Structure and Dynamics of Primary Hydration Shell of Phosphatidylcholine Bilayers at Subzero Temperatures

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ABSTRACT Deuterium NMR relaxation and intensity measurements of the ^{2}H-labeled H_2O/dimyristoyl phosphatidylcholine bilayer were performed to understand the molecular origin of the freezing event of phospholipid headgroup and the structure and dynamics of unfrozen water molecules in the interbilayer space at subzero temperatures. The results suggest that about one to two water molecules associated with the phosphate group freeze during the freezing event of phospholipid headgroups, whereas about five to six waters near the trimethylammonium group behave as a water cluster and remain unfrozen at temperatures as low as −70°C. In addition, temperature-dependent T_1 and T_2 relaxation times suggest that dynamic coupling occurs not only between the phosphate group and its bound water, but also between the methyl group and the adjacent water molecules. Based on these observations, the primary hydration shell of phosphatidylcholine headgroup at subzero temperatures is suggested to consist of two distinct regions: a clathrate-like water cluster, most likely a water pentamer, near the hydrophobic methyl group, and hydration water molecules associated with the phosphate group.

INTRODUCTION

It has been known for decades that the study of unfrozen water is useful for our understanding of the property of water molecules tightly associated with surface residues of biomolecules (Kuntz and Kauzmann, 1974; Kuntz et al., 1969). Recent calorimetric, dielectric, small-angle X-ray and NMR experiments indicate that about seven to eight water molecules per phosphatidylcholine (PC) are trapped in the interbilayer space and are unfreezable at least down to the homogeneous nucleation temperature, T_H, of ice formation, i.e., −40°C (Hsieh and Wu, 1995a; Bronshteyn and Steponkus, 1994; Pissis et al., 1993; Grünert et al., 1984; Please note that T_H for heavy water is around −35°C). At lower temperatures, considerable amounts of water molecules remain unfrozen if a methyl group is attached to phosphatidylethanolamine (PE) in the interbilayer space (Hsieh and Wu, 1995b). Interestingly, the rotational motion of the phospholipid headgroup has been known to freeze below −40°C (Hsieh and Wu, 1995c; Wu et al., 1991). The mechanism responsible for the aforementioned freezing event of the phosphate group and the dynamics of interbilayer unfrozen water molecules remain illusive.

Hydrodynamic measurement of PC vesicles indicates that the hydration is limited to only one layer (Wu, 1996), but force measurement between two PC membrane surfaces suggests a long-range repulsive force propagating to 20 Å or even longer (Parsegian and Rand, 1995; McIntosh and Simon, 1994). Although the long-range repulsive force is consistent with mean-field theory of water-mediated polarization near the membrane surface (Marcelja and Radic, 1976), recent computer simulation fails to indicate any water-mediated long-range interaction (Marrink et al., 1993). Nevertheless, an exponential-like decrease in phospholipid-induced polarization was suggested. An NMR investigation into the range of the surface effect on the rotation of the water molecules suggests that only the first layer or two is effected (Woessner, 1980), but other NMR studies of the lipid-water system tend to suggest a surface-induced long-range order of water molecules (Volke et al., 1994; Ulrich and Watts, 1994). Possible reconciliation of the controversy about the origin and range of the detected repulsive force of phospholipid membrane has recently been suggested (Cevc et al., 1995).

The knowledge of hydration and water structure is important in clarifying the interaction between membrane surfaces (Israelachvili and Wennerstrom, 1996). However, our knowledge of the structure of the primary hydration shell in the phospholipid bilayer is limited despite the tremendous effort devoted to this research area (see, for instance, Takahashi, et al., 1989; Cevc, 1993; Volke and Pampel, 1995; Chen et al., 1996). X-ray studies of phospholipid crystals (Pascher et al., 1992) and the related energetic considerations (Vanderkooi, 1991) provide important information regarding the hydration structure near the phosphate group, but it is not known how these hydrated molecules affect the structure or the dynamics of the lipid headgroup. Most of the information regarding the water molecules near the choline group was obtained from computer simulation (Damodaran and Merz, 1994; Tu et al., 1996). Suggestions that a clathrate-like cluster exists near the trimethylammonium group (Damodaran and Merz, 1994) or that water molecules fill in grooves between the methyl groups (Tu et al., 1996) have been made, but there is yet no experimental evidence, to the best of our knowledge, to imply the existence of these structures in PC bilayers. In this study, we present NMR data to shed light on the structure and dynamics of interbilayer waters at subzero temperatures.

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MATERIALS AND METHODS

Spectra and ^{2}H-NMR relaxation times were obtained on a 7.05 T Bruker MSL-300 spectrometer using a broadband probe with a 5-mm insert as described previously (Hsieh and Wu, 1995a–c). Briefly, ^{2}H-NMR spectra were recorded with a quadripolar echo pulse sequence using a 90° pulse length of 2.2–2.5 μs, and the ^{31}P-NMR spectra were recorded with Hahn echo pulse sequence using a 90° pulse of 4 μs in the presence of ^{1}H decoupling. Because the signal intensity of the obtained ^{2}H-NMR spectra is an important parameter in this study, special care was taken to consider the effect of spin-lattice (T_1) and spin-spin (T_2) relaxation time in attenuation of the D_2O signals. Specifically, several ^{2}H-NMR spectra of interbilayer D_2O for each phospholipid sample with known water content were obtained under various interpulse delay (10–1000 μs) using long repetition time (>$5T_1$; 100s–400s, depending on temperatures). Absolute signal intensities (M_o) were then calibrated by considering the respective effect of interpulse delay on the isotropic and Pake doublet signals according to the equation $M(t) = M_o \exp(-2t/T_{2e})$, where T_{2e} is the spin-spin relaxation time measured by two-pulse echo. Because the T_{2e} of isotropic signal is shorter than that of Pake doublet and the apparent lineshape of the Pake doublet remains largely unchanged, we are able to obtain Pake doublet spectra without the superimposed isotropic signal (see, for instance, Fig. 4 in Hsieh and Wu, 1995b). Intensities of superimposed signal can thus be decomposed to allow the estimation of the amount of unfrozen water molecules in the interbilayer space.

The pulse sequence for T_1 measurement was the combination of the inversion recovery and quadripolar echo pulse sequence ($180°_x$ − VD(τ) − $90°_x$ − t_1 − $90°_y$ − t_2 − FID). Typically, 10–12 delay times were used to obtain suitable T_1 value by fitting data using the equation $M(\mathrm{VD}) = M_o - [M_o - M(0)]\exp(-\mathrm{VD}/T_1)$, where M_o is the signal intensity at equilibrium state and $M(0)$ is the intensity at time 0. The T_1 values obtained are slightly higher than those obtained using the equation $M(\mathrm{VD}) = M_o[1 - 2\exp(-\mathrm{VD}/T_1)]$. We applied the former equation to obtain T_1 because M_o was found to be larger than $M(0)$ when the experiments were performed at low temperature. The ^{2}H T_1 were measured at the perpendicular ($\theta = 90°$) edges of the ^{2}H powder pattern for —$N(CD_3)_3$ (Batchelder et al., 1983), and at the central isotropic signal for D_2O.

The correlation time, τ_c, for methyl group reorientation was obtained by using a three-site jump model to analyze the T_1 data, and the activation energies, E_a, were determined from the temperature dependence of τ_c. It has been demonstrated for methyl reorientation in polycrystalline amino acids and peptides that the correlation time of the methyl group reorientation can be obtained by using T_1 measured at $\theta = 90°$ (Batchelder et al., 1983; Torchia and Szabo, 1982). Briefly, a linear least square analysis of the T_1 data was performed by using the following equations:

$$T_1^{-1} = (\pi^2/3)(e^2qQ/h)^2[J(\omega) + 2J(2\omega)] \quad (1)$$

$$J(\omega) = \tau/(1 + \omega^2\tau_c^2) \quad (2)$$

$$\tau_c = \tau_o\exp(-E_a/RT) \quad (3)$$

where ω is the Larmar frequency.

The spin-spin T_2 relaxation time was measured by the two-pulse quadripolar echo using variable echo delay times (Pauls et al., 1985). The echo intensity of water signals can be fitted by assuming a single relaxation time, but more than one relaxation parameter is needed to fit the signal intensity from lipid. In the latter case, the initial rate of the intensity decay was used for T_2 measurements.

Samples in this work were commercially available (Avanti Polar Lipids Inc; Alabaster, AL): dimyristoylphosphatidylcholine (DMPC), perdeuterated-choline d_{13}-DMPC, and DMPC selectively deuterated at both C_α and C_β methylene segments (d_4-DMPC). Spectroscopic grade D_2O and phosphorylcholine chloride salt selectively deuterated at Cγ methyl group (d_9-phosphorylcholine) were from Cambridge Isotope Laboratory.

Lipids with different hydration states were prepared gravimetrically in preweighed NMR tubes from known amount of lyophilized lipid. Lipid lyophilized overnight in chloroform under high vacuum was assumed to contain about two water molecules per lipid (Ulrich and Watts, 1994; Cevc, 1993). In general, estimated error of the reported hydration value was ±1 water molecule per lipid. The possible error introduced by the remaining amount of water molecules becomes less significant when samples with larger water content (up to 40 water per lipid) were used to quantitate the amount of unfrozen water molecules at subzero temperatures.

The temperature of the samples was controlled by evaporation of N_2 gas from a liquid nitrogen dewer and monitored by a Bruker VT-1000 thermal system. The estimated temperature variation is ±2°C. The temperature-dependent NMR spectra were obtained during both the cooling and heating processes to check the reversibility of the spectra. Generally, if freshly prepared samples were cooled to −120°C at a cooling rate of ~1°C/min, reproducible spectra were always obtained during the heating mode.

RESULTS

Correspondence between the freezing event of the PC headgroup and its associated water molecules

Fig. 1 shows ^{31}P and ^{2}H-NMR spectra obtained from d_4-DMPC and d_{13}-DMPC at a hydration state of eight water molecules per lipid. The spectra represent the dynamic states of three different regions, i.e., phosphate (*left panel*), methylene (*center panel*) and trimethylammonium moiety (*right panel*) of the PC headgroup at the indicated temperatures. Both phosphate and methylene groups can be seen to be devoid of rotational motion at −60°C inasmuch as their spectra exhibit a powder pattern with respective chemical shift anisotropy (CSA) and quadripolar splitting ($\Delta\nu_Q$) values close to rigid limit. The methyl and trimethylammonium groups, however, still undergo significant rotation along their respective symmetric axes (Hsieh and Wu, 1995a). All spectra obtained from freshly prepared samples were reversible during the heating and cooling mode.

The ^{2}H-NMR signals of water from D_2O/DMPC samples with excess water, however, were found to be irreversible (Fig. 2 *A*). This can be seen by the absence of a sharp isotropic signal in the spectra obtained during the heating mode at temperatures above −35°C. These signals are, however, visible in the spectra obtained during the cooling mode. The sample with 10 water molecules per lipid still exhibited a residual sharp isotropic signal (data not shown). This suggests that the bulk water molecules, i.e., those corresponding to the sharp isotropic signal, may be undercooled in the temperature range from 0°C to −35°C, the melting (T_M) and homogeneous nucleation (T_H) temperatures of ice, respectively.

When the sample with a hydration ratio of eight water molecules per lipid was used, there was no sharp isotropic signal for the spectra obtained during both cooling and heating scans (Fig. 2 *B*). Because the spectra obtained during the heating scan were essentially the same as those obtained for samples with excess or with eight water molecules per lipid at temperatures above −35°C, it suggests that the excess water is excluded out of the interbilayer space during the freezing process. For samples with hydration state of six or seven water molecules per lipid, the spectra deviate slightly (data not shown) from the spectra

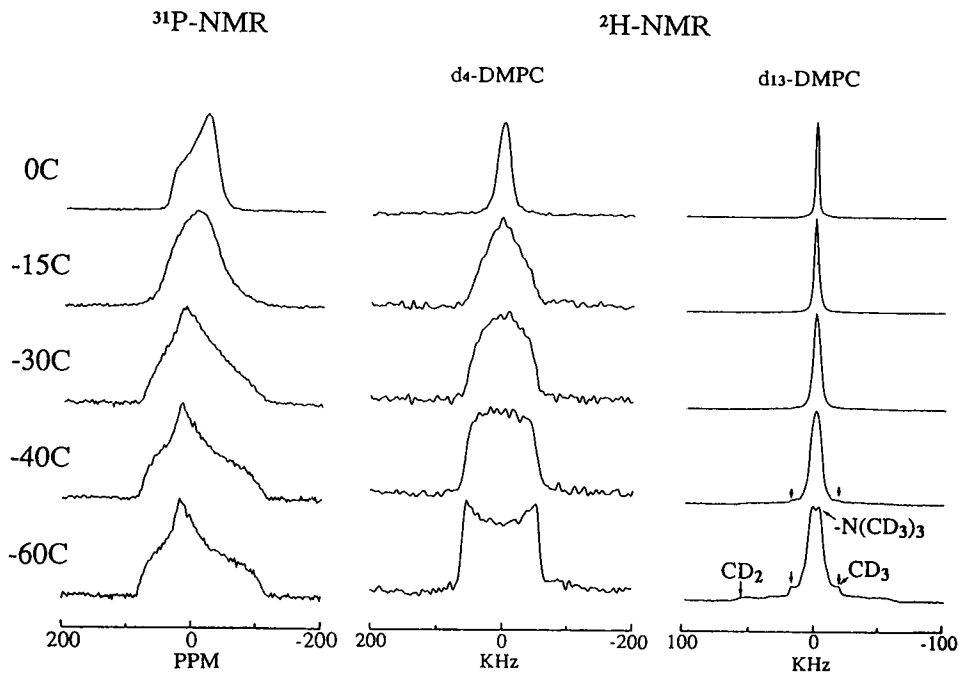

FIGURE 1 Representative ^{31}P and ^{2}H-NMR spectra of selectively deuterated dimyristoyl phosphatidylcholine bilayer with a hydration state of eight water molecules per lipid at indicated temperatures.

obtained from samples after freeze-induced dehydration process, but they are reversible during both the cooling and heating processes. We should point out that the spectra for the sample with eight water molecules per lipid is not completely reversible, although there is no detectable isotropic signal in the spectra (compare right column with left column in Fig. 2 *B*). Taken together, these observations suggest that about seven to eight water molecules per lipid are trapped and remain in equilibrium in the inter-bilayer space during the freezing-induced dehydration process applied in this NMR study. It should be emphasized that a further dehydration of the sample may occur under stronger dehydration force for a prolonged period, e.g., overnight deep-freezing ($<-70°C$).

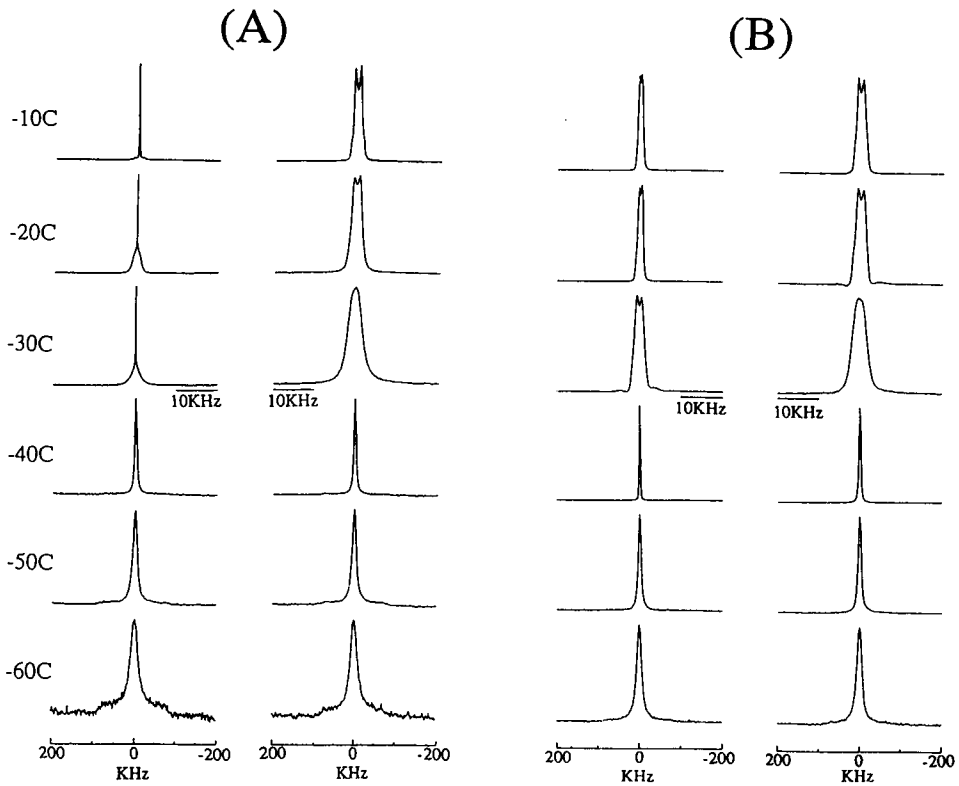

FIGURE 2 ^{2}H-NMR spectra of D_2O/DMPC with hydration states of (*A*) 20 and (*B*) 8 water molecules per lipid. The spectra in the left and right column of each panel were obtained during the cooling and heating process, respectively.

The water content of the freeze-induced dehydration state of our sample is slightly lower than the water content, i.e., ~8.6 ± 0.1 water molecules per DPPC, estimated in the sub-gel, or L_c "crystal," phase of phospholipid bilayers (Nagle and Wiener, 1988). They are much lower than the water content, i.e., 19 and 25 water molecules per DPPC, in the respective $L_{\beta'}$ and L_α bilayer phase (Ruocco and Shipley, 1982). Because the headgroup dehydration is also known to play a predominant role in the formation of sub-gel phase (Wu et al., 1985), it appears that the bilayer organization and the hydrocarbon packing of our sample may share some resemblance with that determined in L_c sub-gel phase.

To understand more about the properties of these trapped unfrozen water molecules, we first characterized the amount of unfrozen water molecules as a function of temperature for sample with eight water molecules per lipid (Fig. 3). As shown by the closed diamond in the figure, the signal intensity of unfrozen water in PC bilayers exhibits two stages of "transition": a sudden decrease in the signal intensity occurs around −35°C, which is followed by a slow decrease in the signal intensity till about −80°C. In the following result, we present evidence to indicate that the initial water freezing event that occurs near −35°C is mainly responsible for the previously identified freezing of the phospholipid headgroup (Hsieh and Wu, 1995c; Wu et al., 1991), whereas the event corresponding to the slow decrease of the isotropic water signal is a general property of water molecules near the hydrophobic methyl group below −35°C.

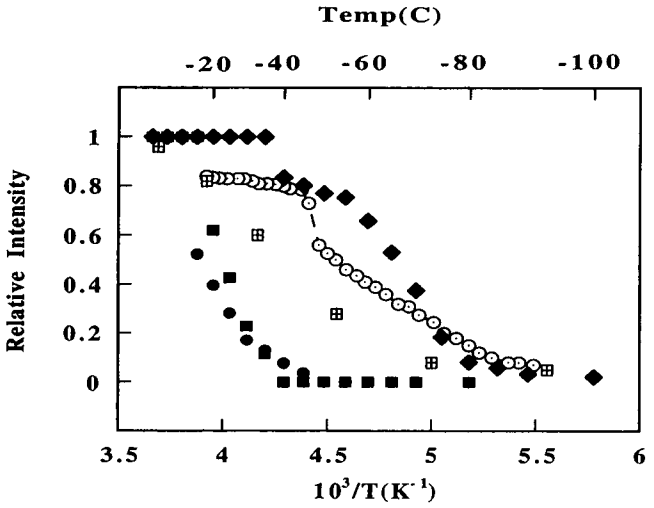

FIGURE 3 Quantitation of the relative intensity of the isotropic ^{2}H-NMR signals of D_2O as a function of temperature: (◆), PC; (●), PE; (■), PA; (⊙), MCM-41; (⊠), crambin polycrystal. The data points for MCM-41 and crambin were obtained from Hansen et al., 1995, and Usha and Wittebort, 1989. The detected apparent "transition" temperature for MCM-41 samples appear to shift to lower temperature than those for PC bilayer. This temperature shift is consistent with isotope effect in the shift of T_H because H_2O was used in MCM-41 pore, whereas D_2O was used in PC bilayers.

First, there is no detectable isotropic D_2O signal for PE and phosphatidic acid (PA) lipid bilayers at temperatures below −35°C (Hsieh and Wu, 1995c; see also closed circles and squares in Fig. 3). In addition, ^{31}P-NMR spectra of PC, PE, and PA all exhibit characteristic axially asymmetric lineshape with CSA values close to rigid limit at temperatures below −40°C, an indication that the rotational motion of phosphate freezes around a similar temperature range.

Second, we exclude the possible involvement of fatty acyl chain in the detected freezing event by showing that phosphorylcholine salt also exhibits similar behavior. As shown in Fig. 4, a dramatic sharpening of the ^{2}H-NMR line width occurs for —$N(CD_3)_3$ in spectra for both phosphorylcholine salt and PC bilayers in the heating mode from −70°C to −30°C.

Finally, Hansen et al. have reported that the signal intensity of H_2O in MCM-41, one-dimensional mesopores from alumino-silicate gels of quaternary ammonium surfactants (C_nH_{2n+1}—$N(CH_3)_3$), decreased slowly until it disappeared in the temperature range −70°C to −80°C (Hansen et al., 1995; open circles in Fig. 3). Because MCM-41 also consists of methylated ammonium group in the mesopore, the slow decrease of the signal intensity appears to be a property of water molecules near the trimethylammonium group in both PC bilayer and MCM-41 pore.

It is interesting to note that the presence of unfrozen water at temperature below T_H is not limited to lipid or mesopore systems. As shown in Fig. 3, there is also detectable unfrozen water signal at temperatures below −40°C for proteins such as crambin polycrystal (Usha and Wittebort, 1989). Interestingly, the water pentamer ring, which we propose to account for the dynamic behavior of unfrozen water molecules near the trimethylammonium group, has been detected near the methyl group of Leu residue by X-ray diffraction (Teeter, 1984).

Quantitation of unfrozen water molecules near the trimethylammonium group

After the determination of the molecular origin of the dynamic behavior of unfrozen water molecules, we now quantitate the number of water molecules involved in the two "transitions" implied by Fig. 3. Because the signal intensity of unfrozen water was found to drop by about 20% from −35°C to −40°C, and about seven to eight water molecules per lipid were found to remain in the interbilayer space during the freeze-induced dehydration process, it can be estimated that about one to two water molecules are involved in the process occurring from −35°C to −40°C. The number of remaining unfrozen water molecules at −40°C can then be estimated to be about six water molecules per lipid.

As further evidence for the estimated amount of unfrozen water molecules, we quantitated the amount of unfrozen water molecules by obtaining fully relaxed NMR spectra of samples with different degrees of hydration (Fig. 5 *A*). After

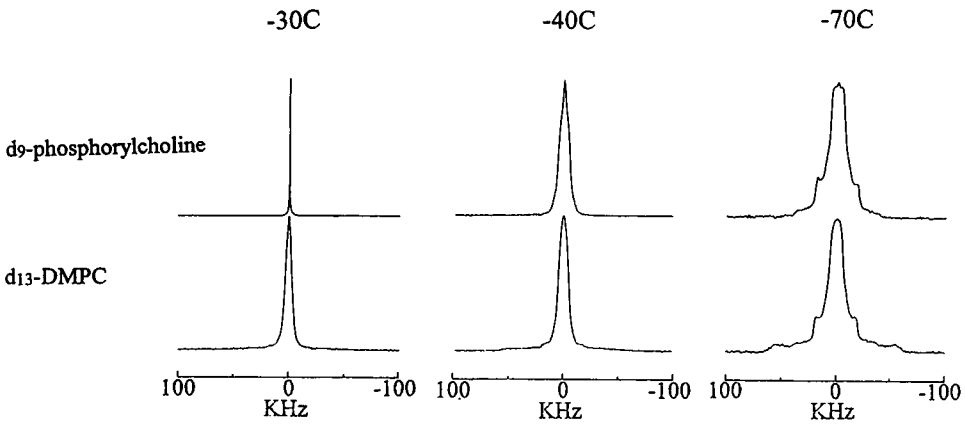

FIGURE 4 Effect of the fatty acyl attachment on the ^{2}H-NMR spectra obtained from deuterated phosphorylcholine salt and PC bilayers at the indicated temperatures. The additional broad signal of the spectra obtained from d_{13}-DMPC is due to its additional deuterated methylene group as compared with d_9-phosphorylcholine. ^{2}H-NMR spectra obtained for —$N(CD_3)_3$ of both deuterated phosphorylcholine salt and DMPC bilayer were similar at temperatures below −40°C, but a much sharper linewidth of d_9-phosphorylcholine was detected at temperatures above −40°C as compared with that of deuterated PC.

correcting for the signal intensity loss due to the spin-spin relaxation process (Fig. 5 *B*; please see Materials and Methods for details), we estimated that about five or six water molecules remained unfrozen at −40°C. This result is in excellent agreement with the previously estimated number of six water molecules per lipid. It can be concluded that about five or six water molecules, which were assigned to be present near the trimethylammonium group, remain unfrozen after the freezing of the one or two water molecules that are associated with the phosphate group occurs.

We also noted that the apparent number of estimated unfrozen water molecules decreases slightly at lower tem

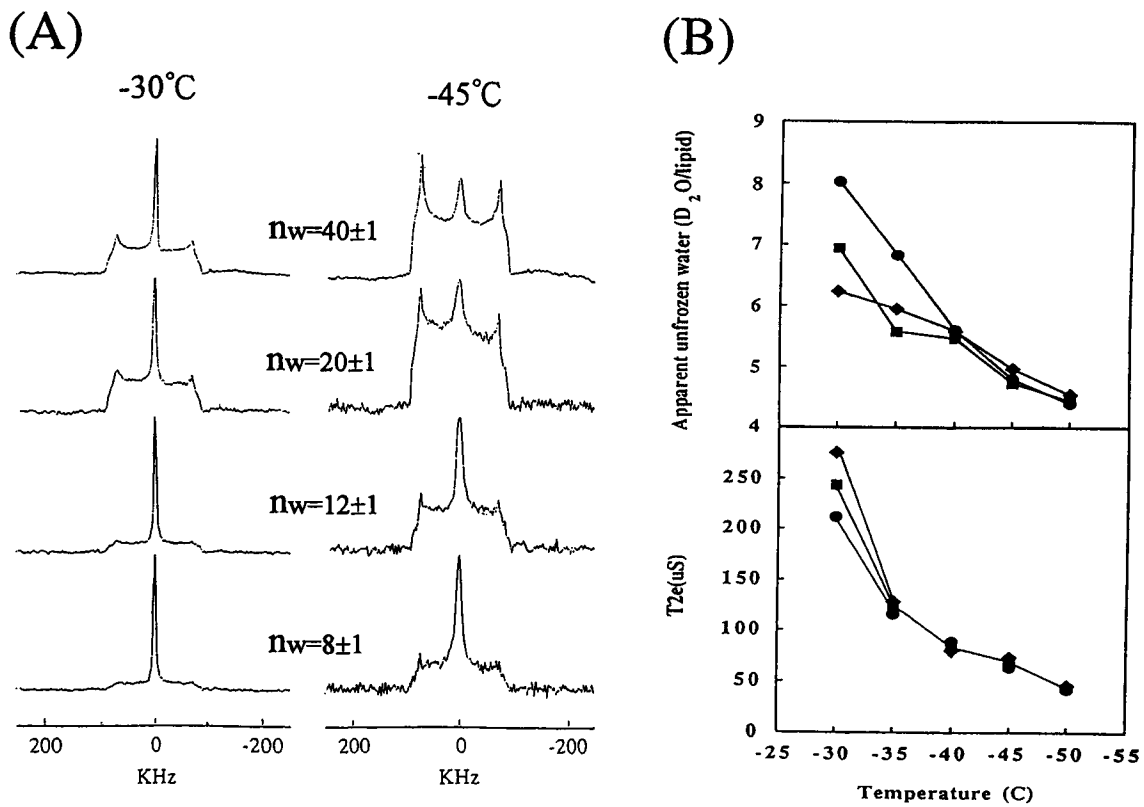

FIGURE 5 Quantitation of the unfrozen water molecules in fully hydrated DMPC bilayers: (*A*) Fully relaxed ^{2}H-NMR spectra of D_2O/DMPC at indicated hydration state and temperature. The repetition time is 100 s and 400 s at −30°C and −45°C, respectively. (*B*) Determination of the number of unfrozen water molecules (*upper panel*) for samples with various hydration contents of nw = 20 (●), 12 (■), and 8 (◆) after correcting for the signal intensity loss of T_{2e} (*lower panel*) effect during the interpulse delay of quadripolar echo.

perature. In fact, a broad component of water signal emerges at lower temperature to replace some of the isotropic sharp component. Moreover, the intensity ratio between the sharp and broad component varies as a function of temperature and may be correlated with the two gel-state bilayer headgroup structure (Hsieh and Wu, 1995a). The water molecules responsible for the broad component must remain in the interbilayer space because not only their T_1 values are the same as the sharp component, but also the signal intensities of both sharp and broad components are immediately reversible. Therefore, much stronger force with prolonged time will be needed if one wants to dehydrate the water content lower than five or six water molecules per lipid. Tentatively, we attribute these waters to constitute the primary hydration layer near the trimethylammonium group. Other evidence based on the surface area consideration and relaxation measurement will be presented later.

Dynamic coupling between PC headgroup and water

Fig. 6 shows studies of T_1 for the interbilayer water molecules in the D_2O/DMPC sample at a hydration state of eight water molecules per lipid. In the spectra shown in panel A ($T = -70°C$), the two overlapping signals (one isotropic and the other anisotropic) exhibit relaxation process with similar T_1 value. At lower temperature, the isotropic signal decreases and an anisotropic powder spectrum with $\Delta\nu_Q$ value of ~146 kHz becomes apparent (*B*) as we pointed out earlier. Interestingly, only one T_1 relaxation process was observed for the entire range of spectra covered by D_2O signals. In addition, the apparent T_1 values, indicated by the τ-dependent profiles of the signal intensity (*M*, panel *C*), are in the 100 ms range and are longer at lower temperature. Therefore, these water molecules behave as a macromolecule, as reflected by the T_1 relaxation measurements, despite the differences in the linewidth of two components of water signals at temperatures below −40°C.

Fig. 7 shows the temperature-dependent profiles of ^{2}H-NMR T_1 relaxation times for the —$N(CD_3)_3$ and D_2O signals in DMPC dispersions in the reported temperature range of −10°C and −70°C. The closed and open square symbols represent relaxation times obtained at two different hydration states in H_2O/d_{13}-DMPC (panels *A* and *B*), while the closed triangle and circle symbols represent those obtained in D_2O/DMPC (*B*). It is surprising to note that there is no detectable break in the temperature − dependent T_1 profile, although the studied temperature range also covers the freezing temperature, i.e., −40°C, of the phospholipid headgroup. The result, however, is consistent with our previous observation that unfrozen water in fully hydrated sphingomyelin bilayers undergoes both fast and slow motions at characteristic NMR time scales (Wu et al., 1991). The slow, but not the fast, motions are affected when the lipid headgroup freezes.

The solid line shown in Fig. 7 *A* represents the simulation curve based on equations described in the Materials and Methods section. The solid line indicates that the T_1 relaxation of —$N(CD_3)_3$ can be described reasonably as a threefold jump of the methyl group with activation energy of 3.2 kcal/mol for the sample with a hydration state of about two water molecules per lipid. It should be pointed out that there is also libration motion for the entire phosphocholine headgroup (Hsieh and Wu, 1995a; Dufourc et al., 1992) and their rates are significantly different from the rotational motion of the phosphate group (Milburn and Jeffrey, 1987). Not only the T_1 of d_4-DMPC were found to exhibit minima (5 ms) at about −15°C (Fig. 7 *C*), but also the quadripolar splitting for —CD_2—CD_2— was reduced from its rigid value in the temperature range of −90°C and −110°C (Hsieh and Wu, 1995a, b). In light of this, care should be exercised in interpreting the T_1 relaxation time in terms of specific motions and activation energies.

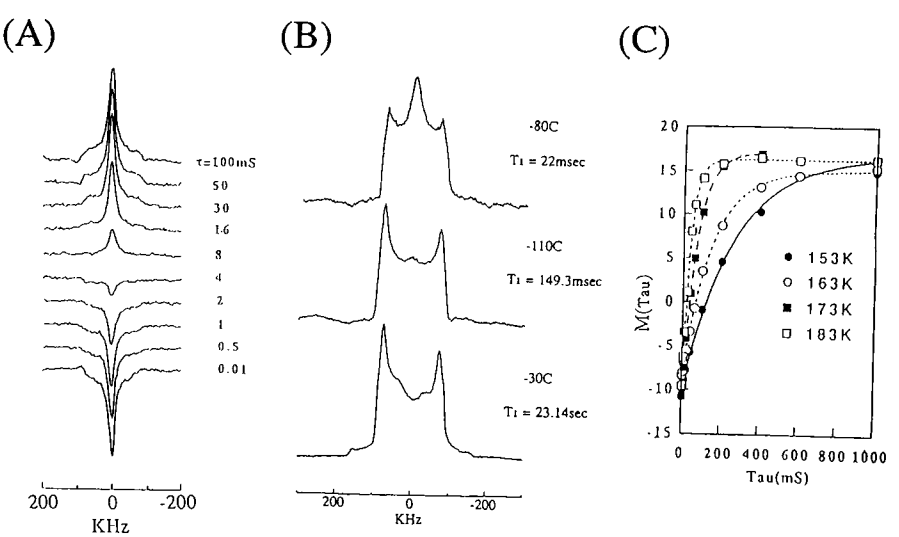

FIGURE 6 Determination of the spin-lattice (T_1) relaxation time of D_2O in D_2O/DMPC sample with hydration ratio of 8, at subzero temperatures: (*A*) representative ^{2}H-NMR spectra at −70°C at indicated interpulse delay, (*B*) representative ^{2}H-NMR spectra at indicated temperatures, and (*C*) the variation of the signal intensity as a function of tau (τ). ^{2}H-NMR spectrum of pure D_2O at −30°C (*bottom trace* in *B*) is also included for comparison. The T_1 of D_2O ice is about 2 to 3 orders of magnitude larger than that of interbilayer D_2O.

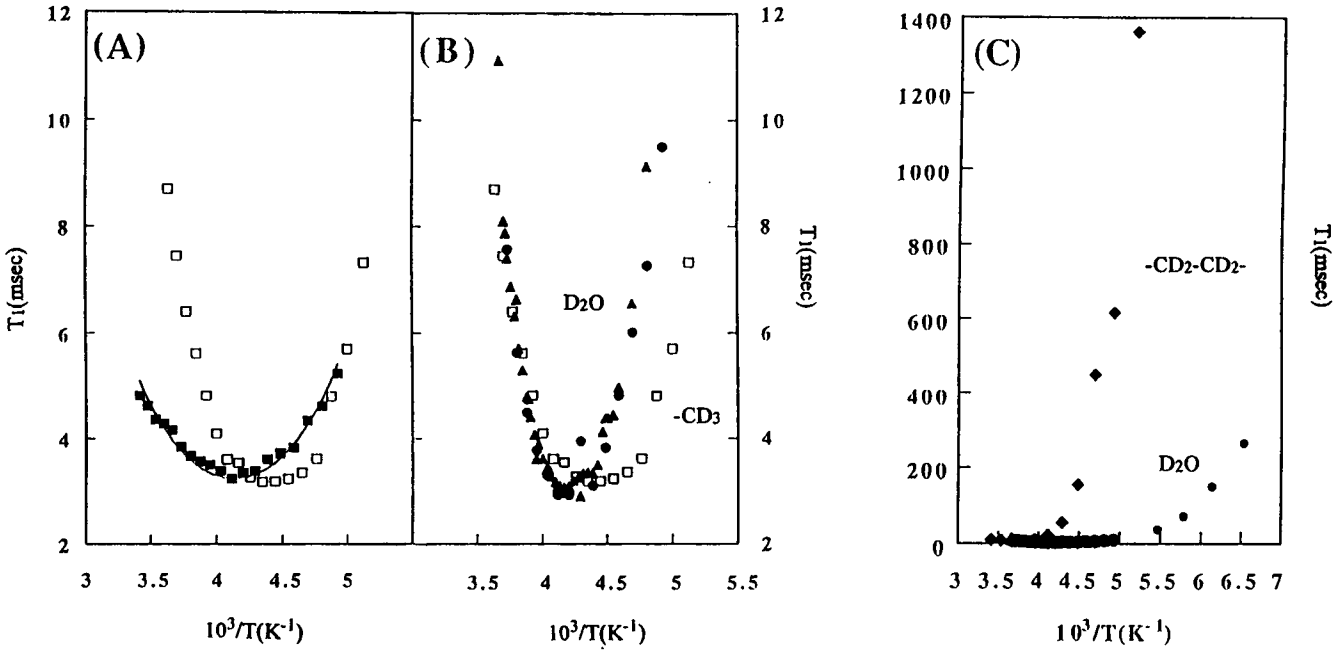

FIGURE 7 Relationship between the mobility of trimethylammonium group and its hydrates as reflected by ^{2}H-NMR spin-lattice (T_1) relaxation studies of hydrated DMPC at 46.073 MHz: (*A*) T_1 plotted as a function of inverse temperatures for H_2O/d_{13} DMPC at two molar ratio. Closed and open square symbols represent T_1 values obtained from samples with hydration ratios of 2 and 8, respectively. Comparison of T_1 between D_2O (●,▲) and —$N(CD_3)_3$ (*B*), and between D_2O and —CD_2— (◆) (*C*) in the interfacial headgroup region of fully hydrated DMPC bilayers.

With an increase in the hydration from two to eight water molecules per lipid, the detected T_1 minima of —$N(CD_3)_3$ shifts to lower temperature (Fig. 7 *A*) by approximately 10°C. The observed T_1 values for the —$N(CD_3)_3$ and D_2O signals in DMPC dispersions are surprisingly similar in the studied temperature range under fully hydrated condition (≥8 water molecules per lipid for PC at subzero temperatures) (*B*). Significant differences in the T_1-dependence profiles can, however, be detected between —CD_2—CD_2— of the methylene group and interbilayer D_2O (*C*). These results suggest that there is a dynamic coupling between the methyl group and the interbilayer water molecules near the trimethylammonium group. The reported T_1 values are in ms range with a detectable minima, indicating that both water and *N*-methyl signals are modulated by a motion with a rate close to its characteristic NMR time scale of 10^{-8}–10^{-9} s. Therefore, these water molecules are, indeed, in close proximity to the trimethylammonium group, and methyl group rotation is mainly responsible for the T_1 relaxation of interbilayer water molecules.

Fig. 8 *A* compares the ^{2}H-NMR T_{2e} values obtained from the —$N(CD_3)_3$ group in H_2O/d_{13}-DMPC and D_2O in D_2O/DMPC samples at hydration state of eight water molecules per lipid. There is a clear transition for the T_{2e} temperature-dependent profile of the trimethylammonium group and interbilayer D_2O occurring at temperature close to T_H. The apparent activation energy estimated at temperatures below −40°C is about 5 kcal/mol, which is significantly lower than the value of 13.5 kcal/mol estimated previously from the dielectric relaxation and tetrahedral reorientation of water molecules of hexagonal ice in the temperature range of T_M and T_H (Wittebort et al., 1988). Although the determined activation energy can only be considered apparent because T_2 measured by the two-pulse method is usually not describable by a single exponential decay (Pauls et al., 1985), there is no doubt that the activation energy at temperatures below T_H is much smaller than that detected above T_H. We interpret this as an indication of the existence of water cluster (Wales, 1996; Liu et al., 1996), which is known to undergo flipping of the deuteron across the ring surface of the water cluster with anomalously low activation energy.

The apparent T_{2e} values detected for the trimethylammonium group is about three- to fourfold higher than those detected for interbilayer water, but they exhibit a process with low activation energy similar to that of unfrozen water. In fact, as shown in Fig. 8 *B*, their temperature dependence is found to be similar below T_H regardless of their water contents. Therefore, the lipid headgroup must also undergo slow motions, such as fluctuation of P-N dipole suggested previously based on the NMR-detected two coexisting gel-state headgroup structures (Hsieh and Wu, 1995a). These motions, by themselves, however, cannot account for the apparent isotropic motions of the interbilayer water molecules because the P-N dipole can only move anisotropically, as indicated by the significant residual quadripolar splitting of the deuterated trimethylammonium group. Both the flipping of the deuteron across the putative water pentamer ring and the fluctuation of the P-N dipole along the membrane surface may contribute to the detected slow isotropic motions of deuterons of interbilayer water molecules.

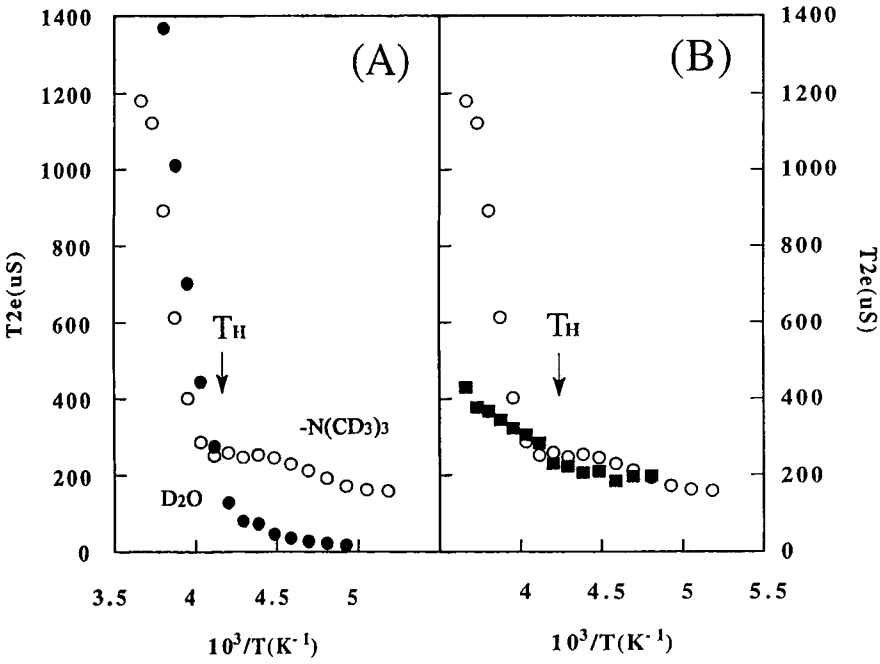

FIGURE 8 Comparison of temperature-dependent profiles of T_{2e} obtained from (*A*) deuterated trimethylammonium group (○) and unfrozen interbilayer waters (●) for DMPC at hydration state of eight water molecules per lipid and (*B*) deuterated trimethylammonium group at a hydration state of eight (○) and two (■) water molecules per lipid.

DISCUSSION

In this communication, we show that freeze-induced dehydration of fully hydrated freshly prepared PC bilayer yields a controllable lipid hydration system with a primary hydration layer of about seven or eight water molecules per lipid. We also quantitate the respective amount of water molecules involved in the interaction with the phosphate and trimethylammonium group by both NMR intensity and relaxation measurements. To show clearly how the dynamic coupling may occur between the PC headgroup and interbilayer water molecules, a structural model, depicted in Fig. 9, of the primary hydration shell of the PC headgroup is proposed: first, a water pentamer intercalates into the interbilayer space (*A*) to cover the trimethylammonium group (*B*); second, two water molecules associated with the phosphate group form an extended intra-bilayer hydrogen bond network to lock the phosphate group rotation (Vanderkooi, 1991). In this proposed model, we adopt the energetically minimized DMPC intra-bilayer dihydrates structure of Vanderkooi to account for the correspondence of the freezing events of the phosphocholine headgroup and its associated water molecules. A water pentamer ring can then pack near the trimethylammonium group without perturbing the original hydrogen bonds network. Furthermore, the freezing of the two water molecules near the phosphate group would allow intra-bilayer hydrogen bond formation, and at the same time induce the formation of a water cluster near the trimethylammonium group.

We propose a pentagonal ring as the water cluster to cover the hydration shell of trimethylammonium group for the following reasons: first, a pentagonal ring of water structures has been observed to be present around the methyl group of amino acids in several proteins. As pointed out earlier, there is also a detectable unfrozen isotropic water signal in crambin polycrystals at temperatures below −40°C. Second, pentagonal rings of hydrogen-bonded water molecules are capable of self-replicating to account for the thermodynamic anomalies, i.e., T_H, of supercooled water and hydrophobic effect in aqueous solution (Speedy, 1984). The anomalous behavior of the T_{2e} temperature-dependent profile is detected near T_H. Third, low energy rearrangement of a water pentamer has been shown to occur both theoretically and experimentally (Wales, 1996; Liu et al., 1996). This explains why the apparent activation energy of the deuteron flipping process detected at temperatures below −40°C is much lower than that of tetrahedral jump in hexagonal ice (Wittebort et al., 1988). Fourth, the isotropic and anisotropic ^{2}H-NMR signals of the interbilayer water molecules appear to be reversible and behave as one entity. Finally, the stoichiometry of the lipid to unfrozen water molecules is consistent with experimentally determined values. If the proposal can stand future tests by techniques such as multiple quantum NMR, PC bilayers may serve as a potential biological system to study the pentagonal water structure.

It is also interesting to point out that recent advances in the phase diagram and structure of water molecules have suggested the presence of low- and high-density amorphous water with anomalous behavior near the T_H and T_g of waters (Tanaka, 1996). The biological relevance of these water structures has been suggested (Angell, 1995). There is no evidence to indicate the two anomalous temperatures for H-bond water and the putative water pentagon near −40°C and −70°C, respectively, can be related to these waters.

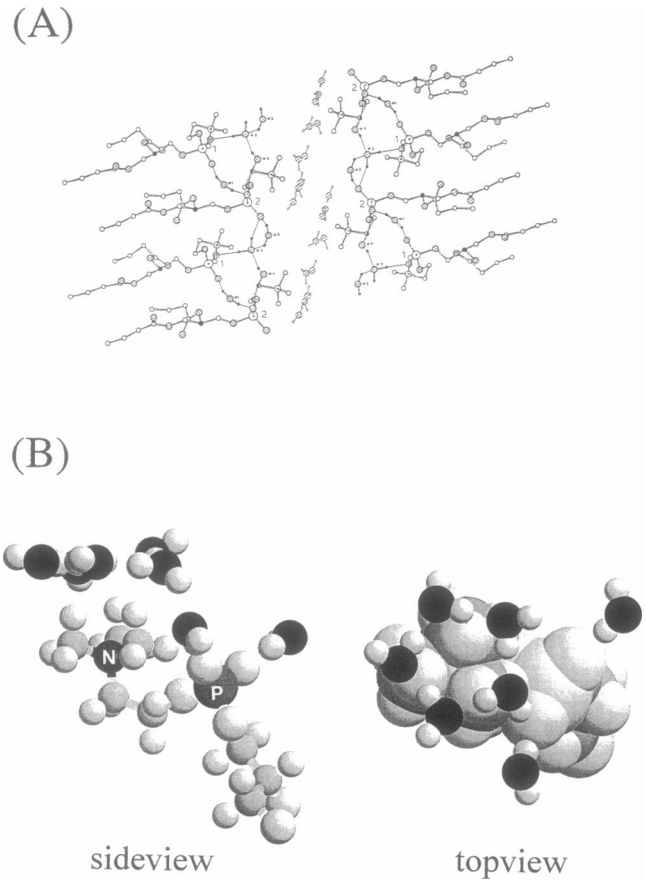

FIGURE 9 Proposed model of primary hydration shell of PC bilayers: (*A*) a schematic picture based on the energy minimized DMPC dihydrate structure II from Vanderkooi, 1991. and (*B*) top and side view of the water/phosphorylcholine complex. We emphasize that the model can only be considered as schematic to show the relative position of the phosphate dihydrate and putative water pentamer near the trimethylammonium group because the packing density and hydrogen bond formation of the hydrates has not been considered quantitatively. The cross-sectional area of putative water pentamer can be seen to match that of trimethylammonium group. The oxygen atom of water molecule is represented by black ball.

But, the reversibility of the relaxation behavior of primary hydration water layers near T_H for both phospholipid and proteins warrants future investigation along this direction.

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