Freezing of Phosphocholine Headgroup in Fully Hydrated Sphingomyelin Bilayers and Its Effect on the Dynamics of Nonfreezable Water at Subzero Temperatures*

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Differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR) spectroscopy are applied to characterize the nonfreezable water molecules in fully hydrated D2O/sphingomyelin at temperatures below 0°C. Upon cooling, DSC thermogram displays two thermal transitions peaked at -11 and -34°C. The high-temperature exothermic transition corresponds to the freezing of the bulk D2O, and the low-temperature transition, which has not previously been reported, can be ascribed to the freezing of the phosphocholine headgroup in the lipid bilayer. The dynamics of nonfreezable water are also studied by 2H NMR T1, (spin-lattice relaxation time) and T2 (spin-spin relaxation time) measurements at 30.7 MHz and at temperatures down to -110°C. The temperature dependence of the T1, relaxation time is characterized by a distinct minimum value of 2.1 ± 0.1 ms at -30°C. T2, is discontinuous at temperature around -70°C, indicating another freezing-like event for the bound water at this temperature. Analysis of the relaxation data suggest that nonfreezable water undergoes both fast and slow motions at characteristic NMR time scales. The slow motions are affected when the lipid headgroup freezes.

In the past 2 decades, the approach to study the mobility of bound water, i.e., water in intimate association with the lipid headgroup, involved mainly the study of NMR parameters as a function of water content (1–3). At low water content, however, the molecular packing of phospholipid molecules in the bilayer could be altered, since the phase transition temperature of phospholipids increases markedly with decreasing water content (4). Furthermore, 31P NMR spectra of dipalmitoylphosphatidylcholine (DPPC) exhibit different line shape and line width at different water content (5), suggesting that the dynamics of the phospholipid headgroup are also perturbed as a result of low water content. Since the dynamics of phospholipid molecules are expected to affect the NMR relaxation behavior of the bound water at ambient temperature, it is essential to monitor the motional state of phospholipid headgroup if the dynamics of the bound water are to be studied.

An alternative approach for the investigation of the bound water dynamics is by gradually freezing out different parts of molecular dynamics in the phospholipid-water systems at subzero temperatures. For instance, when one cooled the hydrated phosphatidylcholine from 25°C to -50°C, the correlation time corresponding to the rotational diffusion of DPPC headgroup increased from 10-4 to ~10-6 s to a value satisfying the rigid lattice condition as revealed by the line shape analysis of 31P NMR spectra (6). Since bulk free water is frozen at a lower temperature, the NMR signals of nonfreezable water, i.e., bound water, of phospholipid molecule will emerge as a result of different spin-lattice and spin-spin relaxation behavior between bulk and bound water. The information about the dynamics of bound water can then be deduced from NMR relaxation measurements as a function of temperature at the water-lipid interface. This approach has been used in NMR studies of supercooled water under high pressure and of nonfreezable water in concentrated electrolyte solution or reversed micelles (7–9).

In this paper, we applied DSC and NMR techniques to study the nonfreezable water in fully hydrated sphingomyelin bilayers at subzero temperatures. Sphingomyelin was chosen because of its low mobility in the gel state due to the amide bond in the interface region (10) and because of its phosphocholine headgroup identical to that of phosphatidylcholine. A new lipid thermal transition was detected by DSC at -34°C. It was shown by 31P NMR as a result of the freezing of phosphocholine headgroup motion. The dynamics of nonfreezable water were also studied by T1 and T2, relaxation measurements as a function of temperature by 2H NMR. The results shed new light on the effect of lipid headgroup freezing on the dynamics of nonfreezable water in lipid bilayers at subzero temperature.

**MATERIALS AND METHODS**

Egg sphingomyelin (Avanti Polar Lipids Inc., Birmingham, AL) samples used in this study contain 28, 36, and 50% D2O by weight (percent D2O weight of total weight of D2O/sphingomyelin mixtures). They were all fully hydrated as indicated by the invariance of lipid phase transition temperature of the samples.

**DSC Measurement—**Calorimetry was performed with a Du Pont 910 DSC system. Hydrated sample (1–5 mg) was hermetically sealed in aluminum DSC pan. A scan rate of 0.5°C/min was used over the temperature range from +55°C to -85°C in both heating and cooling mode. Enthalpy measurements were determined from the area under transition peak by comparison with those of known standard (indium). At the end of DSC scans, lipids were then assayed for phosphate content.

**NMR Measurements—**All NMR spectra were obtained on a Bruker MSL-300 spectrometer with a 4.7-Tesla (30.7 MHz for 2H and 80.9 MHz for 31P) superconducting magnet using a broadband probe head.

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1 The abbreviations used are: DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DSC, Differential scanning calorimetry; T1, spin-lattice relaxation time; T2, spin-spin relaxation time measured by two pulse echo; T1*, spin-spin relaxation time measured by line width.
\(^{3}\)H NMR spectra were recorded by a quadrupolar echo pulse sequence (11) with full phase cycling and recycle delays of 100 ms. Due to spectrometer limitations, the width of a 90° pulse was 7 μs. The wide-line \(^{3}\)H NMR spectra obtained by such soft pulses were distorted (12); consequently, no attempt was made to extract dynamic information from the details of \(^{3}\)H NMR line shapes at temperatures below -70 °C.

\(T_2\) was measured by varying the interpulse delay time from 20 to 1000 μs depending on the studied temperatures. Semilog plots of echo intensities against the interpulse delay times are linear for most temperature studies with the exception of those performed near -70 °C or the freezing temperature of bound water. For temperatures between -11 and -65 °C, \(^{3}\)H NMR spectrum exhibits a single Lorentzian line shape. The apparent spin-spin relaxation time \(T_2^*\) can then be estimated from line width at half-height within this temperature range. \(T_1\) was measured by an inversion recovery method. The NMR signal intensity was determined by integrating the Fourier-transformed absorption spectrum.

\(^{31}\)P NMR spectra were recorded at 80.9 MHz by employing a single pulse. The experimental conditions correspond to 60 ° pulse of 5 μs and 3 s for the recycle delay. Proton decoupling was not used because of the lack of decoupling channel in the probe. Although this experimental condition caused severe line broadening on the \(^{31}\)P NMR spectra, one can still conclude whether the phospholipid headgroup is frozen or not from the invariance of line shape below a certain temperature. The rigid-limit \(^{31}\)P NMR spectrum of phosphocholine powder (13) is known to show the chemical shift tensor with the principal tensor elements \(\delta_1 = -100, \delta_2 = -30,\) and \(\delta_3 = 130 \text{ ppm}\). Upon hydration of phosphocholine lipid molecules (14), the tensor values are partially averaged out, resulting in an axially symmetrical pattern with \(\delta_1 = -40\) and \(\delta_2 = 20 \text{ ppm}\). Therefore, freezing of phospholipid headgroup can be detected if an axially symmetrical powder pattern line shape becomes asymmetric and reaches rigid lattice limit.

**RESULTS**

**Characterization of the Freezing Event of Phosphocholine Headgroup**—Fig. 1 shows representative DSC cooling and heating scans of fully hydrated (36%) D₂O/phosphatidylcholine between +12 and -82 °C. Similar results were obtained using 28 and 50% samples. A separate run in the heating mode on the same sample showed the gel to liquid-crystalline transition of the hydrocarbon chain in the sphingomyelin lamella occurring at 38 °C (data not shown). It should be pointed out that the gel to liquid-crystalline phase transition of D₂O/phosphatidylcholine sample was 1 °C higher than that of H₂O/phosphatidylcholine sample. The freezing and melting of interbilayer free water (15) can be assigned to correspond to the observed transitions at -11 and +4 °C, respectively. An expansion of part of the DSC cooling scan reveals an exothermic peak at -34 °C with a width at half-height \(\Delta T_{1/2}\) of 2 °C.

**Characterization of Bound Water**—As shown in Fig. 2B, water molecules (D₂O) are motionally anisotropic in the studied samples at room temperature as demonstrated by the \(^{2}\)H NMR quadrupolar splitting of interbilayer free water. Similar \(^{2}\)H NMR spectra with a characteristic Pake doublet have been reported for other D₂O/lipid system (3). \(^{31}\)P NMR spectrum of D₂O/phosphatidylcholine at -42 °C, however, exhibits a Lorentzian line shape with much broader line width. By further decreasing temperature to -66 °C, two coexisting resonances of deuterons can be seen as judged from the overlapping broad and sharp signals. Similar spectra with two detectable over-
Freezing of Phosphocholine Headgroup

Fig. 2. Representative $^3$P NMR (A) and $^2$H NMR (B) spectra for sphingomyelin and D$_2$O, respectively, in fully hydrated (28%) condition. Dotted lines in $^3$P NMR spectra are simulated spectra using modified Freed's slow motion NMR line shape analysis (6). A frequency independent distribution of temperature-dependent line broadening was assumed across each spectrum. The rates of axial diffusion for sphingomyelin at 25, 0, and -25 °C were found to be $3 \times 10^5$, $7.5 \times 10^4$, and $3.8 \times 10^3$ s$^{-1}$, respectively. Angular parameters $\theta$ and $\phi$ used for simulation were 76 and 90° for all three spectra. Note that the scales of $^2$H NMR spectra are different.

Lapping signals can be detected between -65 and -75 °C. The rigid limit lattice spectrum of D$_2$O obtained in this study is represented by $^2$H NMR spectrum at -110 °C. Due to the finite pulse width (7 µs) used in the present study, the line shape was distorted and did not show quadrupolar splitting at the two ends of the spectrum. Since a freezing-like event (H$_2$O becoming a solid, but not in normal ice state) occurs at -50 to -80 °C for bound water in a variety of systems, we conclude that spectral changes observed in the temperature range around -70 °C are associated with the freezing of bound water in phospholipid bilayers.

The effects of the freezing of bulk free water detected at -11 °C are also manifest in the line shape and intensity of the $^2$H NMR spectrum. Shown in Fig. 3 are the two representative $^2$H NMR spectra obtained at temperatures above and below freezing. The characteristic quadrupolar splitting (~700 Hz) line shape at -5 °C becomes a single Lorentzian peak at -14 °C. These two spectra are plotted to depict their relative intensity. It is evident that the spectrum recorded at -14 °C shows a significant loss in the intensity as a result of water freezing. The recycle delay used in this study is much shorter than the very long deuterium $T_1$ of frozen water (>9 s). One can then reasonably assume that when the bulk water is frozen below -11 °C, it no longer contributes the signal intensity to the obtained $^2$H NMR spectra due to severe saturation. In addition, the size of the spectral splitting for the frozen water has been known to be in the range of 140 KHz (17), which is much larger than the line width of the presently detected spectra between -11 and -65 °C. The latter explanation, however, may not warrant the invisibility of the bulk water in view of the recent observation of very narrow $^2$H NMR spectrum as a result of tetrahedral reorientation of the D$_2$O (18). Finally, it should be pointed out that the motion of the bound water will affect the signal intensity and therefore would affect any estimate of the number of bound water per lipid based on intensity differences before and after freezing.

Fig. 3. $^2$H NMR spectra of D$_2$O at temperatures above (A) and below (B) freezing for 28% D$_2$O/sphingomyelin. Significant change of the line shape and integrated intensity of $^2$H NMR spectrum can clearly be seen above and below bulk water freezing temperature.

Effect of Headgroup Freezing on the Dynamics of Bound Water—Based on the above observations, dynamics of non-freezable water can now be characterized by $T_1$ and $T_2$, relaxation experiments in $^2$H NMR. In Fig. 4, values of $T_1$, $T_2$, and $T_3$ are plotted against the inverse temperatures. The $T_1$ minimum of 2.1 ± 0.1 ms was observed at -30 °C. $T_3$ values were detected to be consistently smaller than those of $T_2$, since both $T_1$ and other inhomogeneous factors are expected to broaden the line width. The line connecting the $T_2$...
values shows a change in the slope at $-34^\circ C$ or the determined headgroup freezing temperature, suggesting that the water motions monitored by the $T_{2e}$ are affected by the headgroup freezing (Fig. 4). At temperatures below $-70^\circ C$, all $^2$H NMR spectra exhibit the same line shape as the one recorded at $-110^\circ C$ (Fig. 2). Temperature dependence of $T_{2e}$ shows a reverse profile above and below $-70^\circ C$ (Fig. 4), suggesting that bound D$_2$O would still undergo slow motions even at temperatures below freezing.

**DISCUSSION**

In the present investigation, DSC and NMR techniques were applied to study the thermodynamic and structural properties of fully hydrated sphingomyelin at subzero temperatures. By gradually lowering the temperatures of the sample, a stepwise freezing of the bulk water, the sphingomyelin headgroup, and the bound water was detected successively.

A schematic diagram to illustrate the freezing events at various temperature is shown in Fig. 5. The first event occurring at $37^\circ C$ is the liquid-crystalline to gel phase transition of the hydrocarbon chain in the sphingomyelin lamella. Solid-state high-resolution $^1$C NMR studies show an abrupt change in the chemical shift of hydrocarbon chain from $33$ to $31$ ppm (data not shown). Second event at $-11^\circ C$ upon cooling (or $+4^\circ C$ upon heating) is due to the freezing (melting) of interlamellar bulk D$_2$O. This is best represented by the reduced signal intensity and the change in line shape of the $^1$H NMR spectra at $-14^\circ C$ in comparison with those at $-5^\circ C$ (Fig. 3). The cooperative transition detected at $-34^\circ C$ upon cooling, but not heating, has not been reported before. According to our $^3$P NMR results, phosphocholine headgroups become immobilized in this temperature range. This result is consistent with other investigations by using line shape simulation study of $^3$P NMR spectra for $40\%$ H$_2$O/DPPC mixtures at $-25^\circ C$ and $-50^\circ C$ (6). Therefore, we assign this transition as the freezing of the lipid headgroup, in contrast to the freezing of hydrocarbon chains detected at $37^\circ C$. The last event is the condensation or freezing of D$_2$O near the phosphocholine headgroup. $^3$P NMR studies show the coexistence of two overlapping signals with large differences in their line width. The stepwise freezing events for the interfacial system such as the one depicted in Fig. 5 could then open an avenue for the studies of water activity near the biological macromolecules.

In view of our relaxation data being limited to only one frequency (30.7 MHz) and the complexity of the existing motional model for water in heterogeneous systems (19–21), it is perhaps unwise to carry out theoretical simulation. Nevertheless, useful information can still be obtained by simply judging from the values of $T_1$, $T_{2e}$, and their temperature-dependent profiles. In the studies of the $^3$H spin-lattice and spin-spin relaxation times, two NMR characteristic times are of importance: $\tau_1 = \omega_0^{-1}$ and $\tau_2 = (\epsilon_0 q Q / h)^{-1}$. With the present experimental conditions, the values of these are $\tau_1 (\omega_0 / 2\pi = 30.7$ MHz) $= 5 \times 10^{-8}$ s, $\tau_2 (\epsilon_0 q Q / h = 213$ kHz) $= 1 \times 10^{-6}$ s. The observation of a $(T_1)_{\min}$ in the studied temperature range suggests that the detected water signals are modulated by a motion with characteristic NMR time scale of $\tau_1$. It is also evident from the values of $T_{2e}$ that a slower molecular reorientation with NMR time scale of $\tau_2$ is responsible for the loss of signal intensity during the echo delay periods.

The experimental $(T_1)_{\min}$ value of $2.1$ ms is significantly larger than the theoretical $(T_1)_{\min}$ value of $1.14$ ms at $30.7$ MHz based on the isotropic model (20). The $T_1 / T_2$ ratio obtained at the minimum of $T_1$ also deviate from the theoretical value of $1.6$. This indicates strongly that the motions of unfreezable water in lipid bilayers are complex. It has been proposed that a distribution of correlation time caused by processes such as the exchange between free and bulk domain and/or anisotropic motion near the heterogeneous surface (21–24) should be considered for the bound water near the interface.

Line shape simulations of our $^3$P NMR results indicate that phospholipid headgroup undergoes rotational motion at a rate of $3.5 \times 10^6$ s$^{-1}$ before it freezes. Although this rate is much slower than the characteristic rate of water motions detected by $^3$H NMR, we did observe a change in the slope of $T_{2e}$ as a function of temperature (Fig. 4). It suggests that freezing of phosphocholine headgroup motion might change the slow motional mode of bound water detected by $T_{2e}$ relaxation process. The apparent activation energy of $T_{2e}$ obtained for temperatures above and below $-35^\circ C$ can be determined to be $4.5$ and $2$ kcal/mol, respectively. This corresponds roughly to the activation energy for the breaking of a hydrogen bond but is substantially smaller than the activation energy determined from the dynamical and relaxation processes of ice (18). Future studies using other experimental approach such as field-dependent relaxation (19) or $^1$H/$^2$H spin relaxation (22) should provide useful information to shed more light on the interaction of phosphocholine headgroup with water molecule.

At temperatures below $-70^\circ C$, or the freezing temperature of bound D$_2$O, the $T_{2e}$ values are observed to increase with decreasing temperatures. It suggests that bound D$_2$O undergo some motions with time scale smaller than $10^{-6}$ s (25). Assuming that jump motions dominate the relaxation in this temperature range, the correlation time can be shown to have the numerical value of $T_{2e}$ (26). Therefore, jump rate of deuterium in the random network of hydrogen bond can be estimated to be around $10^4$ s$^{-1}$ for frozen bound water, which is comparable with the recent direct observation of molecular reorientation of ice by proton and deuterium NMR (18).

In summary, nonfreezable water near phosphocholine headgroup has been studied by using $^3$H NMR relaxation measurements as a function of temperature at subzero temperature. Temperature-dependent measurements of $T_1$ and $T_{2e}$ on nonfreezable water suggest the presence of both fast and slow motions with characteristic NMR time scales. Freezing of the polar headgroup motion is also detected by DSC and $^3$P NMR. The slow motions are affected when the lipid headgroup freezes.

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