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EFFECTS OF FREEZING AND COLD ACCLIMATION ON THE PLASMA MEMBRANE OF ISOLATED PROTOPLASTS

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SUMMARY OF PROGRESS (May 16, 1992 - January 9, 1993)

The principal goal of our program is to provide a mechanistic understanding of the cellular and molecular aspects of freezing injury and cold acclimation from a perspective of the structural and functional integrity of the plasma membrane—the primary site of freezing injury in winter cereals. We have utilized protoplasts isolated from leaves of winter rye (*Secale cereale* L. cv Puma) to study the cryobehavior of the plasma membrane during a freeze/thaw cycle. Previously, we established that destabilization of the plasma membrane can be effected in several different ways depending on the freeze/thaw protocol and the stage of cold acclimation. The focus of our current studies is on lesions in the plasma membrane that result from severe freeze-induced dehydration and result in the alteration of the semipermeable characteristics of the plasma membrane so that the protoplasts are osmotically unresponsive. In protoplasts isolated from non-acclimated rye leaves (NA protoplasts), injury is associated with the formation of aparticulate domains in the plasma membrane, aparticulate lamellae subtending the plasma membrane, and lamellar-to-hexagonal II phase transitions in the plasma membrane and the subtending lamellae. However, lamellar-to-hexagonal II phase transitions are not observed following severe dehydration of protoplasts isolated from cold-acclimated rye leaves (ACC protoplasts). Rather, injury is associated with the 'fracture-jump lesion', which, in freeze-fracture electron microscopy studies, is manifested as localized deviations in the fracture face of the plasma membrane. The fracture plane 'jumps' from the plasma membrane to either subtending aparticulate lamellae or aparticulate regions of various endomembranes (predominantly chloroplast envelopes) that are in close apposition with the plasma membrane.

The current focus of the project is (i) to provide an understanding of the mechanism by which freeze-induced dehydration effects the formation of aparticulate domains and lamellar-to-hexagonal II phase transitions in the plasma membrane of NA protoplasts and the fracture-jump lesion in ACC protoplasts and (ii) to determine whether differences in the lipid composition of the plasma membrane of winter rye and spring oat are associated with the extreme difference in freezing tolerance of these two cereals. Progress has been made in the following areas in 1992:

1. Hydration characteristics and lyotropic phase behavior of membrane lipids

We have taken a systematic approach to determine the contribution of the different membrane lipid classes to the hydration and phase characteristics of the plasma membrane. We have started by developing a rigorous understanding of the hydration and phase characteristics of relatively simple binary mixtures of unsaturated phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (DOPE and DOPC) (Webb et al., 1993a). The addition of the highly hydrated DOPC to poorly hydrated DOPE results in a non-linear increase in the hydration of the membranes. Similarly, the addition of molar proportions of DOPC as low as 25% to DOPE significantly stabilizes the lamellar phase to lyotropic -induced formation of the H_{II} phase. That is, while DOPE is in the H_{II} phase when fully hydrated at 20°C, the addition of 25 mol% DOPC stabilizes the membrane such that dehydration to approximately 7 wt% water is required for the $L_{\alpha} \rightarrow H_{II}$ phase transition. In addition, X-ray diffraction analysis has demonstrated that the different hydration characteristics of the PE and PC headgroups causes the lateral separation, or demixing, of the lipids into separate fluid-lamellar (L_{α}) domains. Analysis of the variation of lamellar repeat spacing with water content has demonstrated that the dehydration-induced demixing of DOPC from DOPE results in the formation of a DOPE-enriched domain that is more prone to undergo the $L_{\alpha} \rightarrow H_{II}$ phase transition. Therefore, dehydration-induced demixing of DOPE provides a way of understanding how the H_{II} phase may occur even in membranes with relatively low contents of unsaturated PE species.

This analysis of the contribution of the different lipid classes to the hydration and phase characteristics of membranes has been extended to include the free sterols, cerebrosides and acylated sterylglucosides, lipids that are common constituents of the plasma membrane of rye and oat (Webb et al, 1992). The addition of free sterols, predominantly ß-sitosterol, to DOPE:DOPC (1:1) mixtures at levels of 33 or 50 mol% both reduces bilayer hydration and facilitates the lyotropic formation of the H_{II} phase. That is, in the DOPE:DOPC (1:1) dispersion lacking free sterols, the H_{II} phase is first observed at a relative humidity equivalent to an osmotic pressure of 76 MPa. The presence of either 33 or 50 mol% free sterols in the lipid dispersion lowers the threshold osmotic pressure required for the formation of the H_{II} phase to 20 to 39 MPa of osmotic pressure. Similarly, the free sterols also promote the thermotropic $L_\alpha \rightarrow H_{II}$ phase transition in fully hydrated mixtures with DOPE:DOPC. The biophysical cause of the greater propensity of the lipids to form the H_{II} phase during dehydration is not certain, however our data suggests that the sterols promote the phase transition by *both* lowering bilayer hydration and facilitating closer bilayer-bilayer interaction and by decreasing the membrane spontaneous curvature. Interestingly, while the addition of the sterols facilitates the lyotropic $L_\alpha \rightarrow H_{II}$ phase transition, they also preclude the lyotropic increase of the $L_\beta \rightarrow L_\alpha$ phase transition temperature (T_m) observed in phospholipid dispersions. This result is consistent with the promotion of the formation of the H_{II} phase since increased lateral packing pressures within the acyl domain of the bilayer as a consequence of the presence of the sterols will both favor the formation of the H_{II} phase and prevent the decrease in lateral spacing of the lipids and decreased lipid molecular area that accompany increases in T_m due to dehydration.

Recent X-ray diffraction results have demonstrated that the sterol derivatives, the acylated sterylglucosides (ASG), are significantly more effective than the free sterols at promoting the formation of the H_{II} phase in DOPE:DOPC mixtures (Webb et al., 1992). While a mixture of DOPE:DOPC:sterol (1:1:1) is in the L_α phase when fully hydrated at 20°C and requires dehydration at osmotic pressures $\geq$ 20 MPa, mixtures of DOPE:DOPC:ASG (1:1:1) are in the H_{II} phase when fully hydrated at 20°C (i.e., they do not require dehydration to elicit the $L_\alpha \rightarrow H_{II}$ phase transition). Furthermore, the H_{II} phase in fully hydrated mixtures of DOPE:DOPC:ASG (1:1:1) is stable even when supercooled to -5°C. After ice formation occurs at -5°C in this mixture, the H_{II} phase separates into two coexisting L_α and H_{II} phases. Preliminary analysis of the dimensions of the H_{II} phase before and after freezing at -5°C suggests that the H_{II} phase in the presence of ice is more stable than that occurring in supercooled water at -5°C. In the absence of a change in temperature, this is strong evidence for the promotion of the H_{II} phase by freeze-induced dehydration. These results also suggest that ASG strongly promotes the formation of the H_{II} phase and may explain the greater propensity of non-acclimated oat protoplasts to undergo the $L_\alpha \rightarrow H_{II}$ phase transition during freezing.

We have also investigated the ability of the cerebrosides purified from non-acclimated winter rye to facilitate the lyotropic and thermotropic $L_\alpha \rightarrow H_{II}$ phase transition in mixtures of DOPE:DOPC (1:1). At equi-molar proportions of 33% (DOPE:DOPC:CER 1:1:1), the rye cerebrosides do not promote the formation of the H_{II} phase. Rather, dehydration induces a gel-liquid crystalline phase separation that yields a crystalline gel (L_c) phase probably enriched in CER. As CER is phase separated from the DOPE and DOPC in these dehydrated dispersions, it is not possible to directly evaluate the effect of CER on the formation of the H_{II} phase in the total mixture. Experiments are in progress to determine the miscibility of lower proportions of CER in DOPE:DOPC mixtures during dehydration. In addition we have also examined the effects of adding small (5 mol%) and large (20 mol%) proportions of CER to mixtures containing DOPE, DOPC and free sterols (DOPE:DOPC:sterols:CER, 22.5:22.5:50:5 and 15:15:50:20, respectively). Addition of 5 mol% CER to DOPE:DOPC:sterol (1:1:2) had little observable influence on membrane hydration or phase characteristics of the lipid mixture. However, when the proportion of CER was increased to 20 mol% (a value intermediate between those observed

in the rye and oat plasma membranes), with a corresponding decrease in the proportion of the phospholipids, the hydration of the mixture was significantly reduced and the H_{II} phase was observed at lower osmotic pressures (8 to 13 MPa vs. $\geq$ 20 MPa). The presence of the CER in these dispersions may promote the formation of the H_{II} phase by both decreasing bilayer hydration (and presumably promoting close bilayer-bilayer interaction) and by lowering the free energy of acyl chain packing of the H_{II} phase (see Steponkus & Webb, 1992; Steponkus et al., 1993a). However, the $L_{\alpha} \rightarrow H_{II}$ phase transition in these dispersions may also be facilitated by the higher sterol:PL ratio in this mixture. Experiments are in progress to determine if the greater propensity of these mixtures to undergo the $L_{\alpha} \rightarrow H_{II}$ phase transition during dehydration is a consequence of CER addition *per se* or the result of changes in the molar ratio of sterols:phospholipids.

2. Hydration and phase characteristics of NA and ACC rye and oat plasma membrane lipids during freezing

To determine the temperature x hydration interactions that influence the cryobehavior of the plasma membrane lipids of NA and ACC rye we have examined the subzero phases adopted by dispersions of the NA and ACC rye plasma membrane lipids by freeze-fracture electron microscopy and X-ray diffraction.

Fully hydrated dispersions of the plasma membrane lipids isolated from NA and ACC rye leaves are in the lamellar phase at temperatures between 0°C and 20°C as determined by both freeze-fracture electron microscopy (FFEM) and ^{31}P-Nuclear Magnetic Resonance. Following freezing at either -10°C or -20°C for up to 2 hours, the lamellae become very closely appressed between ice crystals as a consequence of freeze-induced dehydration. Preliminary FFEM experiments indicate that both the NA and ACC rye plasma membrane lipid dispersions are in the lamellar phase at both -10°C and -20°C during 2 hours of freeze-induced dehydration (Webb & Steponkus, unpublished results). Experiments are in progress to determine the influence of extended periods of equilibration at these temperatures and of freeze-induced dehydration at lower temperatures. Nonetheless, the NA and ACC rye plasma membrane lipids exhibit differential tendencies to enter the H_{II} phase during osmotic dehydration and to undergo lipid mixing following a freeze-thaw cycle based on resonance-energy transfer measurements (Uemura & Steponkus, unpublished results). Therefore, we believe that the absence of the H_{II} phase in freeze-fracture replicas of frozen NA plasma membrane lipid dispersions is the consequence of technical difficulties in the experimental protocol. Specifically, examination of both the NA and ACC rye plasma membrane lipid dispersions frozen to -10°C to -20°C by X-ray diffraction has indicated that the dispersions have very little long-range order, despite the appearance of closely appressed lamellae by FFEM. The absence of long-range order in these dispersions suggests that free sterol crystals and/or ice crystals have formed between adjacent lamellae during freezing. Free sterols are extremely effective nucleators of ice formation and could trigger the formation of ice between adjacent lamellae during cooling to -10°C. Ice crystal formation between adjacent lamellae would have two consequences. First, ice crystals would impose a physical barrier to bilayer-bilayer interaction that is a prerequisite for the formation of the H_{II} phase, and would therefore preclude the $L_{\alpha} \rightarrow H_{II}$ phase transition. Second, variations in the thickness of the ice crystals would cause wide variability in the lamellar repeat spacing and lack of long range order. This explains the absence of a strong lamellar X-ray diffraction pattern despite the observation of appressed lamellae by freeze fracture electron microscopy. We have also performed similar experiments to determine the subzero phase characteristics of the plasma membrane lipids of NA and ACC oat. However, similar concerns regarding the presence and distribution of ice crystals between adjacent lamellae apply to the oat plasma membrane lipid dispersions.

Our current efforts are to devise protocols by which ice crystal formation between adjacent lamellae is prevented. This will allow bilayer-bilayer interactions that are the prelude to the $L_\alpha \rightarrow H_{II}$ phase transition and should promote sufficient long-range order in the samples for phase determination by X-ray diffraction. Additional experiments include examination of the dependence of the $L_\alpha \rightarrow H_{II}$ phase transition on plasma membrane x endomembrane interactions.

3. Ultrastructural alterations in NA and ACC oat protoplasts during freezing

To determine if the ultrastructural alterations that occur in rye protoplasts and in rye leaves (Webb & Steponkus, 1993) also occur in a freezing-sensitive cereal, we have examined frozen protoplasts of spring oat by freeze-fracture electron microscopy. In protoplasts isolated from non-acclimated seedlings of spring oat, injury due to freezing is manifested as a loss of osmotic responsiveness and is first observed at -3°C (Webb et al., 1993b). Membrane ultrastructural alterations occurring during freezing are first observed at -3°C. Injury is associated with the formation of aparticulate domains in the plasma membrane, aparticulate lamellae subtending the plasma membrane and by the formation of the H_{II} phase in the plasma membrane and closely appressed lamellae. The H_{II} phase was observed in approximately 25% of cells at -3°C, 50% of cells at -5°C and in > 70% of cells at -10°C. Osmotic dehydration of NA oat protoplasts to osmotic pressures equivalent to that exerted by ice at -10°C has indicated that loss of osmotic responsiveness associated with the formation of the H_{II} phase occurs as a consequence of freeze-induced dehydration, rather than exposure to subzero temperatures. In protoplasts isolated from cold-acclimated seedlings of spring oat, freezing injury is also associated with loss-of-osmotic responsiveness. However, the H_{II} phase is not observed. Instead, injury is associated with the occurrence of the fracture-jump lesion in the plasma membrane and subtending lamellae that are brought into close association with the plasma membrane as a consequence of dehydration. That is, the fracture-jump lesion was observed in < 25% of protoplasts at -3°C and ≥ 72% of cells at -10°C.

These results indicate that the ultrastructural alterations occurring in the membranes of rye (e.g. the H_{II} phase in NA, the fracture-jump lesion in ACC) are not unique to rye, but also occur in freezing-sensitive spring oat. Furthermore, despite the extreme differences in the freezing tolerance of rye and oat, the ultrastructural alterations are closely associated with the temperatures at which loss of osmotic responsiveness also occurs. It should be added that we have also performed preliminary experiments to examine the ultrastructural consequences of freezing in protoplasts isolated from leaves of freezing-sensitive tobacco. At temperatures between -2° and -4°C, injury in tobacco protoplasts was associated with the formation of aparticulate domains in the plasma membrane, aparticulate lamellae subtending the plasma membrane, and the H_{II} phase in the plasma membrane and closely appressed lamellae (Webb & Steponkus, unpublished). The temperatures at which the H_{II} phase was observed correspond closely to those at which tobacco protoplasts are injured by freezing (Uemura & Steponkus, unpublished).

4. Ultrastructural alterations in NA and ACC rye leaves during freezing

The freeze-induced formation of the H_{II} phase in NA rye protoplasts and the fracture-jump lesion in ACC rye protoplasts are not unique to isolated protoplasts. We have recently demonstrated that these ultrastructural alterations also occur in NA and ACC rye leaves during freezing at -10° and -35°C, respectively (Webb & Steponkus, 1993a). Freezing of leaves of non-acclimated rye at -10°C is associated with the formation of aparticulate domains within the plasma membrane, aparticulate lamellae subtending the plasma membrane and by the

formation of the H_{II} phase. That is, the ultrastructural alterations occurring in non-acclimated rye leaves are qualitatively identical to those described in NA rye protoplasts. Similarly, in leaves of cold-acclimated rye frozen at -35°C, the H_{II} phase was not observed. Rather, injury was associated with the formation of the fracture-jump lesion in the plasma membrane and closely appressed lamellae. These ultrastructural alterations in ACC rye leaves are also qualitatively identical to those previously described in ACC rye protoplasts. Therefore, these results demonstrate that the ultrastructural alterations characterized in frozen rye protoplasts are not unique to protoplasts, but also occur in the leaves from which the protoplasts were isolated (Webb & Steponkus, 1993).

It should be noted that another group (Pearce & Willison; 1985) did not observe the H_{II} phase in wheat leaves frozen to injurious temperatures. It is likely that the absence of the H_{II} phase in their studies was the result of inadequate equilibration times at subzero temperature to effect cellular dehydration. Cryo-SEM studies of frozen rye leaves have indicated that extracellular ice does not encapsulate the cells, but forms discrete ice crystals. Therefore, it is likely that cellular dehydration occurs by the relatively slow process of vapor phase equilibration. In the study of Pearce & Willison, wheat leaves were frozen after brief periods of exposure to injurious subzero temperature and probably did not undergo sufficient dehydration to effect the $L_{\alpha} \rightarrow H_{II}$ phase transition. We have shown that non-acclimated rye leaves frozen for 30 minutes at -10°C are neither dehydrated nor possess the H_{II} phase (Webb & Steponkus, 1993). Extended exposure to injurious temperatures is required to effect the dehydration sufficient for the $L_{\alpha} \rightarrow H_{II}$ phase transition (Webb & Steponkus, 1993).

5. Plasma membrane lipid composition of winter rye and spring oat

The lipid composition of the plasma membrane isolated from leaves of a spring oat (cv. Ogle) is vastly different from the plasma membrane of winter rye leaves (cv. Puma) (Uemura & Steponkus, 1992). The plasma membrane of nonacclimated leaves of oat contains substantially higher proportions of acylated sterylglucosides (ASG) and glucocerebrosides (CER) and lower proportions of free sterols (FS) and phospholipids (PL). In contrast, the plasma membrane of rye leaves contains FS as the predominant sterols, with lesser amount of ASG; CER are the predominant glycolipid; and the proportion of PL is greater. The proportion of sterylglucosides (SG) is similar in both oat and rye. As a result, the proportion of glycolipids (ASG + SG + CER) in oat plasma membrane is twofold greater than that of the rye plasma membrane. Molecular species analysis revealed additional differences between the plasma membranes of oat and rye. Stigmasterol is the predominant FS in oat, whereas ß-sitosterol is the predominant FS in rye. In addition to ß-sitosterol, the principal sterol moiety in SG and ASG is stigmasterol in oat and campesterol in rye. 2-hydroxynervonic acid (24:1h) is the predominant hydroxy fatty acid associated with CER in both oat and rye, but the relative proportion of 24:1h in oat CER is greater than in rye CER. Although the relative proportion of individual PL, primarily phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and the profiles of the molecular species of PC and PE in oat are quite similar to those in rye, the relative proportions of di-unsaturated species of PC and PE are substantially lower in oat than in rye. After cold acclimation, the proportion of PL increases significantly in both oat and rye. This increase is the result of increases in the proportions of PC and PE with small changes in the other PL. In both PC and PE, the relative proportions of di-unsaturated species increase after cold acclimation, but the increase is higher in rye than in oat. As with rye plasma membrane, the major increase in the proportion of PL in oat occurred during the first week of cold acclimation—when expansion-induced lysis of the protoplasts isolated from the leaves was precluded. The proportion of FS remained relatively constant in both oat and rye. In contrast, the proportions of ASG and CER decreased in both oat and rye after cold acclimation. Nevertheless, the proportion of ASG and CER remained substantially higher in oat than in rye.

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Plans for The Coming Year

The research objectives of the original proposal remain unchanged. In the coming year, emphasis will be placed on the following areas: (i) studies to determine if the fracture-jump phenomenon is a consequence of either the formation of interlamellar attachments and localized fusion or interdigitation of asymmetric lipid species that undergo an L_{α}-to-L_{β} phase transition because of extreme dehydration at low temperatures; (ii) continued studies of the hydration characteristics and force-distance relations of complex mixtures of plasma membrane lipids at subzero temperatures in order to explain the differential behavior of the plasma membrane of rye and oat; (iii) to characterize alterations in the lipid composition of the chloroplast envelope that occur during cold acclimation and genotypic differences between rye and oat (iv) Cryo-SEM studies of ice formation in rye and oat leaves.

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