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Effects of COR6.6 and COR15am Polypeptides Encoded by *COR* (Cold-Regulated) Genes of *Arabidopsis thaliana* on Dehydration-Induced Phase Transitions of Phospholipid Membranes[1]

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Cold acclimation of *Arabidopsis thaliana* includes the expression of cold-regulated (*COR*) genes and the accumulation of COR polypeptides. The hydration characteristics of two COR polypeptides, COR6.6 and COR15am, have been determined and their effects on the dehydration-induced liquid crystalline-to-gel and lamellar-to-hexagonal II phase transitions in phospholipid mixtures have been examined. After dehydration at osmotic pressures between 8 and 150 MPa, the water content of the COR polypeptides was less than that of bovine serum albumin, with COR15am the least hydrated: bovine serum albumin > COR6.6 > COR15am. Neither COR6.6 nor COR15am altered the dehydration-induced gel lamellar → fluid lamellar phase transition temperature of either dipalmitoylphosphatidylcholine or dioleoylphosphatidylcholine (DOPC). In multilamellar vesicles of dioleoylphosphatidylethanolamine:DOPC (1:1, mol:mol) prepared by either freeze-thaw or reverse-phase evaporation methods, neither COR6.6, COR15am, nor bovine serum albumin altered the incidence of the dehydration-induced formation of the inverted hexagonal phase as a function of osmotic pressure. However, a specific ultrastructural alteration—the formation of a striated surface morphology in the lamellar domains—was observed in mixtures of dioleoylphosphatidylethanolamine:DOPC that were dehydrated in the presence of COR15am. Nevertheless, neither COR6.6 nor COR15am appears to participate in a specific protein-phospholipid interaction that alters the dehydration-induced phase behavior of phospholipid vesicles.

Freezing injury in leaves and protoplasts of herbaceous species such as winter rye (*Secale cereale* cv Puma) and spring oat (*Avena sativa* cv Ogle) is primarily a consequence of destabilization of the plasma membrane (Steponkus et al., 1993). The freeze-induced lesions in the plasma membrane that are responsible for increased electrolyte leakage from leaves and loss of osmotic responsiveness of isolated protoplasts are similar in rye (Webb and Steponkus, 1993), oat (Webb et al., 1994), and *Arabidopsis thaliana* (Uemura et al., 1995), but differ in nonacclimated and cold-acclimated leaves. In nonacclimated leaves and protoplasts, the ultrastructural alterations associated with the loss of osmotic responsiveness include the formation of aparticulate domains within the plasma membrane, aparticulate lamellae subtending the plasma membrane, and the H_{II} phase in regions where the plasma membrane is brought into close apposition with various endomembranes (Gordon-Kamm and Steponkus, 1984; Webb and Steponkus, 1993). These alterations in membrane ultrastructure are consequences of interbilayer interactions that result from freeze-induced removal of water that is associated with the headgroups of the membrane lipids (Steponkus and Webb, 1992; Steponkus et al., 1993). After cold acclimation, freeze-induced formation of the H_{II} phase is precluded and injury is associated with the fracture-jump lesion (Fujikawa and Steponkus, 1990; Steponkus and Webb, 1992), which we believe is the result of the formation of interlamellar attachments and fusion of the plasma membrane with various endomembranes in localized domains (Steponkus et al., 1993).

Previous studies have demonstrated that alterations in the lipid composition of the plasma membrane are causally related to its increased cryostability after cold acclimation (see review by Steponkus and Webb, 1992). Also, the extreme difference in the freezing tolerance of winter rye and spring oat is associated with genotypic differences in the lipid composition of the plasma membrane (Steponkus et al., 1993). Nevertheless, freezing tolerance is a multifaceted trait, and differences in lipid composition—be they the

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Abbreviations: COR polypeptides, polypeptides encoded by cold-regulated genes; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; FATMLV, freeze-and-thaw multilamellar vesicle; H_{II}, inverted hexagonal; L_{α}, fluid lamellar; L_{β}, gel lamellar; $L_{\beta'}$, tilted gel lamellar; MLV, multilamellar vesicle; REVMLV, reverse phase evaporation multilamellar vesicle; T_m, liquid crystalline-to-gel phase transition temperature.

result of cold acclimation or genotypic differences—are but one facet. It has been suggested that some proteins that are synthesized during cold acclimation may have cryoprotective effects on cellular membranes (Heber and Kempfle, 1970; Volger and Heber, 1975; Hincha et al., 1989, 1995).

Cold acclimation of *A. thaliana* is associated with the expression of several cold-regulated (*COR*) genes (Thomashow, 1990). Two of these, *COR6.6* and *COR15a*, encode soluble polypeptides that are not precipitated upon boiling, are rich in hydrophilic amino acids, and, on the basis of amino acid sequence analysis, are predicted to be α-helical and amphipathic (Thomashow, 1993). COR6.6 is thought to be located in the cytoplasm; the COR15a polypeptide is targeted to the chloroplasts, and during import is processed to the mature polypeptide COR15am.

The highly hydrophilic nature of some of the COR polypeptides predicted from amino acid sequence analysis has been taken by some workers to imply that they are highly hydrated (Kazuoka and Oeda, 1992). It is also suggested that, since severe cellular dehydration occurs during freezing, the COR polypeptides "hold water molecules on their surface and within their three-dimensional structures" so as to alleviate dehydration-induced damage of proteins and/or membranes (Kazuoka and Oeda, 1992). Alternatively, because some amphipathic proteins have strong interactions with membrane lipids at the interfacial regions of membrane surfaces (McLean et al., 1991), it is conceivable that amphipathic COR polypeptides could associate with the lipids of plant membranes and decrease their propensity for lyotropic phase transitions, including formation of the H_{II} phase, during freeze-induced dehydration.

To determine the influence of COR6.6 and COR15am on dehydration-induced membrane destabilization during freezing, we have determined the hydration characteristics of the polypeptides and their effects on the dehydration-induced $L_\alpha \rightarrow L_\beta$ and lamellar-to-H_{II} phase transitions in phospholipid vesicles.

MATERIALS AND METHODS

Materials

The coding sequences for the COR6.6 and COR15am polypeptides of *Arabidopsis thaliana* were expressed in *Escherichia coli* and purified as described in the accompanying paper (Gilmour et al., 1996). COR polypeptides were lyophilized and stored at −20°C before use. Lipids were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification.

Desorption Isotherms of COR Polypeptides

Aliquots of approximately 1 mg of COR6.6, COR15am, and BSA (Sigma catalog no. A-7030, >98% pure) were dissolved in 15 μL of water and then placed in preweighed DSC pans (model 0219-0062, Perkin-Elmer) and equilibrated at 20°C over saturated salt solutions having known osmotic pressures over the range of 8.3 to 150 MPa. The samples were equilibrated under N_2, initially for 7 d at an osmotic pressure of 8.3 MPa, then for 3 d at each subsequent osmotic pressure up to 150 MPa. Water content of the samples is expressed as wt% water, which was calculated from gravimetric measurements of the mass of the sample after equilibration at each osmotic pressure and the final ("dry") mass after drying the samples overnight over P_2O_5 at 60°C in vacuo.

Preparation of Liposomes

Two different methods were used to disperse the COR polypeptides with phospholipid liposomes. In the first method, lipids or lipid mixtures in $CHCl_3$ were dried under a stream of N_2 at 40°C, and residual solvent was removed by storage overnight under high vacuum. The lipids were dispersed by the addition of an 85 mg mL^{-1} solution of either COR6.6 or COR15am. This represented a final lipid:polypeptide ratio of 2:1 (w/w) or mol ratios of 18:1 for phospholipid:COR6.6 and 25:1 for phospholipid:COR15am. Homogeneous distribution of the polypeptides between the multilayers of the lipid dispersion was facilitated by extensive vortexing and 10 freeze-thaw cycles between −196°C and either 55 (DPPC) or 20°C (DOPC and DOPE-DOPC mixtures). DPPC was also dispersed in the presence of Suc, at a 2:1 (w/w) ratio (0.9:1, mol:mol) of DPPC:Suc, by the same procedure for studies of the effect of Suc on the dehydration-induced increase in T_m. Dispersions resulting from this procedure are referred to as FATMLVs (Mayer et al., 1985). Previous studies have established that repetitive freezing and thawing of multilamellar phosphatidylcholine dispersions facilitates distribution of solutes and results in very high solute-entrapment efficiencies (Mayer et al., 1985).

The second dispersal method used reverse-phase evaporation to facilitate entrapment of COR polypeptides within the liposomes. Approximately 3 mg of DOPE:DOPC (1:1, mol:mol) were dissolved in 45 μL of $CHCl_3$ to which 1.5 mg of either COR6.6, COR15am, or BSA, dissolved in 15 μL of water, was added. The mixture was flushed with N_2 and then sonicated for 5 min at 20°C to generate a stable microemulsion. Chloroform was removed from the emulsion by rotary evaporation at 50°C, after which the sample was rehydrated by the addition of 20 μL of water and the residual solvent was removed by rotary evaporation for 20 min at 50°C. Water was added to the dispersion to generate a final mixture containing approximately 60 to 70 wt% water. Dispersions prepared by this method are referred to as REVMLVs. Previous work has established that reverse-phase evaporation effectively incorporates solutes within liposomes and results in a high-solute encapsulation efficiency (Düzgünes et al., 1983).

Effect of COR Polypeptides on the T_m of DPPC and DOPC

The effect of COR6.6 and COR15am on the T_m of DPPC and DOPC was determined by DSC. The effect of Suc, which minimizes the dehydration-induced increase in T_m, was also determined for comparative purposes. Samples of FATMLVs were placed in DSC sample pans and dehydrated above saturated salt solutions having osmotic pres-

sures between 8.3 and 286 MPa at 20°C. Samples of DPPC and DPPC-Suc were equilibrated for 7 d at each osmotic pressure; the DPPC-COR6.6 and DPPC-COR15am samples were equilibrated sequentially as described above. Control experiments confirmed that the water content of the samples and the temperature and enthalpy of the lipid-phase transition obtained using sequential desorption steps were identical to those obtained using a single 7-d equilibration period. After equilibration, the sample pans were sealed and the phase behavior was determined using a Perkin-Elmer DSC 7. Samples containing DPPC were scanned between 20 and 90°C at 10°C min^{-1}; samples containing DOPC were scanned between −50 and 20°C at a rate of 10°C min^{-1}. This scan rate accurately estimates the phase transition temperature and enthalpy for lipid dispersions having water contents ≥ 5 wt% (Webb et al., 1993). Peak transition temperatures and enthalpies were calculated from the heating scans using DSC 7 software.

Effect of COR Polypeptides on the $L_\alpha \rightarrow H_{II}$ Phase Transition in a DOPE-DOPC Mixture

To determine the influence of COR6.6 and COR15am on the osmotic pressure dependence of the $L_\alpha \rightarrow H_{II}$ phase transition in DOPE-DOPC mixtures, we examined dispersions of DOPE-DOPC dehydrated in the presence of the COR polypeptides. Mixtures of DOPE:DOPC (1:1, mol:mol) were dispersed with either COR6.6, COR15am, or BSA by both the freeze-thaw and reverse-phase evaporation methods. Aliquots of 1 to 3 μL of the concentrated dispersions (containing approximately 60–70 wt% water) were placed on freeze-fracture sample holders and dehydrated over saturated salt solutions having osmotic pressures between 20.4 and 286 MPa. After equilibration for 7 d at 20°C, the samples were cryofixed by plunging into liquid propane supercooled with liquid nitrogen.

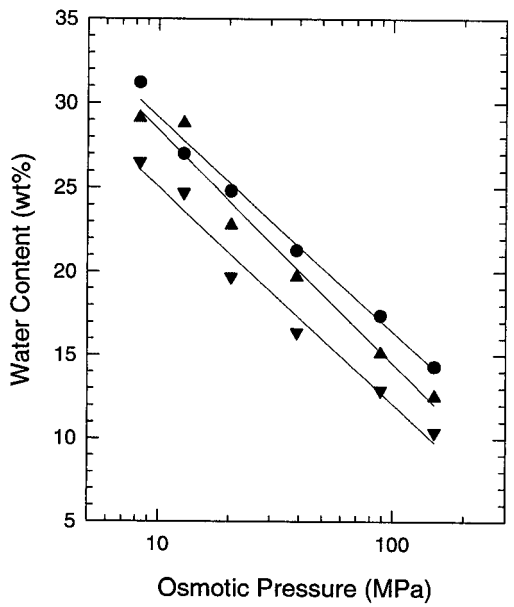

Figure 1. Desorption isotherms of COR6.6 (▲), COR15am (▼), and BSA (●). Aqueous dispersions of the proteins were dehydrated at 20°C over saturated salt solutions with osmotic pressures between 8.3 and 150 MPa. Water contents were determined gravimetrically. Lines represent linear regressions of the data; all regressions have $r^2 \geq 0.975$.

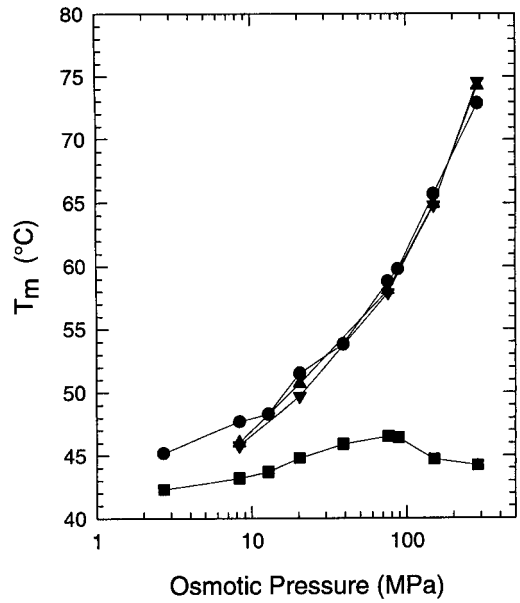

Figure 2. Effect of the COR6.6, COR15am, and Suc on the dehydration-induced increase of T_m of DPPC. FATMLVs of DPPC (●), DPPC-COR6.6 (▲), DPPC-COR15am (▼), or DPPC-Suc (■) were dehydrated at 20°C over saturated salt solutions with osmotic pressures between 2.7 and 286 MPa. T_m values were determined by DSC during a warming scan between 20 and 80°C at 10°C min^{-1}.

Desorbed samples were fractured and replicated in either a Balzers (Liechtenstein) 360 freeze-fracture device at −102°C and $<1 \times 10^{-6}$ torr or a Balzers 400K device at −110°C and $<1 \times 10^{-7}$ torr. Replicas were washed for 2 to 3 d in concentrated H_2SO_4 followed by several hours in Clorox before they were examined in a Philips (Eindhoven, The Netherlands) EM300 electron microscope.

RESULTS

Hydration Characteristics of the COR Polypeptides

The water content of all of the proteins varied linearly with the ln of the osmotic pressure. At all osmotic pressures, the water content of BSA > COR6.6 > COR15am (Fig. 1). Extrapolation of the linear regressions predicts that complete removal of water from the proteins, relative to the water content over P_2O_5, would occur at osmotic pressures of 1900, 1100, and 850 MPa, respectively.

Effect of the COR Polypeptides on the T_m of DPPC and DOPC

Dehydration of DPPC liposomes increased the T_m from 41°C for the fully hydrated lipid to approximately 75°C after dehydration at an osmotic pressure of 286 MPa (Fig. 2). In the presence of Suc, a known cryoprotectant, the dehydration-induced increase of the T_m of DPPC was

minimized (Fig. 2). At all osmotic pressures, the T_m of FATMLVs of DPPC containing either COR6.6 or COR15am was identical to that of DPPC alone (Fig. 2). That is, the COR polypeptides did not influence the dehydration-induced increase of the T_m of DPPC. In addition, the COR polypeptides had no effect on the enthalpy of the $L_\beta \rightarrow L_\alpha$ phase transition (data not shown), indicating that there was no influence on either the energetics or cooperativity of the phase transition of DPPC. Therefore, neither COR6.6 nor COR15am interacted with DPPC in a manner that altered the hydration dependence of the $L_\alpha \rightarrow L_\beta$ phase transition.

A direct interaction between the COR polypeptides and the phospholipid headgroup may require the lateral spacing of the lipid to be characteristic of fluid phase membranes. However, at the hydrations and temperatures used above, DPPC is in the laterally condensed $L_{\beta'}$ phase and its molecular area and lateral spacing are significantly smaller than when it is in the fluid L_α phase. Therefore, the influence of COR15am on the dehydration-induced increase of T_m of DOPC, a diunsaturated species of phosphatidylcholine that is in the fluid phase over the range of hydrations and temperatures used in these studies (Webb et al., 1993), was determined. The T_m of DOPC increased from −21°C in the fully hydrated state to −2.5°C after dehydration at an osmotic pressure of 286 MPa (Fig. 3). In FATMLVs of DOPC-COR15am, there was no effect of the polypeptide on the dehydration-induced increase in the T_m of DOPC (Fig. 3). In addition, COR15am had no effect on the enthalpy of the $L_\alpha \rightarrow L_\beta$ phase transition of DOPC (data not shown).

The absence of an effect of COR15am on the dehydration-induced increase of the T_m of DOPC (Fig. 3) suggested

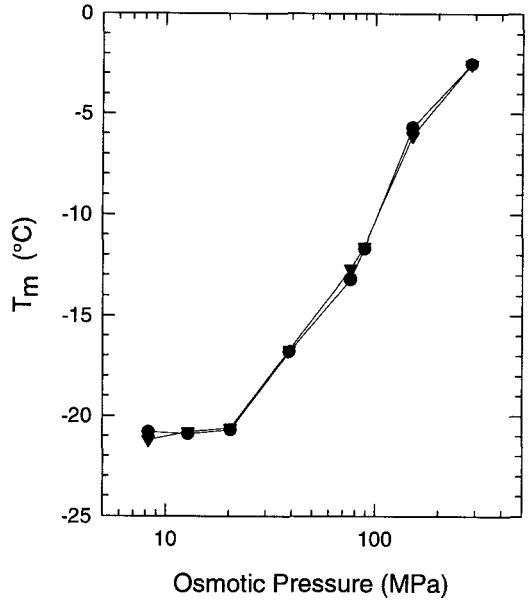

Figure 3. Effect of COR15am on the dehydration-induced increase of the T_m of DOPC. FATMLVs of DOPC (●) or DOPC-COR15am (▼) were dehydrated at 20°C over saturated salt solutions with osmotic pressures between 8.3 and 286 MPa. T_m values were determined by DSC during a warming scan from −50 and 20°C at 10°C min^{-1}.

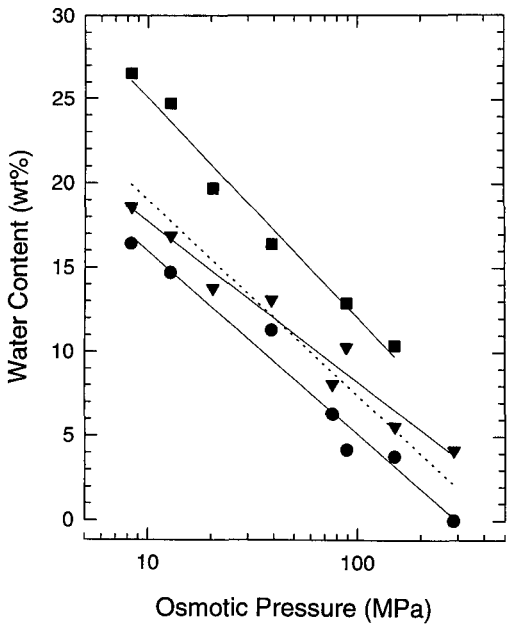

Figure 4. Desorption isotherms of DOPC (●), COR15am (■), and DOPC-COR15am (▼). Aqueous dispersions were dehydrated at 20°C over saturated salt solutions having osmotic pressures between 8.3 and 286 MPa. Water contents were determined gravimetrically. The dotted line represents the calculated water content of the DOPC-COR15am mixture that would be obtained if the protein and lipid components hydrated independently of each other. Solid lines represent linear regressions; all regressions have $r^2 \geq 0.975$.

that the protein had no influence on the water content of the lipid in the dehydrated FATMLVs. To determine if there was an effect of COR15am on the hydration of DOPC in DOPC-COR15am mixtures, the desorption isotherms of COR15am, DOPC, and DOPC-COR15am mixtures were compared (Fig. 4). At all osmotic pressures, the water content of COR15am was significantly greater than that of DOPC. FATMLVs of DOPC-COR15am mixtures had water contents intermediate between those of the individual components. If DOPC did not interact with COR15am (e.g. by hydrogen bonding) but hydrated independently of the polypeptide, then the equilibrium water content of the DOPC-COR15am mixture should be equal to what would be predicted from the hydration of the individual components. Conversely, a DOPC-COR15am interaction may decrease the number of sites for solute-water adsorption and, hence, such a mixture might be expected to have a water content that is lower than that predicted from the individual components. The water content of the DOPC-COR15am mixture was very similar to that predicted for the DOPC-COR15am mixture by assuming that DOPC and COR15am hydrate independently of each other, and correcting for the proportions of the lipid and polypeptide present in the mixture (Fig. 4). Therefore, these results indicate that COR15am does not interact with DOPC in a manner that alters the hydration of the phospholipid (Fig. 4), which is consistent with the observation that COR15am did not alter the hydration dependence of the T_m (Fig. 3).

Effects of COR Polypeptides on the Lyotropic $L_\alpha \rightarrow H_{II}$ Phase Transition of DOPE:DOPC Mixtures

Effect of COR15am on FATMLVs

FATMLVs composed of DOPE-DOPC-COR15am that were dehydrated at an osmotic pressure of either 20 or 39 MPa were exclusively in the lamellar phase; neither the H_{II} phase nor other nonlamellar structures were observed (data not shown). The H_{II} phase was first observed after dehydration at an osmotic pressure of 76 MPa (Fig. 5, A and B) and became the predominant lipid phase after greater dehydration at the higher osmotic pressures of 88 (data not shown) and 150 MPa (Fig. 5C).

In dehydrated suspensions of FATMLVs composed of DOPE-DOPC-COR15am, the lipid lamellae occurred in discrete, multilamellar aggregates; interspersed between the aggregates were domains that were apparently composed of excess COR polypeptide (e.g. Fig. 5). In contrast, in dehydrated lipid suspensions that did not contain added polypeptides, the lipid lamellae appeared as a continuum (e.g. figures 3–5 in Webb et al., 1993). Although a repetitive freeze-thaw dispersal method (Mayer et al., 1985) was used

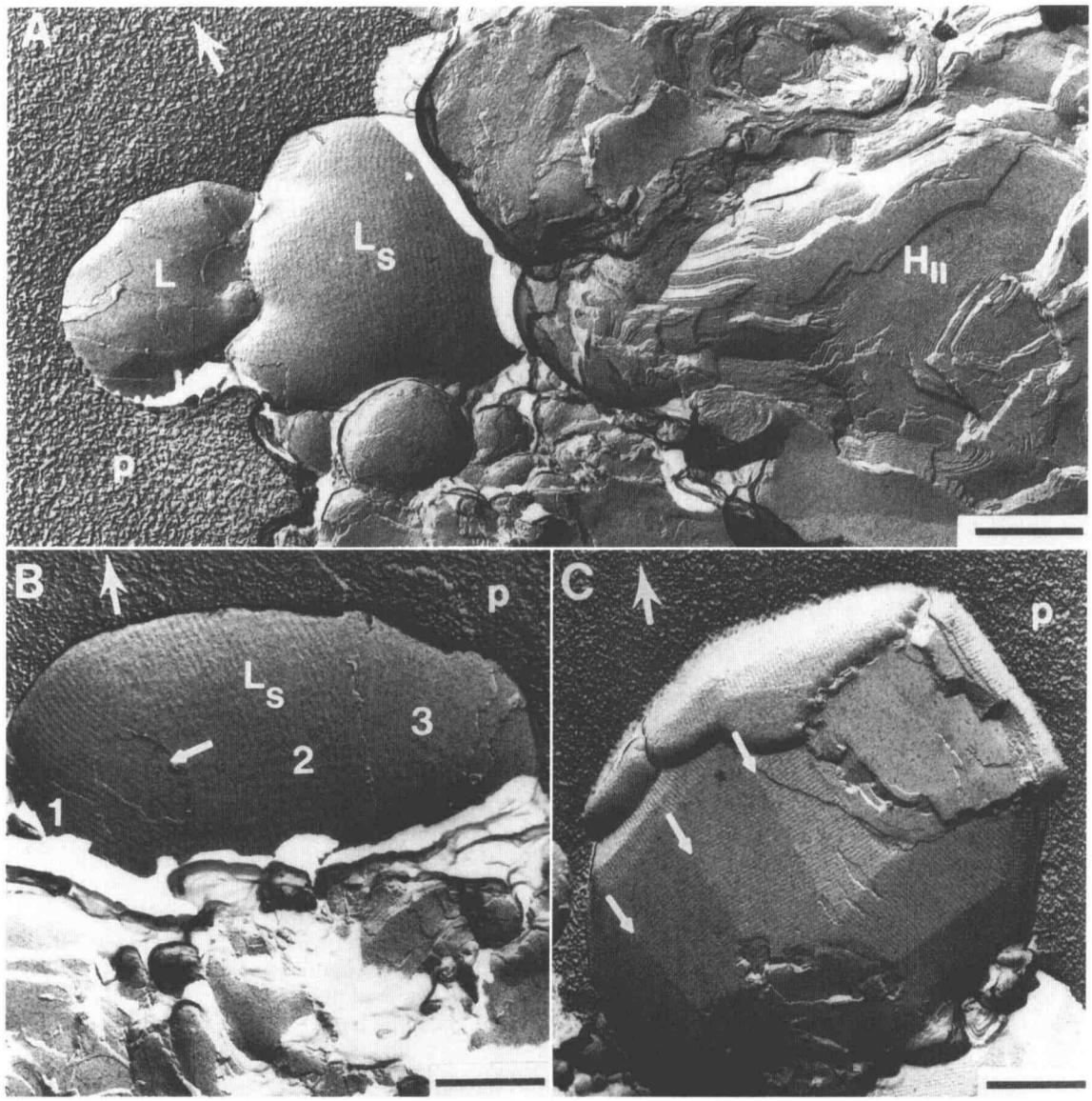

Figure 5. Freeze-fracture electron micrographs of FATMLV dispersions of DOPE-DOPC-COR15am dehydrated at osmotic pressures of 76 (A and B) and 150 MPa (C). Both the lamellar (L) and H_{II} phases were observed at 76 (A and B), 88 (data not shown), and 150 MPa (C). The lipid in close proximity to the COR15am domains (p) was in the lamellar phase, whereas the lipid more distant from the protein regions was in the H_{II} phase (A). A distinctive striated morphology (L_s) was observed in the lamellar regions that were adjacent to the COR15am domains (A–C). The striated morphology was observed to at least the third lamellae (arrow, B) subtending the interface between the lipid and the COR15am domains (numbers, B). In addition, dehydrated lipid aggregates with a polyhedral geometry and distinct "corners" (arrows, C) were observed. Bars represent either 400 (A and B) or 500 nm (C). Large arrows indicate direction of shadowing.

to promote the homogeneous distribution of the COR polypeptides between lamellae, the occurrence of polypeptide domains might suggest a nonhomogeneous and/or partial incorporation of COR15am between the lamellae of the MLVs. However, the COR polypeptide was added in excess, and the existence of isolated regions of COR polypeptide is not necessarily indicative of poor mixing of the lipid and COR polypeptide. Indeed, quantitative analysis of the freeze-fracture EM replicas indicated that the putative polypeptide domains constituted only 3 to 5% of the area of the sample—a proportion of the sample insufficient to account for the majority of the added COR polypeptide. At all osmotic pressures, the lipid lamellae immediately adjacent to the polypeptide domains and many layers subtending the polypeptide/lipid interface were invariably in the lamellar phase (Fig. 5, A–C). That is, the H_{II} phase was observed only in regions of the dehydrated lipid systems that were separated from the polypeptide domains by many intervening lamellae. The occurrence of the lamellar phase proximal and the H_{II} phase distal to the domains of COR15am was consistently observed after dehydration at osmotic pressures of 76 (Fig. 5, A and B), 88 (data not shown), 150 (Fig. 5C), and 286 MPa (data not shown).

In addition, a striking ultrastructural alteration was observed in the lamellar lipid domains of DOPE-DOPC-COR15am FATMLVs that were dehydrated at osmotic pressures of 39 MPa and greater. Frequently, the lamellar domains had a striated surface morphology (Fig. 5, A–C). The striated morphology occurred in lamellae that were immediately adjacent to COR15am domains (see below) and in several layers interior from the polypeptide-lipid interface (Fig. 5B). In some instances, portions of the lipid aggregates in the dehydrated COR15am-lipid dispersions had a polyhedral shape with distinct "corners" (Fig. 5C).

Effect of COR6.6 on FATMLVs

FATMLVs of DOPE-DOPC-COR6.6 that were dehydrated at an osmotic pressure of 39 MPa contained only the lamellar phase (Fig. 6A). At an osmotic pressure of 76 MPa, both lamellar and H_{II} phases were observed (Fig. 6B). The proportion of the sample that was in the H_{II} phase increased with the lower hydrations that occurred after dehydration at osmotic pressures of 88 (Fig. 6C), 150 (Fig. 6D), and 286 MPa (data not shown). As described above for lipid dispersions containing COR15am, dehydrated dispersions of DOPE-DOPC-COR6.6 contained discrete regions attributable to COR6.6 (Fig. 6, B–D). The regions of the dehydrated lipid either in contact with or in close proximity to the COR6.6 domains were exclusively in the lamellar phase. This was observed in samples dehydrated at 76 (Fig. 6B), 88 (Fig. 6C), 150 (Fig. 6D), and 286 MPa (data not shown). In some instances, multiple lamellae separated the COR6.6 domain from regions of the H_{II} phase (Fig. 6C).

The striated surface morphology and polyhedral ultrastructures that were observed in the lamellar regions of dehydrated mixtures of DOPE-DOPC-COR15am were not observed in dehydrated FATMLVs of DOPE-DOPC-COR6.6. Instead, numerous localized, single-step dislocations of the fracture plane to adjacent lamellae (Fig. 6, A and B) were frequently observed. These dislocations in the fracture plane occurred in up-and-down steps that were readily distinguished from the vectorial, unidirectional path of the fracture plane through multiple layers of lamellae in pure lipid systems (Webb et al., 1993).

Effect of COR15am, COR6.6, and BSA on REVMLVs

The observation of domains in the dehydrated suspensions of DOPE-DOPC-COR FATMLVs tentatively attributed to COR polypeptide suggests that the freeze-thaw dispersal method may not have resulted in a homogeneous distribution of the COR polypeptides. Therefore, in an attempt to promote a more uniform distribution of COR15am and COR6.6 between the lamellae of the liposomes, a reverse-phase evaporation method was used to prepare the MLVs. Reverse-phase evaporation is reportedly very effective in encapsulating solutes, including proteins, within liposomes (Szoka and Papahadjopoulos, 1978; Düzgünes et al., 1983).

REVMLVs composed of DOPE-DOPC-COR15am that were dehydrated at an osmotic pressure of 39 MPa were in the lamellar phase (data not shown); however, those that were dehydrated at an osmotic pressure of 76 MPa contained both lamellar and H_{II} phases (Fig. 7A). After dehydration at higher osmotic pressures (88 MPa, Fig. 7C, and 150 MPa, Fig. 7E), the H_{II} phase was the predominant phase. However, small regions of the lamellar phase were observed after dehydration of the REVMLVs at both 88 (Fig. 7C) and 150 MPa (Fig. 7E).

The domains attributed to isolated regions of COR15am in the FATMLV preparations were present at a lower frequency in the REVMLV preparations. This suggests that the reverse-phase evaporation method was more effective at distributing COR15am between the lamellae of the DOPE-DOPC dispersion. However, the incidence of the H_{II} phase in REVMLVs as a function of osmotic pressure was not different from that observed in FATMLVs or in the absence of COR15am. In addition, the striated surface morphology and polyhedral ultrastructures observed in FATMLV dispersions of DOPE-DOPC-COR15am were also observed in REVMLVs of this mixture (Fig. 7, A and C). After dehydration at an osmotic pressure of 76 MPa, REVMLVs that were composed of DOPE-DOPC-COR6.6 contained both lamellar and H_{II} phases (Fig. 7B). After dehydration at either 88 (Fig. 7D) or 150 MPa (Fig. 7F), the H_{II} phase was the predominant phase. However, small regions of the lamellar phase were observed after dehydration at both 88 (Fig. 7D) and 150 MPa (Fig. 7F) in the REVMLVs of DOPE-DOPC-COR6.6. As observed in REVMLVs containing COR15am, the frequency of domains attributable to COR6.6 was lower than that in FATMLVs, again suggesting that the reverse-phase evaporation method was more effective at distributing COR6.6 between the DOPE-DOPC lamellae. Nevertheless, there was no difference in the incidence of the dehydration-induced H_{II} phase that was observed in the REVMLVs and the FATMLVs composed of DOPE-DOPC-COR6.6.

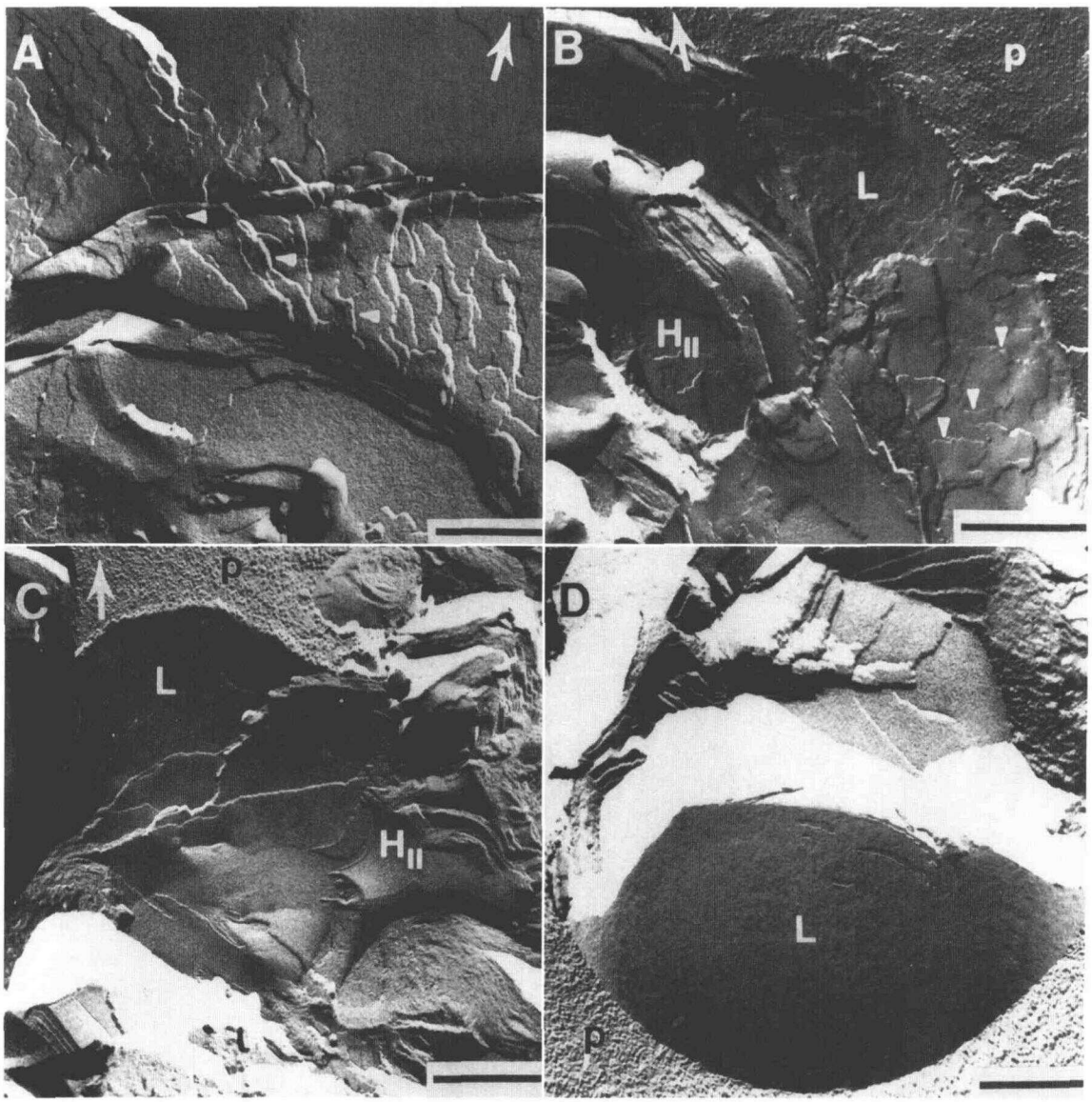

Figure 6. Freeze-fracture electron micrographs of FATMLV dispersions of DOPE-DOPC-COR6.6 dehydrated at osmotic pressures of 39 (A), 76 (B), 88 (C), and 150 MPa (D). Only the lamellar phase was observed after dehydration at 39 MPa (A), but both the lamellar (L) and H_{II} phases were observed after dehydration at 76 (B) and 88 MPa (C). The H_{II} phase was the predominant phase at 150 MPa (D). The lipid in close proximity to the COR6.6 domains (p) was in the lamellar phase, whereas the lipid more distant from the COR6.6 domains was in the H_{II} phase (B–D). In addition, lamellar domains with frequent single-step dislocations of the fracture plane to immediately adjacent lamellae were observed (arrowheads, A and B). Bars represent either 400 (A, B, and D) or 300 nm (C). Large arrows indicate direction of shadowing.

As a control to determine the effect of a protein that is not associated with cold acclimation, REVMLVs of DOPE-DOPC-BSA were dehydrated and examined by freeze-fracture electron microscopy. In dispersions dehydrated at an osmotic pressure of 39 MPa, the lipid was present exclusively in the lamellar phase (Fig. 8A). After dehydration at either 76 (data not shown) or 88 MPa (Fig. 8B), both lamellar and H_{II} phases were present. At 150 MPa, the H_{II} phase was the predominant phase present; however, isolated domains of lipid in the lamellar phase were also observed (Fig. 8C). As observed with FATMLV suspensions containing COR polypeptides, the lamellar phases present at the higher osmotic pressures were either immediately adjacent to or in close proximity to the domains tentatively identified as domains of BSA. It is likely that these domains arise from excess BSA that was not distributed between the lamellae of the liposomes—even after preparation of the MLVs by the reverse-phase evaporation method. The putative domains of BSA were larger and more frequent than the lipid-free domains observed in dehydrated suspensions containing either COR15am or COR6.6; this is probably a consequence of the much larger molecular mass of BSA.

The striated surface morphology and polyhedral structures observed in dispersions containing COR15am did not

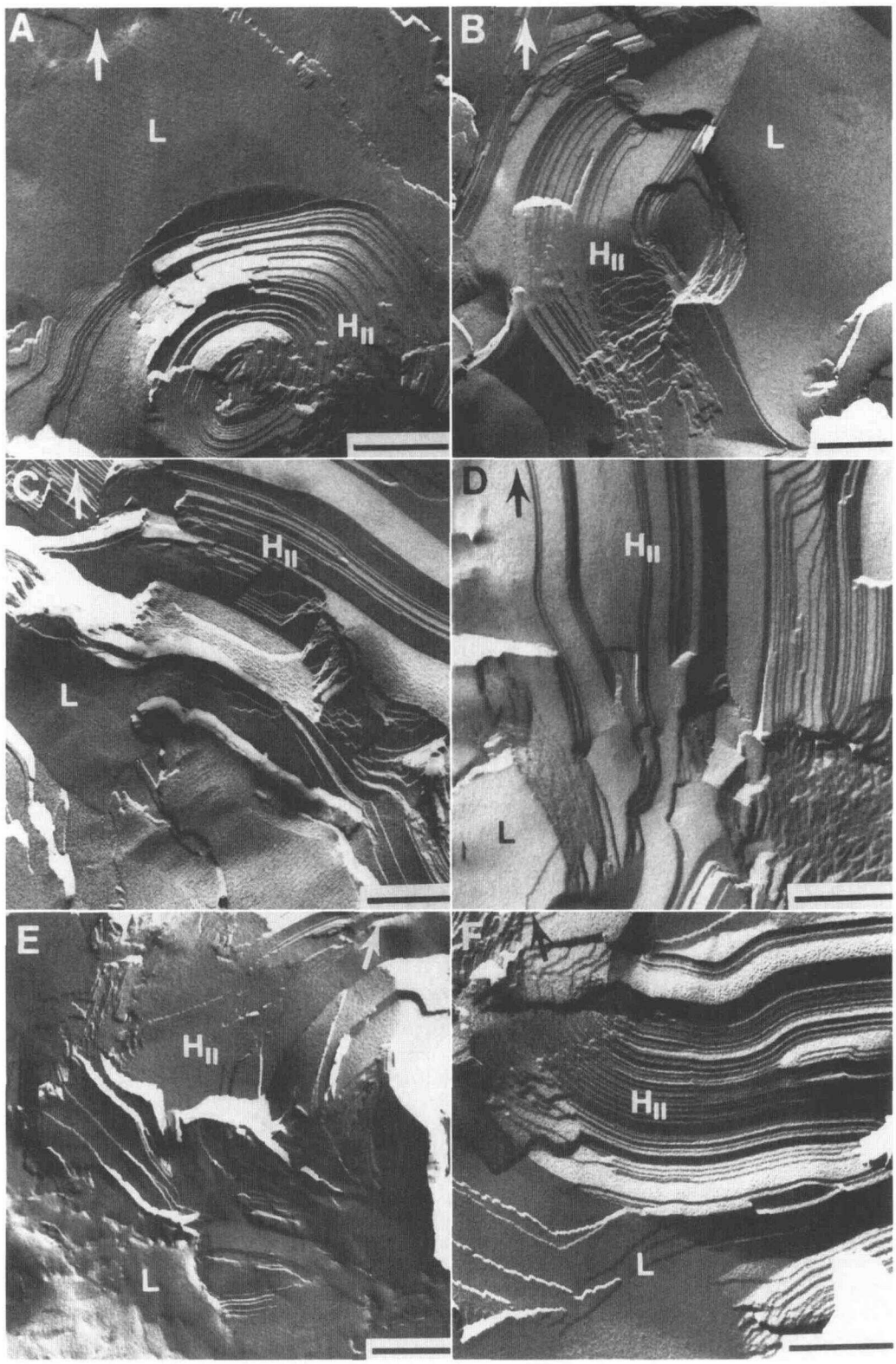

Figure 7. Freeze-fracture electron micrographs of REVMLV dispersions of DOPE:DOPC (1:1, mol:mol) mixtures containing either COR15am (A, C, and E) or COR6.6 (B, D, and F) and dehydrated at osmotic pressures of 76 (A and B), 88 (C and D), or 150 MPa (E and F). Both the lamellar (L) and H_{II} phases were observed at all osmotic pressures in samples containing either COR15am or COR6.6. In addition, the lamellar domains in the DOPE-DOPC-COR15am mixtures frequently contained a striated morphology (L_s, A and C) very similar to that observed in FATMLV dispersions containing COR15am (Fig. 5, A–C). Bars represent either 300 (A, C, and E) or 400 nm (B, D, and F). Large arrows indicate direction of shadowing.

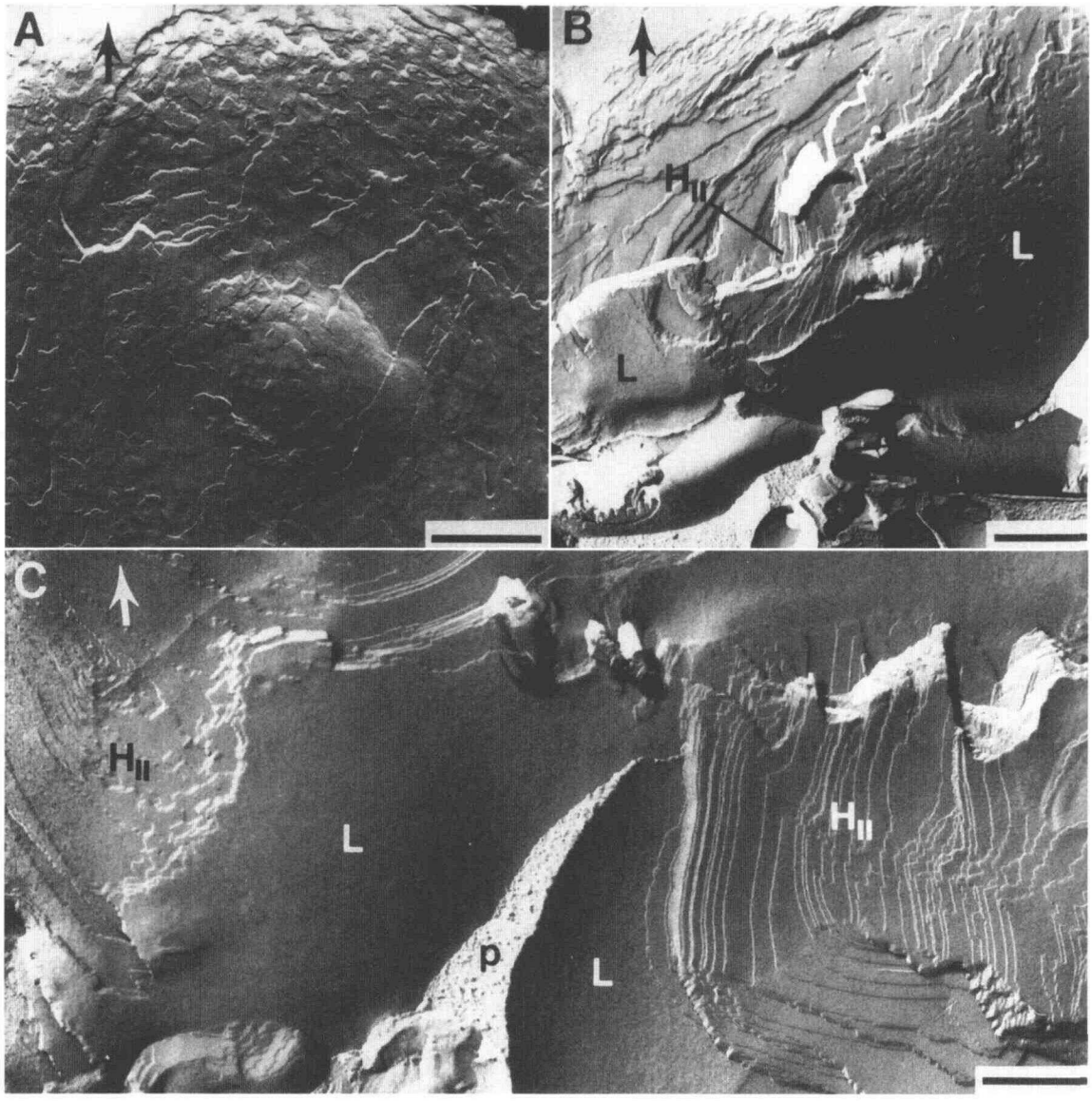

Figure 8. Freeze-fracture electron micrographs of REVMLV preparations of DOPE-DOPC-BSA dehydrated at osmotic pressures of 39 (A), 88 (B), and 150 MPa (C). After dehydration at 39 MPa, the lamellar phase was the only phase present and was characterized by frequent single-step, vertical displacements in the fracture plane (A). At 88 MPa (B), both the lamellar (L) and H_{II} phases were observed, with the lamellar domains characterized by frequent single-step, vertical displacements in the fracture plane. At 150 MPa, the H_{II} phase was the predominant phase; however, regions of lamellar phase lipid were also present immediately adjacent to or in close proximity to the isolated domains of BSA (p). Bars represent either 500 (A and B) or 300 nm (C). Large arrows indicate direction of shadowing.

occur in REVMLVs of DOPE-DOPC containing BSA. However, REVMLVs of DOPE-DOPC-BSA that were dehydrated at 39 (Fig. 8A) and 88 MPa (Fig. 8B) contained frequent, single-step dislocations in the fracture plane that appeared similar to those observed in FATMLV preparations of DOPE-DOPC-COR6.6 (Fig. 6A).

DISCUSSION

Cold acclimation of *A. thaliana* is associated with the expression of several *COR* genes and the accumulation of the COR polypeptides (Thomashow, 1990, 1993). Amino acid sequence analysis indicates that some of the COR polypeptides are highly hydrophilic (Thomashow, 1993); this finding has led some to speculate that these COR polypeptides are highly hydrated (Kazuoka and Oeda, 1992). Our results, however, do not support the notion that the COR polypeptides are highly hydrated. Desorption isotherms of the COR polypeptides and BSA indicated that the COR polypeptides were less hydrated than BSA (Fig. 1). Previous work has established that (horse) serum albumin has a water content intermediately between that of

highly hydrated collagen and poorly hydrated zein (Bull, 1944). Therefore, the desorption isotherms at 20°C indicate that COR6.6 and COR15am have hydration characteristics that are similar to those of many proteins.

Both COR6.6 and COR15am are predicted to form amphipathic, α-helical structures based on the deduced amino acid sequence (Thomashow, 1993). Some cytotoxic, amphipathic, α-helical peptides, such as melittin, interact with phosphatidylcholine liposomes and result in the disruption of vesicle structure and/or a reduction in the enthalpy of the $L_\alpha \rightarrow L_\beta$ phase transition of phosphatidylcholine vesicles without an effect on the T_m (Morrisett et al., 1977; Epand et al., 1987; McLean et al., 1991). Similarly, antifreeze proteins and antifreeze glycoproteins, which also have amphipathic, α-helical structures, increase the leakage of plastocyanin from isolated spinach thylakoids after a freeze-thaw cycle (Hincha et al., 1993). However, neither COR6.6 nor COR15am altered either the T_m or enthalpy of the $L_\beta \rightarrow L_\alpha$ phase transition of either DPPC or DOPC (Figs. 2 and 3). These results suggest that neither COR6.6 nor COR15am significantly interact with either DPPC or DOPC in a manner that would alter the lateral packing of the lipid during dehydration, and hence, alter the dehydration-induced increase of the T_m. This conclusion is consistent with the observation that the water content of a DOPC-COR15am mixture was very similar to that predicted by assuming independent hydration of the lipid and the polypeptide (Fig. 4). Therefore, these data suggest that neither COR6.6 nor COR15am interact with or alter the hydration of liposomes composed of phosphatidylcholine. However, we cannot exclude the possibility of other types of protein-lipid interactions (e.g. hydrophobic, electrostatic, or dipolar), but such putative interactions appear to have no effect on the dehydration-induced formation of the L_β phase. Similarly, we cannot rule out the possibility that the COR polypeptides interact with other membrane lipids (e.g. galactolipids, sterols, and/or cerebrosides).

Although the preceding considerations indicate that the COR6.6 and COR15am polypeptides do not alter the lyotropically induced $L_\beta \rightarrow L_\alpha$ phase transition of phosphatidylcholine vesicles, it is possible that the polypeptides may preclude the $L_\alpha \rightarrow H_{II}$ phase transition. Therefore, the effects of these COR polypeptides on the lyotropic $L_\alpha \rightarrow H_{II}$ phase transition of DOPE-DOPC mixtures were examined. We previously established that in mixtures of DOPE:DOPC (1:1, mol:mol), the H_{II} phase is first observed at approximately 6 wt% water—a hydration that is achieved after equilibration at an osmotic pressure of 76 MPa (Webb et al., 1993). This osmotic pressure represents the threshold pressure for the first occurrence of the H_{II} phase: the H_{II} phase did not occur at the next-lowest osmotic pressure examined (39 MPa) but was the predominant phase at 88 MPa and was the only phase present at osmotic pressures ≥ 150 MPa (Webb et al., 1993). In dehydrated mixtures of DOPE:DOPC (1:1, mol:mol), the $L_\alpha \rightarrow H_{II}$ phase transition is preceded by the close approach of lamellae and the demixing, or lateral segregation, of poorly hydrated DOPE from highly hydrated DOPC as a consequence of differences in their hydration characteristics (Webb et al., 1993). If the COR polypeptides affect either the spatial separation between bilayers during dehydration and/or dehydration-induced lipid-lipid demixing, they could alter the propensity of the lipids to undergo the lyotropic $L_\alpha \rightarrow H_{II}$ phase transition.

Neither COR6.6, COR15am, nor BSA altered the incidence of the dehydration-induced formation of the H_{II} phase as a function of osmotic pressure in DOPE-DOPC mixtures. That is, the threshold osmotic pressure required for dehydration-induced formation of the H_{II} phase in a 1:1 (mol:mol) DOPE-DOPC mixture was 76 MPa in the presence or absence of either COR6.6, COR15am, or BSA, and the proportion of the dispersion containing the H_{II} phase increased at osmotic pressures ≥ 76 MPa. We have been careful to facilitate the entrapment of the polypeptides within the liposomes by the use of freeze-thaw and reverse-phase evaporation dispersal methods. Both methods have been reported to result in large trapped volumes and high encapsulation efficiencies (Düzügenes et al., 1983; Mayer et al., 1985). Although entrapment of the COR polypeptides appeared to be greater in REVMLVs than in FATMLVs on the basis of the frequency of the domains, it should be noted that even in FATMLVs the frequency of the domains attributable to the polypeptides was insufficient to account for the majority of the COR polypeptide that was added to the mixtures. Therefore, it is likely that substantial proportions of COR6.6 and COR15am were entrapped between the lamellae of the FATMLVs, and that the results of the studies of the $L_\beta \rightarrow L_\alpha$ phase transition in which FATMLVs were used are reliable.

Although none of the examined polypeptides precluded the occurrence of the H_{II} phase during dehydration, all of the mixtures (i.e. those containing either COR6.6, COR15am, or BSA) had some lamellar phase lipid after equilibration at higher osmotic pressures—osmotic pressures at which dispersions that did not contain added polypeptides were exclusively in the H_{II} phase. In FATMLV dispersions containing the COR polypeptides, the lamellar regions that existed at higher osmotic pressures were near the peripheral regions of the MLVs—domains where the concentration of the COR polypeptide is expected to be the highest. In REVMLVs containing the COR polypeptides, in which the polypeptide is expected to be more uniformly distributed, the location of the lamellar phase was more random. Although these observations could be interpreted to suggest that the COR polypeptides preclude the dehydration-induced $L_\alpha \rightarrow H_{II}$ phase transition, mixtures containing BSA and prepared by reverse-phase evaporation also had significant proportions of lamellar phase lipid in the peripheral regions of the dehydrated vesicles. Therefore, these results suggest that the apparent ability of the COR polypeptides to preclude the formation of the H_{II} phase in regions of the FATMLVs is not unique to COR polypeptides, but also occurs with BSA. It is possible that both the COR polypeptides and BSA minimize the occurrence of the dehydration-induced $L_\alpha \rightarrow H_{II}$ phase transition via a nonspecific impediment to the close approach of bilayers during dehydration by presenting a steric hindrance to the close approach of bilayers.

Although these studies on the effects of COR polypeptides on lyotropic phase behavior suggest that neither COR6.6 nor COR15am interact with bilayers composed of phospholipids, it should be noted that the polypeptides had different and specific effects on the morphology of the dehydrated dispersions of DOPE-DOPC. In the lamellar domains of dehydrated DOPE-DOPC-COR15am mixtures, the lamellar regions had a striated surface morphology (Fig. 5, A–C), and the presence of a polyhedral topology (Fig. 5C) was consistently observed. Neither of these ultrastructural alterations was observed in dehydrated mixtures of either DOPE-DOPC-COR6.6 (Figs. 6 and 7, B, D, and F), DOPE-DOPC-BSA (Fig. 8), or DOPE-DOPC (Webb et al., 1993). The striated appearance of the lamellar phase resembles the rippled-gel ($P_{\beta'}$) phase formed by saturated species of phosphatidylcholine (Luna and McConnell, 1977, 1978; Parente and Lentz, 1984; Zasadzinski, 1988). However, the formation of a $P_{\beta'}$ phase in dehydrated DOPE-DOPC-COR15am mixtures would represent a significant influence of the polypeptide on the lyotropic phase behavior of the lipid, since the samples were dehydrated at 20°C and the gel phase does not occur at temperatures above 0°C in DOPE-DOPC mixtures without added polypeptide (Webb et al., 1993).

Alternatively, the striated surface morphology of the lamellar phase may represent a structural intermediate in the dehydration-induced $L_\alpha \rightarrow H_{II}$ phase transition (Bradshaw et al., 1989); however, the striated morphology was not observed in mixtures of DOPE:DOPC (3:1, 1:1, and 1:3, mol:mol) in the presence or absence of COR6.6 at water contents intermediate to the $L_\alpha \rightarrow H_{II}$ phase transition. In contrast, the lamellar domains of DOPE-DOPC-COR6.6 dispersions were characterized by the frequent occurrence of single-step dislocations in the fracture plane to adjacent lamellae. However, this alteration was not specific to mixtures containing COR6.6; it was also observed in dispersions of DOPE-DOPC-BSA. Therefore, although there was a difference between COR6.6 and COR15am in their effect on the ultrastructural morphology of the liposomes subjected to severe dehydration, these effects were not manifested as alterations in the dehydration-induced formation of either the L_β or the H_{II} phases.

In summary, the hydration of both COR6.6 and COR15am is lower than that of BSA and likely to be similar to that of many proteins. Neither COR6.6 nor COR15am affects the dehydration-induced increase in the T_m of either DPPC or DOPC, nor do they affect the osmotic pressure at which the dehydration-induced lamellar-to-H_{II} phase transition occurs in DOPE-DOPC mixtures. However, alterations of the surface morphology (a striated appearance) of the lamellar phase after dehydration suggest the possibility of an interaction between the phospholipids and COR15am. Additional studies are required to determine the nature and significance of this interaction.

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