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

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by

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SUMMARY OF PROGRESS (May 16, 1993 to January 29, 1994)

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. Previously, we established that destabilization of the plasma membrane of winter rye (*Secale cereale* L. cv. Puma), which is the most freezing-tolerant winter cereal, can be the result of several different lesions: expansion-induced lysis, lamellar-to-hexagonal II phase transitions, and the fracture-jump lesion (Steponkus *et al.*, 1993a). The occurrence and incidence of these various lesions, which are a consequence of freeze-induced dehydration (Steponkus *et al.*, 1994), depends on the freeze/thaw protocol and the stage of cold acclimation. In non-acclimated leaves and protoplasts, expansion-induced lysis is the predominant lesion at temperatures between -2 and -5°C , whereas freeze-induced formation of the H_{II} phase is the predominant lesion at temperatures below -10°C . Neither of these lesions occurs in acclimated leaves or protoplasts; instead, injury is a consequence of the fracture-jump lesion—which we believe is the result of the formation of interlamellar attachments and fusion of the plasma membrane with various endomembranes. Whereas expansion-induced lysis is a consequence of cell dehydration and the osmotic excursions incurred during a freeze/thaw cycle, freeze-induced formation of the H_{II} phase and the fracture-jump lesion are consequences of freeze-induced dehydration of phospholipids (Bronshteyn & Steponkus, 1993). Although these lesions were first identified and studied in isolated protoplasts, they also occur in leaves from which the protoplasts are isolated (Webb & Steponkus, 1993).

This past year we have established that the same lesions occur in spring oat (*Avena sativa* L. cv. Ogle)—albeit at different temperatures (Webb *et al.*, 1994a). Although there is only a small difference in the temperature range over which expansion-induced lysis occurs in oat (-2 to -4°C) and rye (-2.5 to -5°C), freeze-induced formation of the H_{II} phase and the fracture-jump lesion occur at substantially higher temperatures in oat. In non-acclimated oat leaves, the H_{II} phase occurs at a high frequency at -5°C , whereas in non-acclimated rye leaves it occurs at -10°C . After cold acclimation, the freeze-induced formation of the hexagonal II phase is precluded in both oat and rye; however, survival is limited by the fracture-jump lesion. In cold-acclimated leaves, the fracture-jump lesion occurs at -10°C in oat and -20°C in rye.

Subsequently, we investigated whether the difference in freezing tolerance and the threshold temperatures at which the lesions occur in rye and oat are a consequence of differences in the lipid composition of the plasma membrane (Uemura & Steponkus, 1994). A molecular species analysis of the plasma membrane lipids of each revealed that there are substantial differences between rye and oat—both before and after cold acclimation. The plasma membrane of oat contains greater proportions of acylated sterylglucosides and cerebroside than that of rye, and there is little change in these two lipid classes during cold acclimation. Such an analysis, however, provides only correlative evidence for a causal relationship. Therefore, the lyotropic phase behavior of lipid mixtures that resemble the plasma membrane of rye and oat was studied (Webb *et al.*, 1993; 1994c; 1994d). These studies suggest that the differences in lipid composition of rye and oat are of mechanistic significance because of their influence on (a) the hydration characteristics of the plasma membrane, (b) the propensity for dehydration-induced lipid-lipid demixing, and (c) the intrinsic curvature of the lipid monolayers (Steponkus *et al.*, 1993b). Collectively, these studies suggest that strategies for improving the freezing tolerance of winter cereals should include approaches to modify membrane lipid composition (Steponkus *et al.*, 1993c).

Although this project has focused on the role of membrane lipid alterations, freezing tolerance is a multifaceted trait, and differences in lipid composition—be they the result of either cold acclimation or genotypic differences—are but one facet. For example, there is considerable evidence that endogenous cryoprotectants, such as sugars, increase the cryostability of cellular membranes. There are also suggestions that some proteins that are synthesized during cold acclimation (referred to as COR proteins)

also influence membrane stability; however, the function of these proteins has yet to be determined. The identification of specific lesions in the plasma membrane and a mechanistic understanding of their occurrence during a freeze/thaw cycle and their amelioration by cold acclimation provide for a focused approach to consider the influence of sugars and proteins on the cryostability of membranes. It is from this perspective that we initiated studies, in collaboration with Michael F. Thomashow of Michigan State University, to determine the effect of COR proteins on the cryostability of membranes.

A more detailed discussion of the research in progress that has not been published is as follows:

1. Hydration characteristics and lyotropic phase behavior of membrane lipids. Freeze-induced injury of protoplasts of non-acclimated rye (*Secale cereale* cv. Puma) at -10°C is associated with the formation of the inverted hexagonal (H_{II}) phase in regions of the plasma membrane that are brought into close apposition with various cytoplasmic membranes as a result of freeze-induced dehydration. After 7 to 10 days of cold acclimation, freeze-induced formation of the H_{II} phase does not occur and is a consequence of alterations in the lipid composition of the plasma membrane and various endomembranes. To determine the influence of specific membrane lipids on the propensity of lipid mixtures to form the H_{II} phase, the hydration characteristics and phase behavior of dehydrated mixtures of DOPE:DOPC containing rye cerebroside and sterols were examined by differential scanning calorimetry (DSC), freeze-fracture electron microscopy (FFEM), and x-ray diffraction.

The addition of cerebroside (CER), purified from the leaves of winter rye, to levels similar to those in the plasma membrane of non-acclimated and cold-acclimated rye leaves (DOPE:DOPC: β -sitosterol:CER 22.5:22.5:50:5 and 15:15:50:5, respectively) decreased the total water content of the dispersions below that of comparable mixtures without cerebroside (Webb *et al.*, 1994d; in preparation). Analysis of the water content per acyl lipid suggested that most, if not all, of the water remaining in the dehydrated mixtures was associated exclusively with the phospholipids. The presence of cerebroside in these mixtures at 5 and 20 mol% reduced the $L_{\alpha} \rightarrow L_{\beta}$ phase transition temperature (T_m) of the fully hydrated dispersions by 2 to 3°C and precluded the lyotropic increase in T_m during dehydration. The addition of cerebroside to DOPE:DOPC: β -sitosterol mixtures significantly decreased the osmotic pressure at which the $L_{\alpha} \rightarrow H_{II}$ phase transition occurred. Specifically, the threshold osmotic pressure for formation of the H_{II} phase was 20 MPa in mixtures containing DOPE:DOPC: β -sitosterol (1:1:2); in the presence of 5 mol% cerebroside, the H_{II} phase also first occurred at 20 MPa; however, in mixtures containing 20 mol% cerebroside, the H_{II} phase first occurred at 12.8 MPa. That is, the lipid mixture similar to that of the non-acclimated rye plasma membrane was most susceptible to the lyotropic $L_{\alpha} \rightarrow H_{II}$ phase transition.

Because the increased propensity of the DOPE:DOPC: β -sitosterol:CER (15:15:50:20) mixture to form the H_{II} phase during dehydration could be attributed to the increased sterol:phospholipid ratio in these mixtures rather than a direct effect of the cerebroside *per se*, we examined (by x-ray diffraction) the lyotropic phase behavior of DOPE:DOPC:CER mixtures in the absence of β -sitosterol. At 20 mol%, rye cerebroside (DOPE:DOPC:CER 2:2:1) are completely miscible with DOPE:DOPC during equilibration at osmotic pressures as high as 76 MPa. This mixture remained in the L_{α} phase during dehydration, and there was no evidence of either the H_{II} phase or phase separation of the L_{β} and the L_c phase. Rye cerebroside at 33 mol% (DOPE:DOPC:CER 1:1:1) are miscible in fully hydrated dispersions at temperatures $>20^{\circ}\text{C}$. During dehydration of this mixture, phase separation yielding co-existing L_{α} and L_c phases was observed, however the H_{II} phase was not observed. Similarly, DOPE:DOPC (1:1) mixtures containing either 20 or 33 mol% cerebroside did not undergo the $L_{\alpha} \rightarrow H_{II}$ phase transition during heating of the fully hydrated dispersions to temperatures as high as 60°C . In contrast, DOPE:DOPC mixtures containing either 33 or 50 mol% of β -sitosterol undergo the $L_{\alpha} \rightarrow H_{II}$ phase transition at 45 to 50°C (Webb *et al.*, 1994c; in preparation). Furthermore, fully hydrated lipid mixtures in proportions similar to those in the plasma membrane of non-acclimated and cold-acclimated rye leaves (DOPE:DOPC: β -sitosterol:CER 22.5:22.5:50:5 and 15:15:50:5, respectively) undergo the thermotropic $L_{\alpha} \rightarrow H_{II}$ phase transition at 40 and 35°C , respectively.

These results suggest that the rye cerebrosides by themselves do not promote either the lyotropically or thermotropically induced formation of the H_{II} phase in DOPE:DOPC mixtures. However, in the presence of β -sitosterol, higher levels of cerebrosides did facilitate both the lyotropic and thermotropic $L_{\alpha} \rightarrow H_{II}$ phase transition. The differential propensity of fully hydrated mixtures containing either 5 or 20 mol% CER to form the H_{II} phase thermotropically suggests that the influence of cerebrosides is not restricted to surface hydration characteristics. At 20 mol% cerebrosides—a value intermediate between those found in the plasma membranes of non-acclimated rye and oat—the significantly higher sterol:phospholipid ratio may be primarily responsible for the increased propensity of this mixture to destabilize and undergo the $L_{\alpha} \rightarrow H_{II}$ phase transition. Indeed, acclimation-induced decreases in the content of cerebrosides in the plasma membranes of winter rye and spring oat are also associated with decreases in the sterol:phospholipid ratio (Uemura & Steponkus, 1994). In sum, the results from these studies indicate that the differential propensity of the plasma membranes of non-acclimated and cold-acclimated rye and oat to undergo the $L_{\alpha} \rightarrow H_{II}$ phase transition during freeze-induced dehydration cannot be assigned to a single lipid species. Rather, the tendency of the membrane to form the H_{II} phase during freeze-induced dehydration appears to be a consequence of the summation of several physical characteristics of the membrane lipids, including bilayer surface hydration, packing pressures within the hydrophobic domain of the membrane, and the tendency of the lipid components to demix—all of which are influenced by the relative proportion of the various lipid species.

2. Electron microscopy studies of the fracture-jump lesion. We are continuing electron microscopy studies of the fracture-jump lesion to provide a semi-quantitative analysis of its frequency of occurrence in both oat and rye as a function of the freezing temperature and stage of cold acclimation. We have established that the threshold temperature for the occurrence of the fracture-jump lesion is substantially different for rye and oat. In oat, the threshold temperature for the fracture-jump lesion is -5°C after one week of cold acclimation and -10°C after four weeks of cold acclimation, which results in the maximum increase in freezing tolerance. In rye, the threshold temperature for the fracture-jump lesion is -10°C after one-week of cold-acclimation and -20°C after four weeks of cold acclimation.

In rye, the fracture-jump lesion is apparent in both the frozen state and after thawing the specimens, which suggests that the lesion is a consequence of the formation of interlamellar attachments and localized fusion rather than interdigitation of lipids in the L_{β} phase, which would be expected to be readily reversible upon rehydration of the cells after thawing of the suspending medium. Preliminary studies of the lipid composition of the plasma membrane after a lethal freeze/thaw cycle are consistent with this interpretation: the plasma membrane fraction contains galactolipids such as MGDG and DGDG, which are characteristic of the chloroplast and not normally present in the plasma membrane fractions. This suggests that lipid mixing between the plasma membrane and the chloroplast occurs during a lethal freeze/thaw cycle, which is consistent with the formation of interlamellar attachments and localized fusion.

One difference between oat and rye in relation to the ultrastructural alterations in the plasma membrane that are observed after a lethal freeze/thaw cycle is that in oat there is a high incidence of aggregation of the intramembranous particles, which has not been observed in either protoplasts or leaves of rye (either non-acclimated or cold-acclimated). Neither the physical cause nor the possible injurious consequences of this aggregation is known at this time.

3. Lipid composition of the chloroplast envelope of winter rye. Studies have been initiated to determine the effect of cold acclimation on the lipid composition of the chloroplast envelope, which is of interest because the freeze-induced formation of the H_{II} phase and the fracture-jump lesion are interbilayer events and both occur at a high frequency in regions of the plasma membrane that are brought into close apposition with the chloroplast envelope. Because the frequency of both decreases after cold acclimation, it is very likely that the lipid composition of the chloroplast envelope, like that of the plasma membrane, also changes during cold acclimation. Initial studies have been to develop procedures to

isolate sufficient quantities of purified envelope vesicles so that a molecular species analysis of the lipids can be conducted.

4. COR proteins: their cryobehavior and effects on the cryostability of membranes. In collaboration with Michael F. Thomashow at Michigan State University, we initiated studies to determine the effect of two COR proteins (COR6.6 and COR15am, which are synthesized by *Arabidopsis thaliana* during cold acclimation) on the cryostability of liposomes. We observed that both COR6.6 and COR15am minimize the occurrence of freeze-induced fusion of liposomes composed of either synthetic phospholipids or the total lipid extract isolated from the plasma membrane of rye leaves (Uemura *et al.*, 1993; 1994). When small unilamellar vesicles (SUVs) composed of either POPC (16:0/18:1) or DOPC (18:1/18:1) were frozen to temperatures over the range of -2 to -30°C , liposome fusion occurred at temperatures below -2°C with a substantial increase occurring at temperatures below the T_m of the lipids (-3°C for POPC and -18°C for DOPC). Freeze-induced fusion was not observed in SUVs composed of DL₂PC (18:2/18:2), which has a T_m of -55°C . Addition of either COR6.6 or COR15am significantly decreased the incidence of fusion of SUVs composed of either POPC or DOPC; BSA did not decrease the incidence of fusion. The COR proteins also decreased the incidence of fusion of large unilamellar vesicles (LUVs) composed of DOPC if the proteins were added to the suspension (i.e., extraliposomal) before freezing, but they had no effect if they were encapsulated within the LUVs before freezing (i.e., intraliposomal).

With SUVs formed from the total lipid extract of the plasma membrane lipids isolated from non-acclimated (NA) and cold-acclimated (ACC) rye leaves, there was a differential response to freezing. In NA-SUVs, the incidence of fusion increased after freezing to -5°C and reached a maximum at -10°C ; in ACC-SUVs, the maximum incidence of fusion occurred at -20°C . The difference in the cryostability of the plasma membrane SUVs is attributable to differences in lipid composition after cold acclimation. When frozen in the presence of the COR proteins there was a significant decrease in the incidence of fusion of both NA- and ACC-SUVs. Thus, although the cryostability of the SUVs is primarily influenced by the lipid composition of the bilayers, there is an additive effect of the COR proteins.

Although the COR proteins decreased the incidence of freeze-induced fusion of POPC- and DOPC-SUVs, which occurs predominantly over the temperature range at which the liquid crystalline-to-gel phase transition occurs, the proteins do not affect the lyotropically-induced increase in the liquid crystalline-to-gel phase transition temperature of either DPPC or DOPC (Webb *et al.*, 1994b). Also, the COR proteins do not alter the propensity for the lamellar-to-hexagonal II phase transition in mixtures of DOPC:DOPE. These studies suggest that the decreased incidence of fusion is not the result of direct protein-lipid interactions. Therefore, it is possible that the decreased incidence of fusion may be the result of an indirect effect resulting from amelioration of the freezing stresses. Hence, we initiated studies of the cryobehavior of the COR proteins *per se* (Bronshteyn *et al.*, 1993; 1994).

Although the COR proteins do not have thermal hysteresis activity that is detectable by differential scanning calorimetry (DSC) studies (i.e., they are not "antifreeze proteins"), DSC and EM studies suggest that they influence the size and morphology of ice crystals. During slow cooling ($2^{\circ}\text{C}/\text{min}$), ice formation in solutions of COR proteins is complete at approximately -20°C . This is in agreement with studies in which the T_g' (the temperature at which the maximally freeze-concentrated solution is attained) was determined to be -18°C for both COR proteins and BSA. From DSC thermograms it was calculated that the amount of water remaining unfrozen at the T_g' was approximately 0.47 grams of water per gram of protein for both the COR proteins and BSA. However, at temperatures $\geq -15^{\circ}\text{C}$, the mass of unfrozen water was significantly greater in solutions containing COR proteins than in those containing BSA. It was also higher than that estimated from desorption isotherms determined for the COR proteins and BSA at 20°C (hydration of BSA > COR6.6 > COR15am at osmotic pressures over the range of 7.5 to 150 MPa). This may be because either the hydration characteristics of the COR proteins are greatly different at subzero temperatures or the COR proteins influence the size of the ice crystals differently than

BSA: the total amount of unfrozen water that is associated with the surfaces of ice crystals increases as the size of ice crystals decreases. The latter explanation is consistent with the appearance of recrystallization exotherms during warming of solutions of COR proteins; these exotherms were not observed in solutions of BSA. Therefore, even though the COR proteins are not effective "antifreeze proteins", they appear to affect ice crystal formation and result in the formation of smaller ice crystals. In addition, in EM studies, ice crystals formed in solutions containing COR proteins have a regular hexagonal shape and several "steps" are visible on the prism faces of the crystals; these features are not observed in ice crystals formed in solutions of BSA. Although additional studies are required to resolve these issues, the preliminary studies indicate that the COR proteins alter the cryobehavior of the frozen solution. Therefore, *in vitro* freezing studies to determine the effect of COR proteins on cryostability of membranes or proteins must take this effect into consideration.

These preliminary studies led to the submission of a grant application to the USDA-NRICGP program for continuation of these studies; the grant application was approved with funding for the period of August 1, 1993 to July 30, 1995.

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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) continued 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 phase behavior 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) characterization of alterations in the lipid composition of the chloroplast envelope that occur during cold acclimation (iv) Cryo-SEM studies of ice formation in rye and oat leaves.

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