Nuclear Magnetic Resonance Studies of Lipid Hydration in Monomethyldioleoylphosphatidylethanolamine Dispersions

Zhen-jia Chen,* Leon C. M. Van Gorkom,* Richard M. Epand,‡ and Ruth E. Stark*

*Department of Chemistry, College of Staten Island and the Graduate School of the City University of New York, Staten Island, New York 10314 USA, and ‡Department of Biochemistry, McMaster University Health Sciences Centre, Hamilton, Ontario L8N 3Z5 Canada

ABSTRACT Solid-state proton nuclear magnetic resonance has been used to examine surface hydration in suspensions of monomethyldioleoylphosphatidylethanolamine (MeDOPE). The magic-angle spinning (MAS) ^{1}H spectra for aqueous suspensions of MeDOPE in the L_α phase exhibited two resonances of roughly equal intensity that could be ascribed to water protons, but both their spin-lattice relaxation times and chemical shifts converged upon conversion to the hexagonal phase. Only a single water peak was observed for analogous samples of dioleoylphosphatidylcholine (DOPC). MAS-assisted two-dimensional nuclear Overhauser effect spectroscopy (NOESY) was conducted for multibilayers of both MeDOPE and DOPC. Through-space interactions were identified between pairs of lipid protons, as expected from their chemical structure. For lamellar suspensions of MeDOPE, positive NOESY cross-peaks were observed between the downfield-shifted water resonance (only) and both CH_2N and $NH_2CH_3^+$ protons of the lipid headgroup. These cross-peaks were not observed in the NOESY spectra of MeDOPE in its hexagonal or cubic phases or for lamellar DOPC reference samples. Taken together, the observation of two water peaks, spin-lattice relaxation behavior, and NOESY connectivities in MeDOPE suspensions support the interpretation that the low-field water peak corresponds to hydrogen-bonded interlamellar water interacting strongly with the lipid. Such a population of water molecules exists in association with MeDOPE in the lamellar phase but not for its inverted phases or for lamellar dispersions of DOPC.

INTRODUCTION

There is considerable interest in the role of lipid polymorphism in determining the properties of biological membranes. One indication of the potential importance of this property for the functioning of biological membranes is the observation that microorganisms adjust their lipid composition so as to maintain a nearly constant spontaneous curvature, according to the condition in which the organisms are grown (Lindblom et al., 1993; Rilfors et al., 1994; Rietveld et al., 1994; Österberg et al., 1995). This constant spontaneous curvature is achieved by regulating the relative proportion of bilayer and nonbilayer lipid while maintaining the membrane in a bilayer arrangement.

What physical properties of the bilayer are then altered by the presence of non-lamellar-forming lipids? One such property is the enthalpic stability of the membrane, which is lower for membranes with a greater tendency to form inverted phases (Epand and Epand, 1994). Another change that occurs in a property of the bilayer as the temperature is increased toward that of the bilayer-to-inverted hexagonal phase-transition temperature (T_H) is an alteration in membrane surface polarity as detected with fluorescent probes (Epand and Leon, 1992; Sterk et al., 1996). The properties of membrane surfaces and their interaction with water have long been recognized as important factors affecting the functioning of biological membranes (Watts and van Gorkom, 1992; Rand and Parsegian, 1995).

One of the important aspects of the interfacial properties of membrane bilayers is the nature and extent of hydration of the membrane surface. There have appeared several elegant studies that measure the amount and affinity of membrane-bound water (Rand and Parsegian, 1989; McIntosh and Simon, 1994). Interrelated with hydration is the property of monolayer curvature strain (Gruner, 1992). The extent of hydration, interlipid hydrogen bonding, and steric repulsion are among the factors that affect intrinsic membrane curvature (Cullis et al., 1985). The phenomenon of curvature strain is attracting increased interest as a mechanism for the regulation of biological activity (Epand, 1996).

Among the functions likely to be altered by changes in monolayer curvature strain are the kinetics of interfacial catalysis as well as the rate of membrane fusion. The activity of a number of membrane-bound and amphitropic enzymes (the latter existing in both aqueous and membrane environments) is modulated by membrane curvature (Senisterra and Epand, 1993; Cornell, 1991; McCallum and Epand, 1995). Membrane fusion must also involve changes in membrane curvature during the formation of kinetic intermediates (Siegel, 1993). Finally, it has been found that the channel-opening time of the antibiotic peptide alamethicin is modulated by membrane curvature (Keller et al., 1993).

Of the spectroscopic techniques employed to study the molecular structure of lipid bilayers, nuclear magnetic resonance (NMR) provides complementary information to x-

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Address reprint requests to Dr. Ruth E. Stark, Department of Chemistry, 6S-228, CUNY-Staten Island, 2800 Victory Blvd., Staten Island, NY 10314. Tel.: 718-982-3894; Fax: 718-982-3910; E-mail: stark@postbox.csi.cuny.edu.

The present address of Mr. Chen is Wyeth-Ayerst Research, Pearl River, NY 10965.

The present address of Dr. Gorkom is Unilever Research, Edgewater, NJ 07020.

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ray diffraction, fluorescence, and infrared spectrometry. For multilamellar lipid dispersions and mixtures that mimic natural biological membranes, NMR techniques have been exploited usefully in studies of lipid morphology as well as of molecular dynamics and order (Griffin, 1981; Knowles and Marsh, 1991).

We have now studied the hydration properties of a synthetic phospholipid, monomethyldioleoylphosphatidylethanolamine (MeDOPE), using solid-state ^1H and ^{31}P NMR. Recently, ^2H NMR has been applied to studying the ordering and dynamics of water at membrane surfaces (Gawrich et al., 1992; Volke et al., 1994); magic-angle spinning ^1H NMR has also been used in conjunction with nuclear Overhauser experiments (Forbes et al., 1988) to assess membrane hydration (Volke and Pampel, 1995). The lipid we have chosen to study, MeDOPE, exhibits well-documented polymorphic behavior (Gruner et al., 1988; Siegel and Banschbach, 1990). It is also the model lipid that was used to demonstrate the presence of curvature strain in the L_α phase (Epand and Epand, 1994). In the current work we demonstrate the consequences of inverted phase formation on surface hydration of the lipid. The methodology used in this paper should be generally useful in the study of the solvation of lipid structures.

EXPERIMENTAL PROCEDURES

Materials

The phospholipids used in this study (DOPC, 1, 2-dioleoyl-*sn*-glycero-3-phosphocholine, and MeDOPE, monomethyldioleoylphosphatidylethanolamine) were purchased from Avanti Polar Lipids (Birmingham, AL) and checked for purity by thin-layer chromatography and by their phase-transition behavior. Deuterium oxide (99.996%) was purchased from Aldrich Chemical Company (Milwaukee, WI). To prepare aqueous mixtures, the lipids were first dissolved in 2:1 (v/v) chloroform-methanol, then dried under a stream of nitrogen and at reduced pressure overnight. Aqueous dispersions were made by hydrating the lipid films with 1:1 (w/w) Tris (Tris(hydroxymethyl)aminomethane hydrochloride) buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA) in D_2O. All mixtures were vortex-mixed and freeze-thawed five times to obtain a uniform paste, ~140 mg of which was then loaded into 7-mm cylindrical Zirconia rotors (Varian Instruments, Palo Alto, CA). The samples remained dispersed and showed no phase separation or significant losses in mass for the duration of the NMR experiments.

NMR studies

The NMR measurements were made using a Varian Unity*plus*-300 wide-bore spectrometer equipped for both liquid-state and solid-state NMR. The resonance frequencies were 300.00 MHz (^1H) and 121.44 MHz (^{31}P), respectively. Speeds for magic-angle spinning (ν_{rot}) were adjusted to 2.000 ± 0.002 kHz, and the sample temperature was regulated to ±1°C over the range 10–75°C.

Static ^{31}P NMR was conducted with direct polarization (Bloch decays), using 5-μs 90° pulses and 42-kHz decoupling (dipolar decoupling) gated on during signal acquisition. Typically, the data were obtained using 1000 acquisitions and 5-s recycle delays.

Magic-angle spinning (MAS) ^1H NMR spectra were acquired with 8-μs 90° pulses, 10-kHz spectral widths, and 1-s recycle delays. In a typical experiment, 32 transients composed of 8K time-domain points were collected, zero-filled to 16K points, and subjected to Fourier transformation. Spin-lattice relaxation times (T_1) for protons were measured using an inversion-recovery pulse sequence (Vold et al., 1968).

MAS-assisted nuclear Overhauser effect spectroscopy (NOESY) (Jeener et al., 1979) was performed using the standard pulse sequence $(90°\text{-}t_1\text{-}90°\text{-}\tau_M\text{-}90°\text{-acquire }[t_2])_n$ with $n = 16$, 128 t_1 values, and the States method for phase-sensitive acquisition (States et al., 1982). To optimize the acquisition conditions, the mixing times τ_M were varied between 100 and 800 ms in separate 4-h NOESY experiments. A mixing time of 300 ms was used to make comparisons among different aqueous phospholipid dispersions. The resulting two-dimensional NMR data were analyzed with VMRX software (Varian Associates, Palo Alto, CA).

RESULTS AND DISCUSSION

Lipid morphology: ^1H and ^{31}P NMR spectra

To examine the phase behavior and morphology of model membranes, ^1H and ^{31}P NMR data were acquired in parallel for both MeDOPE and dioleoylphosphatidylcholine (DOPC) over the temperature range 25–75°C. MeDOPE has a known propensity to form hexagonal and cubic phases at elevated temperatures, whereas DOPC is expected to remain in the lamellar liquid-crystalline phase under these conditions.

Fig. 1 displays the 300-MHz MAS ^1H NMR spectra for aqueous dispersions of both phospholipids as a function of temperature, and Table 1 summarizes the resonance

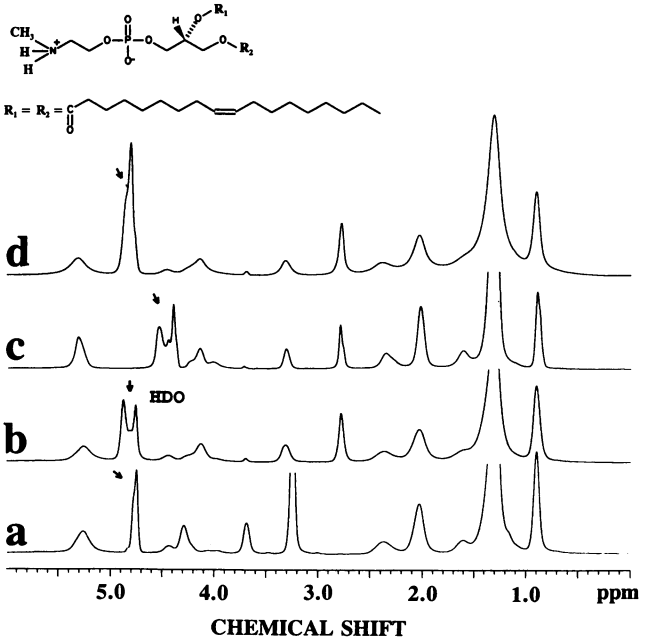

FIGURE 1 ^1H NMR spectra of aqueous phospholipid dispersions obtained with magic-angle spinning (MAS) at 2.000 kHz. The molecular structure of MeDOPE is shown, with corresponding resonance assignments summarized in Table 1. (*a*) DOPC at 25°C (similar spectra are obtained at elevated temperatures); (*b*) MeDOPE at 25°C; (*c*) MeDOPE heated to 75°C but not equilibrated; (*c*) MeDOPE kept at 75°C for ~2 h and then returned to 25°C. The lamellar-to-hexagonal transition for MeDOPE has been reported to occur at 65°C (Gruner et al., 1988; Siegel and Banschbach, 1990).

TABLE 1 ^{1}H NMR resonance assignments for MeDOPE*

Proton type	Chemical shift (ppm)‡	Chemical shift (ppm)§
ω-CH_3	0.90	0.90
$(CH_2)_n$	1.30	1.31
β-CH_2	1.61	1.61
$CH_2CH{=}CHCH_2$	2.05	2.04
α-CH_2	2.36	2.32
NCH_3	2.80	2.71
CH_2N	3.32	3.16
CH_2O, $POCH_2$	4.14	3.98, 4.18
CH_2O	4.44	4.40
HOD	4.75, 4.88	
CHO, CH=CH	5.24	5.24, 5.36

*The chemical structure for this phospholipid is shown in Fig. 1.
‡300-MHz data obtained on an aqueous dispersion of MeDOPE with 2.00-kHz magic-angle spinning at a temperature of 25°C. Shifts are referenced to the ω-CH_3 peak at 0.90 ppm (Li et al., 1993).
§600-MHz data obtained for MeDOPE dissolved in $CDCl_3$ at a temperature of 25°C. The assignments were confirmed with double-quantum-filtered correlated spectroscopy (data not shown).

assignments made by analogy with prior work (Halladay et al., 1990). The corresponding 120-MHz ^{31}P static spectra are shown in Fig. 2. At room temperature, both DOPC and MeDOPE exhibit ^{31}P spectral patterns typical of a fully hydrated lamellar (L_α) phase, in which the anisotropy of the phosphorus chemical shift tensor is partially averaged by axially symmetric rotational motion of the phospholipid headgroup (Knowles and Marsh, 1991). The magnitude of the residual chemical shift anisotropy is −45.1 ppm, in good agreement with previous reports. These results are as anticipated, because the 40:1 molar ratio of water to lipid is sufficient for complete hydration, and the homogeneity of the sample preparation is ensured by at least five freeze-thaw cycles. Surprisingly, the ^{1}H spectrum of MeDOPE exhibits two well-resolved resonances (4.75 and 4.88 ppm) that may be attributed to water (Fig. 1 *b*).

When the sample is heated above the established lamellar-to-hexagonal phase transition temperature for MeDOPE (Gruner et al., 1988; Siegel and Banschbach, 1990; van Gorkom et al., 1992), conversion of the lipid to the hexagonal (H_{II}) phase is confirmed by changes in its ^{31}P spectrum (Fig. 2 *c*). Both water signals in the corresponding ^{1}H spectrum also shift upfield by ~0.4 ppm (Fig. 1 *c*), suggesting a decrease in hydrogen bonding (Schneider et al., 1958). If the MeDOPE is again cooled to room temperature, only one water resonance is resolved (Fig. 1 *d*), indicating the formation of a (metastable) phase that allows rapid exchange between lipid-associated and bulk water. Concomitantly, the ^{31}P NMR pattern narrows and becomes more symmetric (Fig. 2 *d*), suggesting that the recooled state involves an inverted cubic phase such as that reported previously (Gruner et al., 1988). To restore the ^{31}P and ^{1}H spectral features typical of lamellar phospholipid dispersions, a freeze-thaw cycle is required (data not shown).

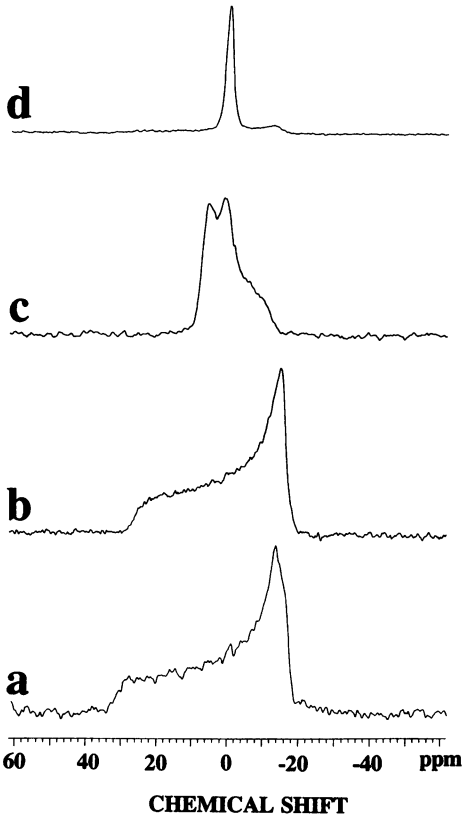

FIGURE 2 Static ^{31}P NMR spectra of the phospholipid samples described in Fig. 1. The averaged chemical shift of the cubic phase has been set to 0 ppm (Yeagle, 1990). (*a*) DOPC at 25°C; (*b*) MeDOPE at 25°C; (*c*) MeDOPE equilibrated at 75°C; (*d*) MeDOPE kept at 75°C for ~2 h and then cooled to 25°C. Heating of MeDOPE to 75°C is accompanied by a rapid disappearance of the spectrum typical for L_α-phases (*a*, *b*), then a slow reduction of the shift anisotropy to 21.1 ppm and the appearance of a primarily hexagonal-phase powder pattern in spectrum *c*. The L_α pattern may be recovered if the recooled sample undergoes freeze-thaw treatment.

Lipid hydration: ^{1}H-NMR spectra and spin-relaxation times

The two slowly exchanging populations of water present in the room-temperature lamellar phase of an aqueous MeDOPE dispersion may be ascribed to a) bulk water outside of the multilamellar vesicles (4.75 ppm) and b) interlamellar water (4.88 ppm). The chemical shift of the latter population is altered because of strong interactions with the lipid, likely by hydrogen bonding. The exchange rate between populations a) and b) is less than ~400 s^{-1}, presumably because of the multiple membrane barriers. It should be noted, however, that MLVs of dioleoylphosphatidylcholine do not exhibit two separate water peaks, nor does MeDOPE when it is present in an inverted phase. This phenomenon is therefore diagnostic of strong interactions between solvent and membrane.

Are the relative signal intensities of the two lamellar-phase water peaks in accord with the above description? Our samples have a 40:1 water-to-phospholipid molar ratio. Approximately 20 water molecules are required to fully

hydrate MeDOPE in the L_α phase (Gruner et al., 1988). This number would then correspond to the interlamellar water, in rapid exchange with a) water molecules that are hydrogen-bonded to the lipid headgroup through its protonated amine and/or phosphate groups; and b) NH_2s of the MeDOPE headgroup. The remaining water comprises the extralamellar bulk water and thus would be present at a level comparable to that of the interlamellar water. Our spectra exhibit an intensity ratio of about 1:1 for the two water resonances, in agreement with these assignments.

Measurements of the ^1H spin-lattice relaxation time (T_1) were also used to verify the identification of bulk and lipid-associated water. Fig. 3 summarizes the temperature dependence of T_1 for both water resonances. Because the T_1s rise (with roughly an Arrhenius dependence) as the temperature rises, the motions responsible for the spin relaxation must be in the fast correlation time limit (Farrar and Becker, 1971). The downfield water signal exhibits a considerably shorter T_1 at all temperatures, indicating reduced mobility of water molecules in that environment and supporting the notion of strong hydrogen bonding. The upfield water has a chemical shift and T_1 similar to those of pure water (data not shown).

At temperatures above 55°C, the proton T_1 increases more sharply for the downfield (interlamellar) water and actually decreases for the upfield (bulk) water—trends that cause their respective spin-lattice relaxation times to approach a common intermediate value above 65°C. Greater motional freedom is certainly expected at higher temperatures, because of simple reorientation (bulk water) or weakening of the hydrogen bonds and lowering of the bilayer barriers (interlamellar water). Both T_1s should then continue to rise, but at the same time they begin to be averaged by more efficient chemical exchange. A single water resonance and spin-lattice relaxation time are finally observed as the lamellar dispersion is converted to a hexagonal arrangement.

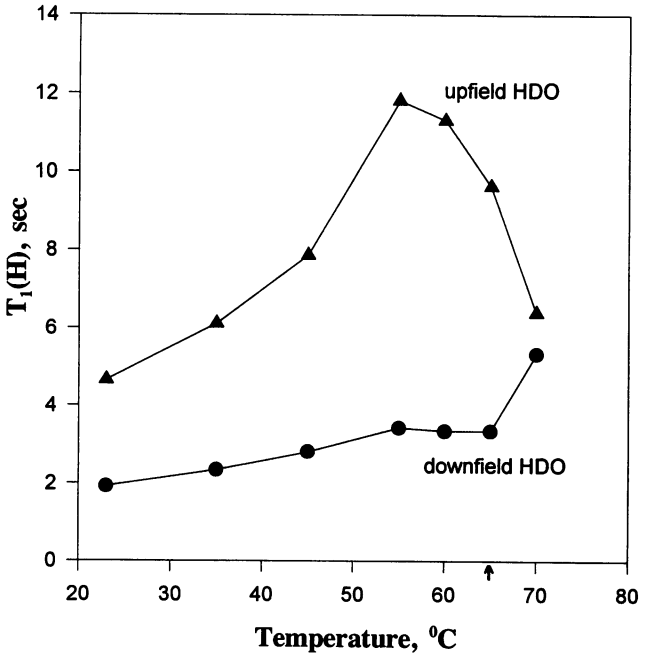

FIGURE 3 Variation of the ^1H spin-lattice relaxation times (T_1) with temperature for the two water peaks observed in MeDOPE dispersions. The arrow on the temperature axis indicates the value of the phase-transition temperature T_H.

Lipid hydration: ^1H two-dimensional nuclear Overhauser effect spectroscopy

To establish which portions of the MeDOPE lipid molecule are involved in specific through-space interactions with water, ^1H NOESY experiments were performed while rotating the samples at the magic angle. For the DOPC standard in the bilayer phase, there are no observable (positive or negative) NOESY cross-peaks between the water resonance and any lipid functional groups (Fig. 4 *a*). This result indicates that, as reported for a concentrated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) dispersion, water diffuses freely (1–10 ns^{-1}) at the lipid-water interface and thus exhibits no positive NOEs in the 2D spectrum (Volke and Pampel, 1995).

By contrast, Fig. 4 *b* illustrates strong dipole-dipole interactions between those water molecules that resonate downfield and the headgroup lipid protons of lamellar MeDOPE. (The upfield water peak in MeDOPE multibilayers displays no cross-peaks, suggesting that excess bulk water diffuses rapidly.) Negative NOEs (positive NOESY cross-peaks) are observed to both NCH_3 and CH_2N segments of the phospholipid headgroup, implying a diffusion rate slower than 0.1 ns^{-1} for interlamellar water in the less shielded environment. This motional restriction is consistent with our observations of two resolved water peaks and a short T_1 for the downfield water resonance; it also supports the hypothesis that the downfield water population is more hydrogen bonded. The NMR signal of interlamellar water is distinct from that of bulk water, but it may include contributions (up to one part in 20) from rapidly exchanging headgroup NH_2s. Thus in addition to water-lipid interactions, the proximity of NH_2 and NCH_3 protons in the lipid headgroup itself could contribute noticeably to NOESY cross-peaks involving the resonance at 4.88 ppm.

Upon heating of the MeDOPE dispersion, NOESY cross-peaks between the downfield water resonance and the lipid headgroup gradually diminish in intensity (Fig. 5); they disappear entirely at temperatures above the lamellar-to-hexagonal phase transition. Both motional averaging and weakening of the lipid-water hydrogen bonds may account for this trend. If hexagonal-phase MeDOPE is again cooled to room temperature, the metastable isotropic phase that is formed displays one water resonance and no NOESY cross-peaks to the lipid (Fig. 4 *c*). This last observation suggests that water in the cubic phase is no longer involved in very strong hydrogen bonding with the phospholipid headgroup. (If the NOESY cross-peaks in Fig. 4 *b* were attributable to

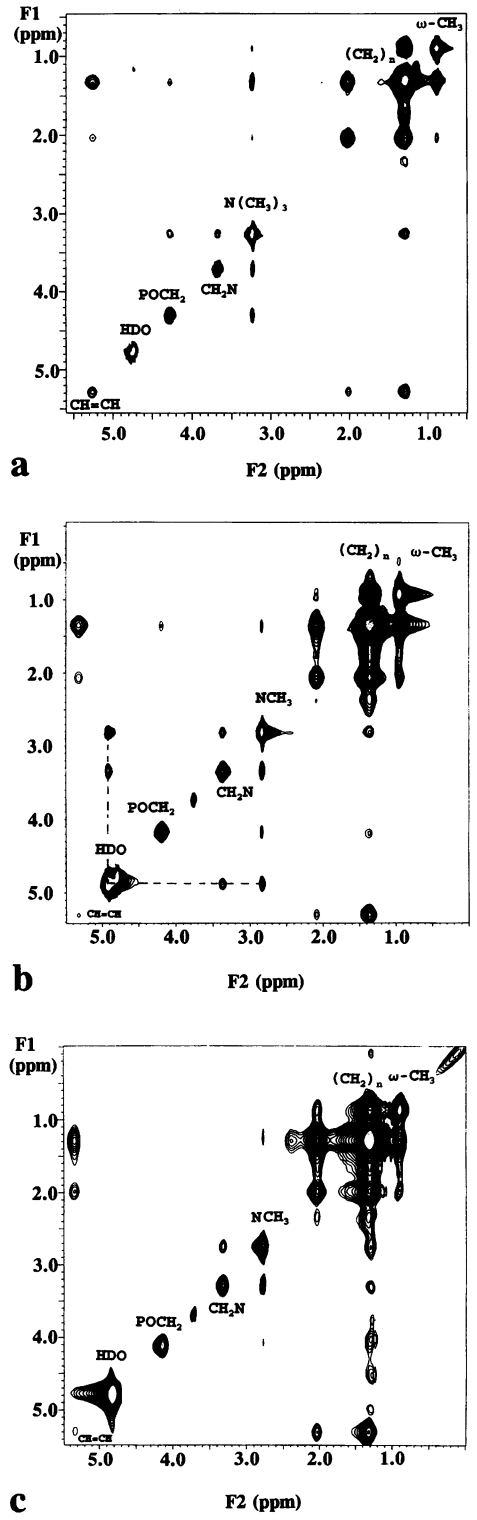

FIGURE 4 MAS-assisted ^1H-^1H NOESY positive-contour plots for aqueous phospholipid mixtures. All experiments were run with 2.000-kHz magic-angle spinning and a mixing time of 300 ms. The frequency axes refer to acquisition (F2) and evolution (F1) periods, respectively, of the two-dimensional NMR experiment. (*a*) DOPC at 25°C; (*b*) MeDOPE at 25°C; (*c*) MeDOPE heated to 75°C and then cooled to 25°C. The dashed lines show NOESY connectivities between the downfield water resonance and protons of the phospholipid. An examination of lower contour levels reveals no additional positive cross-peaks, and no negative cross-peaks are present in the spectrum.

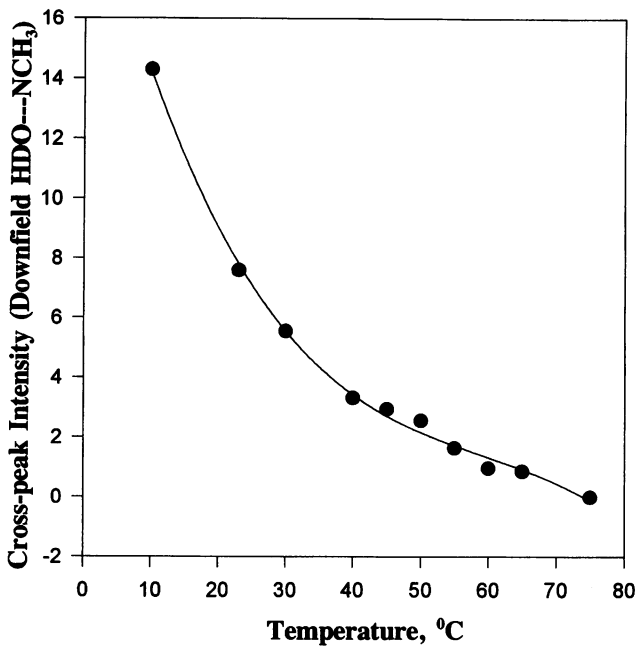

FIGURE 5 Variation of water-lipid MAS-NOESY cross-peak volumes with temperature for MeDOPE dispersions. The integrated intensities for cross-peaks between the downfield water and headgroup methyl peaks are plotted as a percentage of the diagonal peak intensity for NCH_3 protons.

interactions among the lipid headgroup protons, they would be retained in the hexagonal and cubic phases.)

The possible water-lipid interactions in MeDOPE dispersions may be summarized as follows. In the lamellar liquid-crystalline phase, hydrogen bonding may occur through a) interaction between water hydrogens and PO_4^-; b) interaction between water oxygens and $NH_2CH_3^+$; or c) formation of water bridges between $NH_2CH_3^+$ and PO_4^- groups of neighboring phospholipid molecules. Water bridges between oppositely charged lipid headgroups have been suggested previously (McIntosh and Simon, 1986). However, the formation of such water bridges may be precluded for DOPC because of steric hindrance and the inability of $N(CH_3)_3^+$ groups to form hydrogen bonds. In the hexagonal phase of MeDOPE, the acyl chains should occupy more space and the lipid headgroups will be squeezed closer together; thus water-lipid interactions may occur less readily and the molecules at the membrane surface will diffuse more rapidly. Finally, the cubic phase formed upon cooling of the sample should displace the water molecules that may be bridging adjacent phospholipid molecules and gain extra stability from direct hydrogen bonding between oppositely charged $NH_2CH_3^+$ and PO_4^- groups of any neighboring lipid molecules.

It should be noted parenthetically that both DOPC and MeDOPE dispersions exhibit NOESY cross-peaks between various pairs of lipid protons. Many of these connectivities are expected based on the molecular structure of the respective phospholipids: $POCH_2$, CH_2N, and NCH_3 interactions within the headgroup; ω-CH_3, $(CH_2)_n$,

and HC=CH interactions along the acyl chains. More surprising are the observed cross-peaks between the headgroup NCH_3s and both $(CH_2)_n$ and ω-CH_3 portions of the acyl chains. Such magnetization exchange has been attributed previously to chain bendback (Xu and Cafiso, 1986), interdigitation (Halladay et al., 1990), and the confounding effects of spin diffusion (Gabriel and Roberts, 1987; Forbes et al., 1988; Chen and Stark, manuscript submitted for publication) in various organized phospholipid assemblies. Because the spin-lattice relaxation times of various protons in our MeDOPE system differ by a factor of 1.5, and modest spinning speeds (~0.6 kHz) are sufficient to narrow the ^1H spectral lines, it is tempting to attribute these headgroup-chain cross-peaks to bilayer defects associated with negative curvature strain (Epand and Epand, 1994). Nonetheless, NOESY experiments must be performed on selectively deuterated materials to evaluate possible spin-diffusion effects definitively.

CONCLUSIONS

This study demonstrates the usefulness of one- and two-dimensional NMR spectroscopy for the study of water-lipid and lipid-lipid interactions on an atomic level. In addition to the well-documented use of ^{31}P spectral lineshapes to confirm phospholipid phase behavior, the results presented here show how ^1H spectral characteristics may provide detailed information on packing and organization of these physiologically important states.

In the present study we have shown that lipids with a high negative spontaneous curvature, such as MeDOPE, are characterized by a population of slowly exchanging water molecules that appear to be hydrogen-bonded to the lipid. This type of lipid hydration is not exhibited for the stable bilayer DOPC. The ability to measure this population of water molecules will allow for further assessment of the role such lipids play in membrane function.

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