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Lyotropic phase behavior of unsaturated phosphatidylcholine species: relevance to the mechanism of plasma membrane destabilization and freezing injury *

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The phase behavior and hydration characteristics of phosphatidylcholine (PC) species having one or two unsaturated acyl chains (i.e., 1-palmitoyl-2-oleoyl-, 1-palmitoyl-2-linoleoyl-, dilinoleoyl-, and dilinolenoylphosphatidylcholine) were characterized. Using differential scanning calorimetry, it was determined that the phase transition temperatures (T_m values) of these PC species increased by 30–40 C° over the range of water contents between 20 wt% and 5 wt%. To determine the relevance of dehydration-induced phase transitions to membrane destabilization during freezing, the hydration characteristics of PC species were investigated by constructing desorption isotherms. For all the species, the water content decreased from an average 17 wt% to 4 wt% water over the range of water potentials from −16 MPa to −160 MPa. The results of these and related studies of mixtures of 1-palmitoyl-2-linoleoyl-PC and dilinoleoyl-PC indicate that under the conditions of temperature and dehydration encountered during freezing and which result in membrane destabilization, unsaturated species of PC would be expected to remain in the liquid-crystalline phase.

Introduction

Destabilization of the plasma membrane is a primary cause of freezing injury in most biological systems; and, under conditions of slow cooling that preclude intracellular ice formation, injury is a consequence of freeze-induced cell dehydration (see Refs. 1 and 2). In the case of plant cells, considerable insight into the cryobehavior of the plasma membrane in relation to osmotic stress has been gained from studies of isolated protoplasts [1,2]. When suspensions of isolated plant protoplasts are frozen to temperatures over the range of −5°C to −10°C, the osmolality of the suspending medium increases from 0.5 osmolal in the unfrozen samples to 2.69 osmolal at −5°C and to 5.37 osmolal at −10°C with approx. 80% of the osmotically active water removed from the protoplasts at −5°C and approx. 90% at −10°C. Under these conditions injury in protoplasts isolated from nonacclimated rye leaves (NA protoplasts) is manifested as a loss of osmotic responsiveness because of the disruption of the semipermeable characteristics of the plasma membrane [1]. This form of injury is associated with several changes in the ultrastructure of the plasma membrane, including the formation of apparent lateral phase separations, aparticulate lamellae subtending the plasma membrane, and lamellar-to-hexagonal H_{II} phase transitions in the plasma membrane and subtending lamellae [3]. These changes are a consequence of the severe osmotic stresses encountered during freezing rather than exposure to the subzero temperatures per se (i.e., they can be elicited in protoplasts held at 0°C and subjected to 5.3 osmolal sorbitol, but not in protoplasts supercooled to −10°C). These ultrastructural alterations are not observed in protoplasts isolated from cold-acclimated leaves (ACC protoplasts). Thus, one facet of the cold acclimation process is to increase the cryostability of the plasma membrane by decreasing the propensity for lyotropic phase transitions. Because the differential propensity for dehydration-induced lamellar-to-hexagonal H_{II}

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Abbreviations: DSC, differential scanning calorimetry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; T_m, gel-to-liquid crystalline phase transition temperature; T_{BH}, bilayer-to-hexagonal H_{II} phase transition temperature; $X:Y/X':Y'$, acyl chains located at the *sn*-1 and *sn*-2 positions of phosphatidylcholine (For each acyl chain, the number preceding the colon represents the number of carbon atoms in the acyl chain and the number following the colon indicates the number of double bonds present.).

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phase transitions is also observed in liposomes formed from the plasma membrane lipid extracts of nonacclimated and cold-acclimated rye leaves [4], alterations in the lipid composition (see Ref. 5) are a major factor in determining the cryostability of the plasma membrane.

To provide a molecular explanation for the differential propensity for dehydration-induced lamellar-to-hexagonal H_{II} phase transitions in the plasma membrane of NA versus ACC protoplasts, it is necessary first to provide an understanding of the mechanism of dehydration-induced lamellar-to-hexagonal H_{II} phase transitions in NA protoplasts. Although the plasma membrane of both NA and ACC protoplasts contains many nonbilayer-forming lipid species [5], they remain as a bilayer when mixed with other lipid components. In such mixtures, dehydration-induced formation of nonbilayer structures is thought to be preceded by demixing of the membrane components [2,6,7]. It has been proposed that lyotropic liquid crystalline-to-gel phase transitions in membrane phospholipids such as PC result in demixing of the total lipid mixture and the localized enrichment of species such as PE, which, upon further dehydration, would undergo a lamellar-to-hexagonal H_{II} phase transition [6,7]. The ultrastructural changes that occur in the plasma membrane of NA protoplasts as a consequence of freeze-induced dehydration appear qualitatively consistent with this model in that (i) the large aparticulate areas could represent lateral phase separations resulting from liquid crystalline-to-gel phase transitions and (ii) the formation of the apparent lateral phase separations precedes the observed lamellar-to-hexagonal H_{II} phase transitions [3]. Although dehydration is known to raise the T_m of saturated PC species [8], little information is available concerning the lyotropic phase behavior of mixed-chain and di-unsaturated PC species common to rye plasma membrane. Further, most studies report T_m as a function of water content without any information on the hydration characteristics of the phospholipids. For the model to be of relevance to the changes observed in rye protoplasts, water potentials of approx. -12 MPa (equivalent to freeze-induced dehydration at $-10\,^{\circ}$C, when the protoplasts are subjected to a 5.4 osmolal solution) must be sufficient to lead to a lyotropic liquid crystalline-to-gel phase transition of PC, the major phospholipid of the plasma membrane.

Although the ultrastructural changes observed in the plasma membrane during freezing may be a consequence of demixing of gel phase and nonbilayer-forming lipids, lateral segregation (demixing) of membrane components may also occur in the absence of a liquid crystalline-to-gel phase transition. It has been demonstrated that structural changes occur as water is removed from between adjacent bilayers [9,10]. Segregation of membrane components and bilayer-to-nonbilayer phase transitions have been predicted to occur during the close approach of bilayers as a consequence of differences in the equilibrium separation distances of different lipid (and protein) components [9–11]. Based on studies of model systems, it has been predicted that PC, having a relatively large equilibrium separation distance, would diffuse out of regions of close approach, resulting in an enrichment of PE (which has a smaller equilibrium separation distance). This mechanism does not require a lyotropic phase transition to facilitate demixing.

A present limitation to distinguishing between these two possible mechanisms lies in the paucity of information concerning the phase behavior of lipid species found in the plasma membrane. The objective of this study was to characterize the phase behavior and hydration characteristics of PC species containing both one and two unsaturated acyl chains. The influence of dehydration on the phase behavior of individual molecular species of PC and binary mixtures of PC species common to the plasma membrane of rye was examined using differential scanning calorimetry and the relationship between water content and water potential for the various PC species was determined by constructing desorption isotherms.

Materials and Methods

For these experiments, 16:0/18:2-PC was obtained from Sigma Chemical Company (St. Louis, MO). All other lipids were obtained from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Purity of lipids was determined by thin-layer chromatography. For construction of desorption isotherms and differential scanning calorimetry, lipids were dried under nitrogen and in vacuo and dispersed in high purity distilled water by vortexing. Dispersions containing 1–2 mg lipid in excess water were placed into preweighed sample pans and equilibrated for at least five days at $35\,^{\circ}$C over distilled water or saturated salt solutions in sealed chambers providing atmospheres having the following water potentials: KNO_3, -15.7 MPa; KCl, -24.7 MPa; NaCl, -40.8 MPa; NH_4NO_3, -72.4 MPa; and $MgCl_2$, -159.4 MPa. Values for water potential (ψ) were calculated from published values for the respective relative humidities using the relationship $\psi = -RT \cdot \ln[\text{relative vapor pressure}]$ [12]. Note that the water potential of a solution (e.g. unfrozen fraction) is calculated using the relationship $\psi = -RT \cdot [\text{osmolality}]$. It was determined that the T_m and water content of lipids remained constant after three days of equilibration over the saturated salt solutions. To determine the dry weight of the samples, sample pans were opened following the calorimetry studies and the lipids dried for at least 24 h in vacuo over fresh phosphorus pentoxide at $70\,^{\circ}$C. Anhydrous lipid samples for DSC were prepared by drying the lipids directly from a chloroform solution in DSC pans, holding in vacuo over phosphorus pentoxide at 70 °C for 16–20 h, and then sealing in a dry nitrogen atmosphere. Differential scanning calorimetry was performed using a Perkin Elmer DSC-7. Transition temperatures given represent the temperatures of peak maxima determined using the computer software program available for the DSC-7.

Results

The T_m values for unsaturated species of PC in the fully hydrated state were all below 0 °C (Table I). Following dehydration over P_2O_5, the T_m values of all the species of PC examined increased by approx. 70 C°. When fully hydrated, both 18 : 2/18 : 2-PC and 18 : 3/18 : 3-PC exhibited a broad endotherm, with the onset and completion of the transition spanning 30 C°, similar to that reported previously [13]. The other three species of PC, including 18 : 1/18 : 1-PC, exhibited relatively sharp endotherms (data not shown). Anhydrous samples exhibited boarder transition endotherms in comparison to those of fully hydrated lipids. The T_m values for samples in excess water were similar to those reported previously by others for the same species [13,14], and the differences in T_m of approx. 70 C° between anhydrous and hydrated unsaturated PC species are consistent with the 60–70 C° difference reported for saturated PC species under similar hydration conditions [14].

The T_m values for 16 : 0/18 : 1-PC, 16 : 0/18 : 2-PC, 18 : 2/18 : 2-PC and 18 : 3/18 : 3-PC at intermediate levels of hydration were determined (Fig. 1). For all species of PC, decreasing the water content between 20 wt% and 5 wt% water increased the respective T_m values by approx. 40 C°. Removal of the remaining water increased the T_m values by an additional 30 C°. Similar experiments using 16 : 0/16 : 0-PC at lower water contents suggested that an abrupt increase in T_m occurred at water contents below 1 wt% (data not shown). The relationship between T_m and water content for PC species having one or two unsaturated acyl chains was similar to that of 16 : 0/16 : 0-PC (data not shown; see Ref. 8). This suggests that the interaction of water with the polar head group and its influence on T_m is not markedly affected by the type or combination of acyl chains associated with PC.

The relevance of the lyotropic behavior of PC species to dehydration-induced phase transitions during freezing requires information on the relationship between water potential and water content, i.e., the hydration characteristics. Desorption isotherms were constructed for the individual species of PC. Over the range of −16 MPa to −160 MPa the water content of PC decreased from 17 wt% to 4 wt% (Fig. 2). Equilibration over saturated salt solutions at higher water potentials produced variable results and precluded characterization of the relationship at higher levels of hydration. It is not known if this relationship is valid at very low levels of

TABLE I

Transition temperatures of unsaturated phosphatidylcholine species

Hydrated samples were prepared by dispersing lipids in excess water. Anhydrous samples were prepared by drying lipids from chloroform solution in vacuo over P_2O_5.

Phosphatidylcholine molecular species	T_m (°C)	
	hydrated	anhydrous
16:0/18:1	−3	68
18:1/18:1	−18	48
16:0/18:2	−20	37
18:2/18:2	−53	27
18:3/18:3	−63	7

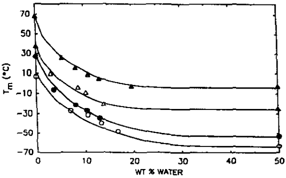

Fig. 1. Influence of water content on gel-to-liquid crystalline phase transition temperatures (T_m values) of phosphatidylcholine species. Lipids were dispersed in water and equilibrated at different relative humidities to obtain water contents between 20 wt% and 5 wt%. The values given for 50 wt% water represent the T_m values of samples in excess water and are the same as shown in Table I. Dry lipids (zero wt% water) were prepared by drying in vacuo over P_2O_5. ▲, 16:0/18:1-PC, △, 16:0/18:2-PC; ●, 18:2/18:2-PC, ○, 18:3/18:3-PC.

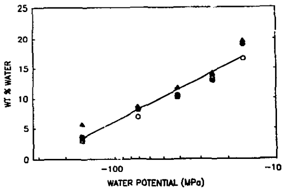

Fig. 2. Water content of phosphatidylcholine species as a function of water potential (expressed as a log scale). Lipid dispersions were equilibrated over saturated salt solutions and the water content determined as described under Materials and Methods. ▲, 16:0/18:1-PC; △, 16:0/18:2-PC; ●, 18:2/18:2-PC; ○, 18:3/18:3-PC.

hydration, given the affinity of PC for water (see Refs. 7 and 8). Nevertheless, the desorption isotherms indicate that the hydration characteristics of the different PC species were essentially identical over the range tested (Fig. 2) and can be described by the equation: $w = -13.09P + 32.61$, where w = water content and P = (log | water potential |). From this equation, the predicted water potential required to remove all water from PC is −310 MPa. The predicted water content at −1 MPa is 32.6 wt%, which is close to the minimum water content required to produce limiting lamellar repeat spacings for PC/water systems [9].

The relationship between T_m and water potential is presented in Fig. 3. Between −16 MPa and −160 MPa the respective T_m values of the PC species increased by 30–40 C°. Although the T_m values of 18:2/18:2-PC and 18:3/18:3-PC were raised by approx. 10 C° at high (but limiting) hydration levels compared to those in excess water, the T_m values of 16:0/18:1-PC and 16:0/18:2-PC were not significantly influenced at these water potentials. At hydration levels corresponding to a water potential of −12 MPa, the T_m values of 16:0/18:2-PC, 18:2/18:2-PC, and 18:3/18:3-PC were below −15°C. Thus, freezing to −10°C would not be expected to induce a phase transition in any of these species. Although the T_m of 16:0/18:1-PC was above −10°C even at full hydration, it is a minor constituent of the plasma membrane [5].

The lyotropic phase behavior of binary mixtures of the major plasma membrane PC species having one or two unsaturated acyl chains was also examined. Mixtures of 16:0/18:2-PC and 18:2/18:2-PC in excess water or at hydration levels effected by equilibration at water potentials between −16 MPa and −160 MPa exhibited a single endotherm at all concentrations, indicative of ideal mixing (data not shown). The respective T_m values increased nearly linearly with increasing proportions of 16:0/18:2-PC, and dehydration raised the T_m values of the mixtures (Fig. 4). In the plasma membrane of nonacclimated rye leaves the mol% of PC species containing one unsaturated acyl chain is 4-fold greater than that of PC species containing two unsaturated acyl chains. Mixtures containing up to 80% 16:0/18:2-PC exhibited T_m values below −20°C at water potentials above −25 MPa. Assuming that these two species are representative of the two major types of species of plasma membrane PC, the results suggest that demixing of selected PC species as a consequence of dehydration is unlikely.

The results of these studies indicate that under conditions of temperature and dehydration encountered during freezing to −10°C, the major species of PC common to the plasma membrane would be expected to

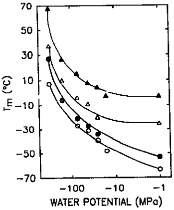

Fig. 3. Transition temperatures (T_m values) of phosphatidylcholine species as a function of water potential. Note that values at −1 MPa represent fully hydrated samples with excess (freezable) water. The T_m values at the lowest water potentials are those of samples dried over P_2O_5. Since this was assumed to be zero wt% water, these T_m values are placed at a water potential extrapolated for zero wt% water from Fig. 2. ▲, 16:0/18:1-PC; △, 16:0/18:2-PC; ●, 18:2/18:2-PC; ○, 18:3/18:3-PC.

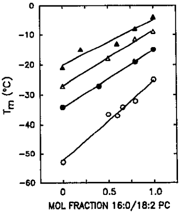

Fig. 4. Transition temperatures of binary mixtures of 16:0/18:2-PC and 18:2/18:2-PC as a function of water potential. Lipids were combined in chloroform in the appropriate ratios, dried, dispersed in water, then equilibrated over saturated salt solutions as described under Materials and Methods. The T_m for the lot of 16:0/18:2-PC used in these experiments was −25°C. (compare to Table I). No apparent chemical difference between these two lots was detected. ○, excess water; ●, −25 MPa; △, −41 MPa, ▲, −72 MPa.

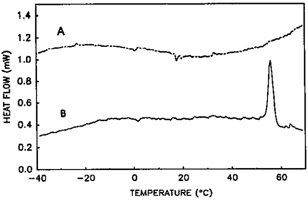

Fig. 5. DSC thermograms of rye plasma membrane total lipid extracts equilibrated at water potentials of −16 MPa (A) and −160 MPa (B). Samples were prepared as described under Materials and Methods. Scanning rate was 10 C°/min.

remain in the liquid-crystalline phase. Although small amounts of PC species having relatively high T_m values (e.g., 16:0/18:1-PC) are present in the membrane [5], the experiments with binary mixtures suggest that they would not phase separate. Other plasma membrane lipid constituents, e.g., glucocerebrosides, may be involved in such a mechanism; however, no apparent transition was observed over the range of −40°C to 70°C with plasma membrane total lipid dispersions in excess water or equilibrated at a water potential of −16 MPa (Fig. 5). Only at lower water potentials was an endotherm observed. For lipids equilibrated at a water potential of −160 MPa, an endotherm was observed at 54°C (Fig. 5). This was attributed to the phospholipid components of the plasma membrane inasmuch as an endotherm at 54°C was also observed for plasma membrane total phospholipids at low hydration (not shown).

Discussion

Freeze-induced cell dehydration results in destabilization of the plasma membrane of isolated rye protoplasts and is a primary cause of freezing injury in nonacclimated protoplasts frozen to −10°C. Destabilization of the plasma membrane is manifested as apparent lateral phase separations, aparticulate lamellae, and inverted cylindrical micelles [3]. To develop an understanding of dehydration-induced lamellar-to-hexagonal H_{II} phase transitions in relation to freezing, the mechanism of demixing of the membrane lipid components preceding bilayer-to-nonbilayer transitions must be established. Specifically, we wished to determine whether a lyotropic phase transition in the PC species is involved in demixing of plasma membrane lipid components.

It has been proposed that during dehydration lyotropic liquid crystalline-to-gel phase transitions in phospholipids such as PC result in demixing of the total lipid mixture and the localized enrichment of species such as PE, which can undergo a lamellar-to-hexagonal H_{II} phase transition [7]. This hypothesis was originally proposed to explain changes in membrane permeability and ultrastructure observed during lyophilization or desiccation [7]. The model has been suggested as a possible explanation for freezing injury as a consequence of freeze-induced dehydration [2,6] based on observed changes in membrane structure and properties: It was inferred from studies of the thermotropic properties of membranes that the aparticulate regions represent domains of gel phase lipid [3,6]. Based on the phase diagrams of a few (saturated) lipids it was proposed that the dehydrative stresses encountered during freezing would effect an increase in T_m and a decrease in T_{BH} of the membrane lipids. It was also thought that individual species of lipids would undergo a transition and subsequently phase separate. The results presented here argue against this model: The extent of dehydration required to raise the T_m of PC is greater than that encountered during freezing, and studies of mixtures suggest that unsaturated PC species do not readily undergo phase separations. These results, taken together with the lyotropic phase behavior of plasma membrane lipid extracts, suggest that the dehydration-induced redistribution of membrane particles (and hexagonal H_{II} structures) observed in nonacclimated protoplasts frozen to −10°C are not a consequence of liquid crystalline-to-gel phase transitions in the PC species (or other lipid components) of the plasma membrane.

An alternate mechanism, based on the studies of Parsegian, Rand and coworkers [9,10], may account for changes in plasma membrane ultrastructure during freeze-induced cell dehydration. The ultrastructural alterations observed (apparent lateral phase separations, aparticulate lamellae and lamellar-to-hexagonal H_{II} phase transitions) may be a consequence of differences in the hydration forces of the various lipid species [2,9,10,11,15]: As water is removed and adjacent bilayers are forced closer together, components within the bilayer pack more closely, creating a compressive stress (i.e., increased lateral pressure) and decreasing molecular area. Several changes in bilayer structure have been predicted to occur when bilayers are forced together [9–11]. These include (a) liquid crystalline-to-gel phase transitions, (b) demixing of lipid mixtures and segregation into separate coexisting lamellar phases, and (c) bilayer-to-nonbilayer transitions. Different lipids (and proteins) exhibit different equilibrium separation distances, i.e., bilayer separations for a given force. For example, egg PE and 18:1/18:1-PC were calculated to have equilibrium separations of 1.7 nm and 2.2 nm, respectively [9]. Consistent with this, Marra and Israelachvili [16] have shown that the hydration repulsion of DPPC is orders of magnitude greater than that of DPPE at separations of ⩽ 2 nm. Thus, lateral diffusion of lipids into or out of regions of close approach is expected in multicomponent bilayers that are being forced together as a consequence of dehydration [9–11]. Forces on the order of 10 MPa are sufficient to effect the close approach of bilayers, and consequently alter bilayer structure [9]. Based on these measurements, it is suggested that freeze-induced dehydration encountered at −10°C (equivalent to −12 MPa) may be sufficient to induce demixing in certain lipid mixutres in the absence of a liquid crystalline-to-gel phase transition. Similar calculations/predictions have been made for protein/lipid systems [11]. The hydration repulsion between proteins anchored in and extending from opposing bilayers has not been measured directly, but assuming it is similar to that of lipids and taking into consideration size, shape, and distance the protein protrudes from the bilayer, demixing of the lipid and protein components of a bilayer may occur at pressures of 5–15

MPa. Thus, aparticulate regions of the plasma membrane and demixing of plasma membrane lipids (and proteins) as well as bilayer-to-nonbilayer phase transitions may occur in the absence of a liquid crystalline-to-gel phase transition of a subpopulation of plasma membrane lipid species.

Although we have considered the possible role of hydration forces and the close approach of bilayers in the mechanism of lipid demixing as a consequence of freeze-induced dehydration, a similar mechanism may be invoked to explain injury as a consequence of desiccation. Although the dehydrative stresses associated with the (final) dry state of anhydrobiotic organisms are sufficient to induce pronounced changes in the phase behavior of lipid species (i.e., a liquid crystalline-to-gel phase transition of PC) as suggested by Crowe and Crowe [7], demixing of lipid components as a consequence of the close approach of bilayers may occur at higher hydration levels encountered during early stages of drying, particularly in sensitive biological systems where injury is observed at relatively high moisture contents.

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