of $S_{\text{CD}}^{(i)}$ were derived from the quadrupolar splittings using equations (1) and (2) of the text. The segmental ordering in the reversed hexagonal, H$_n$, phase (a) is lower compared to the L$_a$ phase, and the change in $S_{\text{CD}}^{(i)}$ is more gradual as a function of chain position.

for the L$_a$ phase. Similar behavior has been observed for 1-perdeuteriopalmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE-d$_{31}$) in the H$_n$ phase (21).

One can hypothesize that the dissimilarities in the segmental order profiles originate from differences in packing of the acyl chains associated with the geometries of the two phases. Israelachvili et al. (33) introduced the idea that lipids have certain characteristic average shapes such as cones, truncated cones, and cylinders, which reflect which phases will be formed. Others have argued that these average shapes reflect the actual phases instead of properties of the lipids themselves (7). When packed in a bilayer arrangement, the lipid average shape approximates a cylinder, in which the cross-sectional area of the acyl chains is nearly the same as that at the lipid/water interface. However for lipids packed in the H$_n$ phase, with their polar headgroups inside and hydrocarbon chains outside the cylindrical aggregates, the average lipid shape would then approximate a frustum of a right circular cone, in which the area farther down the chain is now greater than at the lipid/water interface. We propose that the difference in average molecular shape as governed by the aggregate geometry corresponds to the differences in the order profiles between the two phases. If the area at the lipid/water interface is approximately the same in the two cases, and the volume is constant, the acyl chains would become shorter on average and occupy greater cross-sectional area, in order to pack in the H$_n$
phase compared to the $L_a$ phase. The larger area allows for a greater number of possible conformations of the acyl chains in the $H_n$ phase compared to the $L_a$ phase, leading to a reduction of the order parameters in the $H_n$ phase.

We propose that the differences in the order parameters between the $H_n$ and the $L_a$ phases reflect a change in the cross-sectional area of the acyl chains, and that the order profiles can be used to determine some of the structural features of the $H_n$ phase relative to the $L_a$ phase. It is assumed that the average shape of a lipid in the $H_n$ phase is a frustum of a right circular cone. The molecular volume can be approximated from density measurements and x-ray studies (34-38). The height is the sum of $<L>_m$ as determined from the $^2$H NMR order profile in the $H_n$ phase and the projected length of the headgroup $<L>_{head}$ (37). We assume that the area at the lipid-water interface, $<A>_{L_a}$, is the same in the $H_n$ phase as in the $L_a$ phase, and that one can obtain this quantity from the $^2$H NMR order profile in the $L_a$ phase (20). Thus knowledge of the $^2$H NMR order profiles for both the $H_n$ and $L_a$ phases is necessary. For the $H_n$ phase, the smallest cross-sectional area of the frustum is the area at the lipid-water interface. Using simple geometrical relations one can compute the largest cross-sectional area of the frustum and the height of the corresponding right circular cone. We calculate for PLPE-d$_{31}$ in the $H_n$ phase that the molecular volume (34,37) is approximately 1415 Å$^3$, the total projected molecular length is $<L>_m + <L>_{head} = 11.3 + 7.6 = 18.9$ Å, and that the cross-sectional area at the lipid/water interface, $<A>_{L_a}$, is 71.8 Å$^2$. It follows that the height of the cone is 37.0 Å; subtracting the total projected length of the molecule gives $R_1 = 18.1$ Å as the radius of curvature of PLPE-d$_{31}$ in the $H_n$ phase at 60°C. This approach may overestimate the radius of curvature because we are ignoring the curvatures at the bases of the frustum. Furthermore we do not have a complete understanding of the order profiles in the $H_n$ phase, and thus cannot evaluate the cross-sectional area at any given segment, nor can we calculate the curvature at any position along the chain. An alternative as discussed by Thurmond et al. (20) is that only the plateau carbons of the $L_a$ phase order profile (cf. part a of Figure 3) reflect the cross-sectional area at the lipid/water interface. If this is true then the value for the cross-sectional area, $<A>_{L_a}$, would decrease from 71.8 Å$^2$ to 62.5 Å$^2$, leading to a decrease in $R_1$ of 2.6 Å. Either of the calculated values for $R_1$ are in qualitative agreement with low-angle x-ray diffraction data on a closely related lipid, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) in the $H_n$ phase (8,9).

To conclude, the segmental order parameters reflect differences in the packing of lipids in the reversed hexagonal phase relative to the $L_a$ phase. In addition, the experimental $^2$H NMR order parameters can be used to calculate the radius of curvature in the $H_n$ phase. The calculations presented here are model-dependent and rely on major but we believe reasonable assumptions. However the most important idea is the method used to calculate the radius of
curvature and not the actual numbers themselves, which we view as only rough estimates of the actual value. The fact that the results agree with x-ray diffraction measurements for related systems, which depend on separate assumptions, is comforting. Consequently it appears that $^2$H NMR spectroscopy is a promising tool for investigating the structural properties of hexagonal phases.

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