Direct Determination of Hydration in the Interdigitated and Ripple Phases of Dihexadecylphosphatidylcholine:
Hydration of a Hydrophobic Cavity at the Membrane/Water Interface

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ABSTRACT   Hydrophobic cavities at the membrane/water interface are stably expressed in interdigitated membranes. The nonsolvent water associated with 1,2-di-O-hexadecyl-sn-glycero-3-phosphocholine (Hxdc$_2$Gro$P$Cho) in the interdigitated (L$_\beta$I) and ripple (P$_{\beta'}$) states and with its ester analogue 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (Pam$_2$PtdCho) in the gel (L$_{\beta'}$) and P$_{\beta'}$ states are determined directly. In the L$_\beta$I state at lower temperatures (4–20°C), 16–18 water molecules per phospholipid are bound, consistent with water-filled cavities and hydrated headgroups. At 28°C, the nonsolvent water decreases to 12, consistent with a reduction of the cavity depth by 0.34 nm due to increased chain interpenetration. This geometric lability may be a common feature of hydrophobic cavities. Only 5.4 waters are bound in the noninterdigitated P$_{\beta'}$ (40°C), whereas the ester bound 8.1 waters in its P$_{\beta'}$ (37°C), a difference of about one water per ester carbonyl. The relative dehydration of the ether linkage is consistent with it promoting more densely packed structures, which in turn, accounts for its ability to interdigitate.

INTRODUCTION

It can be argued that the most dominant force determining membrane and biomolecular structure is the hydrophobic effect and its drive to minimize the exposure of nonpolar moieties to the aqueous milieu (Tanford, 1980). Transient expression of nonpolar moieties or nonpolar cavities to the aqueous milieu and the avoidance thereof are important factors in membrane assembly (Tanford, 1980), membrane fusion (Siegel, 1999), the insertion of proteins into membranes (Jähnig, 1983), and protein folding (Fersht et al., 1993). In lipids, internal hydrophobic cavities play an important role in lipid polymorphism (Turner and Gruner, 1992) and fusion mechanisms (Siegel, 1999). In proteins, internal hydrophobic cavities have been implicated as important in structure/function/stability relations (Fersht et al., 1993), with their propensity to bind water or other small solutes being a significant consideration (Buckle et al., 1996; Takano et al., 1997; Baldwin et al., 1998). Whether and under what circumstances these cavities accommodate water has been widely debated (Matthews et al., 1995; Yu et al., 1999). According to one estimate, the probability that a purely hydrophobic cavity will accommodate water is only 1 in 20,000 (Wolfenden and Radzicka, 1994), although the probability increases with cavity size and polarity (Williams et al., 1994). The concept that interfacial packing defects, such as hydrophobic cavities at the membrane/water interface, are targets of alcohols and anesthetics has received heightened scrutiny (Channareddy et al., 1996). In this

formulation, small amphiphiles bind to and displace water from a high-energy interfacial site, act to stabilize that structure, and render it insensitive to external energetic stimuli.

The interdigitated membrane is a simple well-defined example of hydrophobic packing defects stably presented at the membrane/water interface. The interdigitated structure is a symmetrical lipid monolayer with the chain ends and phosphocholine headgroups exposed to water on each face (reviewed by Slater and Huang, 1988). The chain-end methyl groups are slightly recessed from the neighboring headgroups to form an amphipathic cavity. Small amphipathic molecules are thought to bind within the cavity and shield the hydrophobic methyl chain ends from water (McDaniel et al., 1983). As a result, the formation of these cavities is significantly promoted by small amphiphilic molecules at clinically relevant concentrations (Veiro et al., 1988). As expected for a cavity of defined geometry, and as observed for many biological processes, there is a sharp cutoff in the ability of the longer alcohols to stabilize interdigitation (Rowe and Campion, 1994). The stability of these sites is highly sensitive to their hydration (Kim et al., 1987; Ohki, 1991). A prerequisite to an understanding of the interactions of amphiphiles with interfacial cavities is an understanding of the changes in hydration that occur when the cavities are expressed.

In this study, a dual radiolabel centrifugal technique is used to determine directly the amount of bound water in the interdigitated (L$_\beta$I) and the ripple (P$_{\beta'}$) structures of 1,2-di-O-hexadecyl-sn-glycero-3-phosphocholine (Hxdc$_2$Gro$P$Cho) and in the P$_{\beta'}$ and the gel (L$_{\beta'}$) states of its ester analogue 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (Pam$_2$PtdCho). The latter does not stably interdigitate without the presence of an inducer, such as a short-chain alcohol, that ameliorates the unfavorable energetics associated with the exposure of the

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hydrophobic chain ends to water. The explanation for the different structural preferences of these linkages is unknown, as is whether differences in their hydration might account for their respective behavior.

One distinction between the properties of bulk and bound water is their ability to act as a solvent for solutes (Katz and Diamond, 1974; Channareddy et al., 1997). Bound water that is sequestered by the lipid structure is unavailable for solvation and is termed "nonsolvent water." Experimentally, the presence of bound water is reflected by a water-to-solute ratio in a lipid pellet that exceeds the ratio in the supernatant, when an ideal polar 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. The bound water determined in this manner typically is less than the water needed to maintain lipid samples at full hydration, as assessed by an invariance of structural parameters to further increases in water content, owing to a structural requirement for bulk (free) water that varies with lipid morphology.

MATERIALS AND METHODS

Hxdc$_2$Gro$P$Cho (Fluka, Milwaukee, WI) and Pam$_2$PtdCho (Avanti Polar Lipids, Alabaster, AL) were monitored for purity by thin-layer chromatography (>99%). [^{3}H]H$_2$O (1 mCi g^{-1}), 1,3-[^{14}C]glycerol (100 μCi ml^{-1}), and U-[^{14}C]sucrose (3.1 mCi mmol^{-1}) were obtained from NEN Research Products (Boston, MA).

The powdered Hxdc$_2$Gro$P$Cho was dissolved in chloroform at a concentration of 20 mg ml^{-1}; Pam$_2$PtdCho was obtained in chloroform. Typically, 60 mg of the lipids were dried to a thin film under N$_2$, evacuated overnight (<5 mTorr), and hydrated with 5 ml of buffer (radiolabels, 20 mM nonradiolabeled sucrose or glycerol, 10 mM Hepes, 100 mM KCl, pH 7.4) at 55°C for 20 min. The sample was annealed by cycling between 10°C (20 min) and 55°C (20 min) three times. Before the hydration assay, the samples were equilibrated at the experimental temperature overnight. The phase behavior of the hydrated Hxdc$_2$Gro$P$Cho was characterized by electron paramagnetic resonance studies of 2,2,6,6-tetramethyl-1-piperidinyloxy (Tempo Aldrich, Milwaukee, WI) binding (Wang et al., 1993) and was consistent with prior studies (Ruocco et al., 1985b; Kim et al., 1987; Laggner et al., 1987). The hydration assay is a dual radiolabel centrifugal approach developed by Katz and Diamond (1974) with the modifications described previously (Channareddy et al., 1997), based on the principles briefly described above.

RESULTS AND DISCUSSION

The ideal marker for solvent water is a solute that does not interact with membranes. Sucrose proved to be an appropriate nonsolvent water marker for bilayers and inverted phases (Katz and Diamond, 1974; Channareddy et al., 1997). To corroborate results obtained using sucrose, we have also employed glycerol as a nonsolvent water marker. Similar to sucrose, the lipid solubility of glycerol is negligible (Katz and Diamond, 1974; Hansch and Leo, 1979). Neither solute altered the phase behavior at the concentrations used (20 mM). Yet, at high concentrations all solutes will modify the lipid phase behavior by altering the activity or structure of water (Lehtonen and Kinnunen, 1995; Takahashi et al., 1997). Glycerol (McDaniel et al., 1983) and

sugars (Takahashi et al., 1997) are known to alter the stability of the interdigitated phase, but in an opposite sense. Glycerol at high concentrations promoted the formation of interdigitated structures in Pam$_2$PtdCho presumably by substituting for water at the interface (McDaniel et al., 1983), whereas the sugar trehalose promoted the formation of bilayer structures in Hxdc$_2$Gro$P$Cho through a kosmotropic action on water (Takahashi et al., 1997). Thus, the use of both water markers in tandem served as a check for the experimental fidelity.

Nonsolvent water of Hxdc$_2$GroPCho

Fully hydrated 1,2-di-O-hexadecyl-sn-glycero-3-phosphocholine exhibits four phases (Ruocco et al., 1985b; Kim et al., 1987; Laggner et al., 1987). A reversible transition between interdigitated phases of similar structure occurs at 5°C. A transition between the interdigitated gel and ripple phases occurs at 32.3°C. A transition between the ripple and fluid bilayer (L$_\alpha$) phases occurs at 43.5°C. The ester-linked analogue, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, exhibits four phases at similar temperatures with the gel structure (L$_{\beta'}$) being a noninterdigitated bilayer.

The fraction of water in a Hxdc$_2$Gro$P$Cho dispersion that is unavailable to solvate sucrose and glycerol is shown in Table 1. Similar values were obtained for both sucrose and glycerol solvent water markers. Using the average of the two markers, the nonsolvent water associated with the interdigitated state varied between 17.5 (4°C) and 16.4 (20°C) water molecules per phospholipid. A previous study has shown that a minimum of 16.8 waters are required to support interdigitation at 22°C (Kim et al., 1987). Below that threshold, Hxdc$_2$Gro$P$Cho was shown to adopt the noninterdigitated L$_{\beta'}$ structure. Between 16.8 and 18.5 waters both the L$_{\beta'}$ and the L$_\beta$I phases coexisted. The L$_\beta$I structure continued to swell until about 23.5 waters per phospholipid. Thus, the nonsolvent water determined in the

TABLE 1 Nonsolvent water

Lipid	Solute*	T (°C)	Phase	Water/lipid[#]	N[§]
Hxdc$_2$Gro$P$Cho	Sucrose	4	L$_\beta$I$_o$	19.5 ± 5.5	3
		10	L$_\beta$I	18.9 ± 5.9	3
		20	L$_\beta$I	17.7 ± 1.9	6
		28	L$_\beta$I	11.4 ± 2.0	6
		40	P$_{\beta'}$	5.1 ± 1.7	7
	Glycerol	4	L$_\beta$I$_o$	15.5 ± 3.8	3
		20	L$_\beta$I	15.1 ± 2.9	3
		28	L$_\beta$I	12.6 ± 0.9	3
		40	P$_{\beta'}$	5.8 ± 1.0	3
Pam$_2$PtdCho	Sucrose	25	L$_{\beta'}$	4.7 ± 1.5	3
		37	P$_{\beta'}$	8.2 ± 0.9	3
	Glycerol	25	L$_{\beta'}$	5.2 ± 0.8	5
		37	P$_{\beta'}$	8.0 ± 1.3	3
Myr$_2$PtdCho	Sucrose	30	L$_\alpha$	8.6 ± 1.9	9[¶]

*Solute concentration 20 mM.
[#]Mean and standard deviation.
[§]Number of determinations.
[¶]From Channareddy et al., 1997.

present study closely correlates with the minimum water necessary to support interdigitation and is slightly less than that observed at full hydration. This evidences a structural requirement for bulk (free) water that is modest, in contrast to the situation observed for the inverted hexagonal phase (Channareddy et al., 1997).

The nonsolvent water in $L_\beta I$ decreased from 16.4 waters per phospholipid at 20°C to 12.0 waters per phospholipid at 28°C. The possibility that this decrease arose from an admixture of $P_{\beta'}$ was ruled out by parallel electron paramagnetic resonance experiments that showed the sharp thermotropic $L_\beta I \rightarrow P_{\beta'}$ and $P_{\beta'} \rightarrow L_\alpha$ transitions consistent with published reports and not consistent with a broad range of phase coexistence (Ruocco et al., 1985b; Kim et al., 1987; Laggner et al., 1987). Several studies support the concept that the $L_\beta I$ of $Hxdc_2GroPCho$ is a mostly static structure below 20°C, with 20°C representing the onset of more rapid structural changes. The ^2H line shapes of chain-labeled $Hxdc_2GroPCho$ were invariant from -20°C to 20°C but began to change above 20°C (Ruocco et al., 1985a). The fluorescence intensity of 1,6-diphenyl-1,3,5-hexatriene in $Hxdc_2GroPCho$ was nearly constant below 20°C but increased above 20°C (Veiro et al., 1988). The spectrum of a chain-labeled pyrene-PtdCho in $Hxdc_2GroPCho$ showed marked changes above 20°C (Komatsu and Rowe, 1991). The integral small-angle diffraction intensity of $Hxdc_2GroPCho$ was stable from 10 to 20°C but began to increase above 20°C (Lewis et al., 1986). The CH_2 scissoring bands of $Hxdc_2GroPCho$ were distinct below 20°C but merged above that temperature (Laggner et al., 1987). A likely explanation arises from the observation that the location of the chain methyl groups (cavity depth) is not subject to any strong energetic tether or restraints. With the onset of spectroscopic changes occurring at 20°C, increases in the rate of chain isomerizations reduce the van der Waals' forces. To offset this reduction, the methyl terminus of the chain extends further toward the interface to maximize chain overlap and minimize hydrophobic exposure, resulting in a reduced cavity depth. Related variations have been demonstrated in the alcohol-induced $L_\beta I$ structure as a function of alcohol chain length (Adachi et al., 1995).

Taking the molecular volume of bulk water, 0.03 nm^3, a decrease of 4.4 water molecules corresponds to a decrease of 0.13 nm^3 in cavity volume. The surface area of interdigitated $Hxdc_2GroPCho$ has been calculated to be 0.762 nm^2 (22°C) (Kim et al., 1987) and 0.79 nm^2 (20°C) (Ruocco et al., 1985b). The cross-sectional area of the hydrophobic defect structure then would be 0.388 nm^2 per phospholipid (using the average of the two determinations). Considering the cavity depth as the dominant factor in determining the cavity volume, then the cavity depth is decreased and the chain interpenetration is increased by 0.34 nm. Using 0.125 nm as the separation of fully extended methylenes (Tardieu et al., 1973), the increased chain interpenetration between 20°C and 28°C amounts to 2.7 methylene units. Two assumptions deserve comment. The approach is sensitive to the molecular volume chosen for water and to the tilt of the

$L_\beta I$ structure. Smaller volumes for bound water are not unreasonable (Wolfe and Brockman, 1988; Williams et al., 1994). Most (McDaniel et al., 1983; Ruocco et al., 1985b; Kim et al., 1987; Adachi et al., 1995), but not all (Laggner et al., 1987; Vierl et al., 1994) studies have evidenced a nontilted $L_\beta I$ structure. Changes in tilt, in principle, could provide an alternative mechanism of modulating the volume of the interfacial cavity.

The nonsolvent water consists of contributions from the cavity and from the glycero-phosphocholine headgroup. The cavity depth (phosphorus to chain methyl) in the induced $L_\beta I$ of $Pam_2PtdCho$ has been estimated to be 0.7 nm from geometric considerations (McDaniel et al., 1983) and 0.49 nm from x-ray diffraction (by extrapolation to the notional alcohol of zero chain length) (Adachi et al., 1995). Such a cavity would accommodate 6.3–9.1 water molecules per cavity. From the total nonsolvent water of 16.4 molecules at 20°C, the difference yields the phosphocholine headgroup hydration as 7.3–10.1 waters. This compares with the nonsolvent water (8.6 ± 1.9) previously found for the fluid bilayer (L_α) state of 1,2-dimyristoyl-sn-glycero-3-phosphocholine ($Myr_2PtdCho$). (The L_α phase of $Hxdc_2GroPCho$ is less dense than water (Laggner et al., 1987) and the centrifugal hydration assay was less reliable. The ether should be slightly less hydrated than the ester, as discussed below.) The choice of the L_α state as a reference for headgroup hydration is justified on the grounds that the large interfacial area of $L_\beta I$ is more closely mimicked in the expanded L_α structure than in the condensed $P_{\beta'}$ structure.

A sharp decrease to 5.4 nonsolvent water molecules per phospholipid is observed in the noninterdigitated $P_{\beta'}$ structure of $Hxdc_2GroPCho$ (40°C) consistent with the loss of the interfacial cavities and a densely packed headgroup region.

Nonsolvent water of $Pam_2PtdCho$

The cause of $Hxdc_2GroPCho$'s ability to interdigitate has remained obscure. To determine whether hydration may play a role in its ability to interdigitate, the nonsolvent water associated with the gel ($L_{\beta'}$ and $P_{\beta'}$) structures of $Pam_2PtdCho$ was obtained (Table 1). $Pam_2PtdCho$ in isolation does not form an interdigitated structure at any temperature or level of hydration in the absence of elevated external pressure. As observed for $Hxdc_2GroPCho$, the amount of nonsolvent water did not depend on whether glycerol or sucrose was employed as a marker for solvent water. At 25°C $Pam_2PtdCho$ adopts a planar gel structure ($L_{\beta'}$) with tilted chains. At 37°C, the $Pam_2PtdCho$ adopts a rippled gel structure ($P_{\beta'}$) in which the interfacial area is increased (Cevc, 1991). The nonsolvent water associated with the $L_{\beta'}$ structure was 5.0 water molecules per phospholipid. In the $P_{\beta'}$ structure, the nonsolvent water increased by 3.1 water molecules per phospholipid. The results are consistent with the expectation that the $L_{\beta'} \rightarrow P_{\beta'}$ transition involves an increase in hydration (Cevc, 1991).

The ether linkage is less hydrated than the ester linkage. The amount of nonsolvent water associated with the $P_{\beta'}$ structure of Pam$_2$PtdCho is 8.1 waters per phospholipid. In Hxdc$_2$Gro$P$Cho, it drops to 5.4. Approximately one additional water molecule is bound for each ester carbonyl.

A decreased level of hydration in a noninterdigitated bilayer structure is generally indicative of tighter lipid packing (Lehtonen and Kinnunen, 1995) and vice versa (Smaby et al., 1983). Several reports are consistent with the ether linkage promoting more tightly packed noninterdigitated structures than those with the ester linkage (Smaby et al., 1983; Ruocco et al., 1985a; Levin et al., 1985; Lewis et al., 1986; Laggner et al., 1987; Lehtonen and Kinnunen, 1995). This may arise from changes in the chain orientations that stem from the sp^2 ester linkage being replaced by the sp^3 ether linkage (Ruocco et al., 1985a) or directly from the hydration of the ester carbonyl (Smaby et al., 1983). A more compact $L_{\beta'}$ structure is energetically less stable due to unfavorable steric effects in the headgroup region. The $L_{\beta'}$ structure of Pam$_2$PtdCho undergoes chain tilt to increase the headgroup area and thereby lessen the steric constraints. At higher temperature the crowding is relaxed further by rippling in the $P_{\beta'}$ structure. Hxdc$_2$Gro$P$Cho forms the $L_{\beta'}$ only at low water. Here, the dehydration associated with the ether linkage is demonstrated in the $P_{\beta'}$ structure. However, the concomitant headgroup crowding effects are expected to have destabilized the $L_{\beta'}$ structure and result in its replacement with the interdigitated $L_{\beta}I$, which in turn is stabilized by more densely packed chains (Braganza and Worcester, 1986). Thus, the noninterdigitated ether structures are less hydrated, more tightly packed, more sterically constrained in the headgroup region, and therefore prone to interdigitation.

Interdigitation as a model for interfacial packing defects

Our primary interest in the interdigitated phase of Hxdc$_2$Gro$P$Cho is that it provides a stable and dense population of hydrophobic cavities at the aqueous interface. The $L_{\beta}I$ phase is unknown in biology, but interfacial packing defects analogous to those that are presented in this structure may be more broadly relevant. The probability that water occupies small and purely hydrophobic cavities is thought to be quite low (Wolfenden and Radzicka, 1994), but that probability increases considerably with the size of the cavity and with the number of polar contacts available to water (Williams et al., 1994). The $L_{\beta}I$ cavities are large and being interfacial possess a cylinder of polar contacts. Thus, their ability to accommodate water is not unexpected. By contrast, the wholly nonpolar interstitial cavities expressed between the cylinders of hydrocarbon chains in the inverted hexagonal phase are not hydrated significantly (Channareddy et al., 1997). The hydration status of the cavity is important in assessing the interaction of nonpolar solutes with the interfacial cavities. Solute transfer to a hydrated nonpolar cavity is likely to be considerably more favorable than is solute transfer to an unoccupied cavity (Buckle et al., 1996).

We were initially surprised at the lability of the cavity as evidenced by the decrease in bound water as a function of temperature in this work and as evidenced by the change in cavity dimensions as a function of n-alcohol chain length in the work of Adachi et al. (1995). Was this a peculiarity of the $L_{\beta}I$ structure that might mitigate against its use as a cavity model or is dimensional lability likely to be common feature of hydrophobic cavities? A recent study of the hydrophobic cavity in the maize nonspecific lipid transfer protein supported the latter formulation (Gomar et al., 1998). That cavity, comparable in volume to the cavity studied here, was observed to possess considerable dimensional plasticity and was observed to swell upon binding its hydrophobic ligand. Hydrophobic cavities engineered into protein cores, although typically smaller and not interfacial, also exhibit structural plasticity (Buckle et al., 1996; Baldwin et al., 1998) and a broad selectivity or permissiveness in ligand binding that far exceeds that found for engineered polar cavities (Baldwin et al., 1998). Hydrophobic cavities, presented as interstices between the hydrocarbon chains of the cylinders that comprise inverted hexagonal lipid phases, also exhibit dimensional lability and swell upon filling with nonpolar solutes (Turner and Gruner, 1992). These observations suggest that a static picture of a structurally circumscribed cavity of rigidly defined dimensions may be inadequate in general. Rather, interfacial cavities may prove to be more accurately depicted as geometrically dynamic and labile structures.

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