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Direct Determination of Hydration in the Lamellar to Inverted Hexagonal Transition of Phosphatidylethanolamine†

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Abstract: The bound water associated with phosphatidylethanolamine (PtdEtn) in the lamellar and inverted hexagonal structures is determined directly. Bound water is considered as that water which is unavailable for solvation of the polar solute sucrose. In the fluid lamellar (L_α) state of dioleoyl PtdEtn (at 2 °C), 7.2 water molecules per phospholipid are bound and unavailable as a solvent for sucrose. In the inverted hexagonal structure (16 °C and 30 °C), 5.4 and 5.6 water molecules per phospholipid, respectively, are unavailable for solvation. Similar results are obtained for egg PtdEtn (L_α, 15 °C, 7.5 water per lipid; L_α, 28 °C, 6.9; H_{II}, 40 °C, 5.1). Weakly binding polar solutes (glycine and acetate) yield comparable trends that support a dehydration at the lamellar to inverted hexagonal phase transition of approximately 2 water molecules per PtdEtn. This is the first direct determination of the changes in hydration that occur in the lamellar to inverted hexagonal transition.

Introduction

Evidence that the curvature energy of membranes is a determinant of protein function[1] and membrane fusion[2] has focussed interest in the role of bound water in modulating the effective lipid shape[3] and the intrinsic radius of membrane curvature.[4] Studies of curvature often employ the thermotropic transition between the planar bilayer structure (L_α) and the highly curved inverted hexagonal structure (H_{II}) of lipids, generally using the biologically abundant phosphatidylethanolamine (PtdEtn), as a facile paradigm for the structural changes that result with the expression of curvature.[5]

The highly curved H_{II} structure is known to take up more water than the planar L_α structure.[6,7] By contrast, recent studies using fluorescence[8] and infrared spectroscopies[9] were interpreted as evidence for a dehydrated H_{II} structure, suggesting that the excess water taken up is bulk water that fills the core of the H_{II} cylinders. However, no quantitative measurement of bound water in the H_{II} phase has been made.

In this study, a dual radiolabel centrifugal technique is employed to determine directly the amount of bound water in the $L_\alpha \rightarrow H_{II}$ transition. One key distinction between the properties of bulk water and bound water is their ability to act as a solvent for solutes.[10] Bound water that is sequestered by the lipid structure is unavailable for solvation and can be termed "nonsolvent water". Experimentally, the presence of bound water is reflected by a water-to-solute ratio in the lipid pellet that exceeds the ratio in the supernatant, when an ideal polar

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solute is chosen that does not interact with lipids. The extra pellet water is bound water, whereas the remainder is bulk water trapped within the pellet. Although a sharp distinction between bulk and bound water pools is somewhat arbitrary and dependent on the experimental approach taken, measures of bound water obtained through the $L_\alpha \rightarrow H_{II}$ transition provide a consistent means to evaluate the dependence of hydration on structure.

Results

The ideal marker for solvent water is a solute that does not interact with membranes, either through lipid solubility or through surface effects. Previous studies have established sucrose as an ideal noninteracting solute for the determination of nonsolvent water in liposomes.[10] The nonsolvent water in dimyristoylphosphatidylcholine (Myr_2PtdCho) at 30 °C using sucrose as a solvent water marker was 0.23 ± 0.05 g of water per g of lipid and is in good agreement with the published result of 0.24 g of water per g of lipid.[10]

The nonsolvent water associated with phosphatidylethanolamine in the lamellar and inverted hexagonal structures is tabulated in Table 1. Dioleoylphosphatidylethanolamine (Ole_2PtdEtn) undergoes a thermotropic transition from the lamellar to the inverted hexagonal structure ($L_\alpha \rightarrow H_{II}$) with a midpoint temperature (T_H) of 8−10 °C.[11] Egg PtdEtn undergoes the $L_\alpha \rightarrow H_{II}$ transition at 36 °C as established by wideline ^{31}P NMR on the same lot. It exhibits a broad main transition that spans from 0 to 13 °C.[12]

The nonsolvent water in Ole_2PtdEtn bilayers (2 °C) was 0.18 g of water per g of lipid (7.2 mol ratio). In the H_{II} structure (16 °C), the value decreased to 0.13 g of water per g of lipid (5.4 mol ratio) and was maintained at 30 °C, indicating that the amount of nonsolvent water in the H_{II} state was not strongly dependent on temperature.

The situation in egg PtdEtn was similar. Nonsolvent water associated with the bilayers was 0.18 (15 °C) and 0.17 (28 °C) g of water per g of lipid without evidence for a significant temperature dependence. Nonsolvent water in the H_{II} structure of egg PtdEtn was 0.12 g of water per g of lipid and is not significantly different from the Ole_2PtdEtn value. In both systems, the $L_\alpha \rightarrow H_{II}$ transition of PtdEtn was accompanied by a decrease in hydration of about 1.7−2.1 water molecules per phospholipid, or a decrease of about 25% in the bound water.

Two additional solvent water markers were examined: anionic acetate and zwitterionic glycine. As noted in Methods, the determination of nonsolvent water relies on the difference between solute binding and water binding. For sucrose, solute binding is considered nil and water binding is obtained. For less ideal water markers, a weak affinity for the lipids makes the difference smaller. With the acetate marker, water binding exceeded the acetate binding in the L_α state of Ole_2PtdEtn at 2 °C by 0.13 ± 0.03 molal unit. In the H_{II} structure the value dropped to 0.08 ± 0.03 at 16 °C and 0.08 ± 0.01 at 30 °C. The change, −0.05 molal unit, was the same as the change observed for sucrose. For glycine in the L_α state at 2 °C of Ole_2PtdEtn, the water binding exceeded the glycine binding by 0.10 ± 0.01 molal unit. In the H_{II} structure, the value dropped to 0.03 ± 0.01 at 16 °C and 0.05 ± 0.02 at 30 °C. The change, −0.06 molal unit, was not significantly different from the change observed for sucrose. Results with acetate and glycine were consistent with a decrease of about 2 waters/lipid in the transition from the planar L_α to the highly curved H_{II} structure.

Table 1. Nonsolvent Water

lipid	T (°C)	phase	$f^{a,b}$	water/lipid
Ole_2PtdEtn	2	L_α	0.18 ± 0.01	7.2 ± 0.3
	16	H_{II}	0.13 ± 0.02	5.4 ± 0.7
	30	H_{II}	0.14 ± 0.03	5.6 ± 1.4
Egg PtdEtn	2	L_α	0.17 ± 0.05	7.1 ± 2.2[c]
	15	L_α	0.18 ± 0.01	7.5 ± 0.4[c]
	28	L_α	0.17 ± 0.00	6.9 ± 0.1[c]
	40	H_{II}	0.12 ± 0.02	5.1 ± 0.6[c]
Myr_2PtdCho	30	L_α	0.23 ± 0.05	8.6 ± 1.9
	30	L_α	0.24[d]	9.0[d]

[a] Weight ratio of bound water. [b] $n \geq 3$. [c] Molecular weight for (18:0, 18:1) species. [d] From ref 10.

Discussion

The nonsolvent water associated with PtdEtn decreased from about 7 waters per lipid in the planar L_α state to about 5 waters per lipid in the highly curved H_{II} structure. Published values for water bound to the L_α state of PtdEtn are similar to our values. Calorimetric analysis of the dependence of the main transition on water indicated about 7 waters/lipid.[13] Geometric analysis of diffraction studies of dilauroyl PtdEtn yielded about 9 waters/lipid with half of the water intercalated within the headgroup region and the other half at the membrane surface between lamellae.[14,15] Nuclear magnetic resonance studies of motionally restricted water indicated the presence of 11 bound waters/lipid in Ole_2PtdEtn.[16]

The H_{II} phase of PtdEtn is known to take up much more water than the L_α state. The T_H stabilized at approximately 18 waters/lipid.[6,16,17] Seddon and co-workers[6] postulated that this increased water content was necessary for the maintenance of the H_{II} geometry by filling the water cores of the cylinders. At low hydration in the H_{II} state, however, both they and Rand and co-workers[17] noted a deviation in the diffraction data below about 5 waters/lipid, which was consistent with the presence of a pool of water that was more tenaciously held than the water responsible for the filling of the cylindrical core. Subsequently, evidence from infrared[9] and fluorescence[8] spectroscopies was interpreted as suggestive of a less hydrated headgroup region in the H_{II} state than in L_α state. Our results indicate that the amount of nonsolvent or bound water was decreased by almost 2 waters/lipid or about 25% in the curved H_{II} state. Such a decrease is consistent with the greater geometric constraints and increased packing density in the headgroup region of the highly curved H_{II} structure.[6]

Phosphatidylcholine (PtdCho) bilayers take up more water than do PtdEtn bilayers. Nagle and Wiener[15] estimated that dipalmitoyl PtdCho takes up 23 mol of water per mol of lipid in the L_α state, whereas dilauroyl PtdEtn takes up only 9. This difference in water uptake was due predominantly to greater spacings between the PtdCho lamellae. Estimates of the water bound in the headgroup region of PtdCho were much more similar to those for PtdEtn. Headgroup intercalated water amounted to 4.5 waters/lipid in dilauroyl PtdEtn bilayers. By contrast, three diffraction data sets for dipalmitoyl PtdCho yielded 3.7, 5.5, and 7.2 intercalated waters/lipid.[15] The PtdCho values overlapped with the PtdEtn value, but the median and average values were greater. In this study, the nonsolvent water in Myr_2PtdCho at 30 °C was 0.23 ± 0.05 g of water per g of lipid or 8.6 ± 1.9 water/PtdCho. Agreement with the value

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obtained by Katz and Diamond[10] at the same temperature using the same method (0.24 g/g, 9.0 mol/mol) was good. This further supports the concept that fluid bilayers of PtdCho bind more water than fluid bilayers of PtdEtn, but the difference in bound water is less than the difference in water uptake.

Experimental Methods

Materials. [2-^{14}C]Glycine (DuPont NEN, Boston, MA), [^{14}C(U)]-sucrose (Amersham, Arlington Heights, IL), [2-^{14}C]sodium acetate (NEN), and [^{3}H]H_2O (Amersham) were assayed for purity using alcohol/water partitioning[18] at two alcohol/water (octanol/water or butanol/water) ratios (1:1 and 1:10). Pure radiolabels exhibited partitioning that was independent of the alcohol/water ratio.

Phospholipids stored in chloroform (Avanti Polar Lipids, Alabaster, AL), and assayed for purity by thin layer chromatography (>99%), were dried to a thin film under dry N_2, evacuated overnight (< 5 mTorr), and hydrated (100 mM KCl, 10 mM Hepes, pH 7.4, with radiolabeled and unlabeled solutes) at a concentration of 20 mg/mL by vigorous vortexing. Total solute concentrations were 20 mM of sucrose (PtdEtn), 5 μM of sucrose (PtdCho), 25 mM of glycine, 30 μM of acetate. In biological membranes, the need to saturate dilute specificity for these solutes makes concentrations in the millimolar range preferable.[19] For PtdCho, micromolar levels of sucrose were employed to reproduce published work.[10] Preliminary experiments revealed no difference between the millimolar and micromolar solute levels in these lipid systems.

^{31}P NMR. The ^{31}P NMR spectra were acquired on a Bruker AM-360 spectrometer operating at 145.8 MHz (8.5 T) on nonspinning samples with a 10 mm double resonance probe as described.[5]

Water Binding Assay. Binding was determined using a modification of the dual-radiolabel centrifugal technique described by Katz and Diamond[20] and Janes and co-workers.[19,21] Briefly, after 2 h of equilibration the liposomal suspension was transferred to 10-mL sealed Oak Ridge polycarbonate tubes (Nalge Co., Rochester, NY) and centrifuged at the desired temperature for 60 min at 130 000 g in a Beckman Ti-75 rotor and L5-65B ultracentrifuge (Palo Alto, CA). Pellet and supernatant samples (~100 mg) were transferred to tared scintillation vials and weighed. After addition of 1 mL of water and 9 mL of Biosafe II scintillation fluid (Research Products International, Mount Prospect, IL), the samples were stored in the dark overnight. Separations (>99%) were monitored by a phosphorus assay.[22]

The radiolabels were counted in a Packard Tri-Carb model 1900CA liquid scintillation analyzer (Packard Instrument Co., Downers Grove, IL) equipped with a barium-133 external γ-ray source, using a dual-window analysis (0−9.1 keV; 9.1−156 keV). Quench standards were prepared using water (250-mL increments centered at 1.0 mL, 9 mL Biosafe) as a quenching agent. The instrument was calibrated weekly. Typical counting times were 5 min.

Binding Analysis. The determination of nonsolvent water follows the derivation of Katz and Diamond.[20]

$$f = -\frac{(C_{bp}/C_{bo}) - (C_{tq}/C_{to})}{1 - (C_{tq}/C_{to})} \quad (1)$$

f is the nonsolvent water expressed in molal units [g of nonsolvent water/g of lipid]/[g of free water/g of buffer], which reduces to the weight ratio of bound water. C is the radioactivity in units of dpm min^{-1} g^{-1}. The subscripts p and o refer to the lipid pellet and supernatant, respectively. The subscript q refers to the dpm from pellet water per gram of pellet. The subscripts t and b refer to the radiolabel markers for water and noninteracting solute, respectively.

The concept is that a polar solute that does not interact with the membrane marks bulk water, but bound water is inaccessible. As a result, the water/solute ratio is larger in the pellet than in the supernatant by an amount that reflects the bound water. We follow Katz and Diamond in employing sucrose as an ideal solute marker. For solutes that do interact with the membrane and provide less ideal nonsolvent water markers, eq 1 yields the difference between water binding and solute binding.[10,20] Acetate and glycine are the less ideal markers used here to corroborate the results by showing similar trends. Changes in hydration in the $L_\alpha \rightarrow H_{II}$ should be reflected by a change in f that is commensurate to that observed with sucrose given that the weak affinity of the solute for the two phases is similar.

Tritium Exchange. The tritium marker for water is exchangeable and will mark all exchangeable sites. This correction is usually small because of the high density of exchangeable sites in water (110 mol kg^{-1}) and the low density of readily exchangeable sites in lipids (4 mol kg^{-1} for PtdEtn, none for PtdCho). In the present application, however, it is not negligible. An appropriate expression is obtained by replacing C_{tq} with a more general expression for total pellet tritium, C_{tp}.

$$C_{tp} = C_{tq} + C_{tl}\,(L_p/M_p) \quad (2)$$

$$M_p = W_p + L_p \quad (3)$$

$$\beta = C_{tl}/C_{to} \quad (4)$$

$$C_{tq} = C_{to}(W_p/M_p) \quad (5)$$

C_{tl} refers to the exchanged tritium in the lipid (dpm min^{-1} g^{-1}). W_p, L_p, and M_p represent the mass of pellet water, pellet lipid, and total pellet, respectively. The density ratio of exchangeable tritium sites is represented by β. Substitution of eqs 3, 4, and 5 into eq 2 yields the following expression to be substituted into eq 1:

$$C_{tq}/C_{to} = ((C_{tp}/C_{to}) - \beta)/(1 - \beta) \quad (6)$$

This correction, while small, is not insignificant in the present application. Without it the hydration is overestimated by approximately 0.04 g of water per g of lipid or 1.6 waters per lipid.

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