Effect of a Freeze-Thaw Cycle on Properties of Microsomal Membranes from Wheat

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ABSTRACT

A freeze-thaw cycle to -12°C induced several physical and compositional changes in the microsomal membranes isolated from crown tissue of winter wheat (Triticum aestivum L. cv Frederick). Exposing 7-day-old, nonacclimated seedlings to a single freeze-thaw cycle prevented regrowth of the crown and resulted in increased membrane semipermeability. The phospholipid and protein content of microsomal membranes isolated from the crowns decreased by 70 and 50%, respectively. Microsomal membranes isolated after the lethal freeze-thaw stress, and liposomes prepared from total membrane lipids, exhibited greater microviscosity, measured by fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene. The number of free thiol groups per milligram membrane protein, measured using the specific fluorescent probe, N-dansylaziridine, decreased after freezing. In contrast, acclimated wheat seedlings which showed increased freezing tolerance, as indicated by survival and ion leakage, suffered almost no effects from the freeze-thaw treatment as determined by measurements of membrane microviscosity, phospholipid content, protein content, or dansylaziridine fluorescence. An examination of membranes isolated from frozen tissue showed that most of the changes occurred during the freezing and not during the thawing phase.

Exposing plants to subzero temperatures frequently results in injury and death. However, acclimation of many plants at low, above freezing temperatures is known to render them more resistant to subsequent freezing stress (4, 6, 7, 13, 14, 16, 19). Cell membranes are one of the major sites affected by both acclimation and freezing stress (13, 14, 17, 19). For example, in winter wheat, acclimation leads to accumulation of phospholipids (4, 18) and, based on indirect evidence, it has been proposed that freezing involves their degradation (5). Metabolic activity in wheat crowns, as measured by triphenyltetrazolium chloride reduction, declined significantly after freezing, although cells within the tissue differed in their relative freezing tolerances (16).

The loss of membrane integrity, expressed as an increase in the passive leakage of solutes out of the tissue, is a common symptom of freezing injury (6, 7, 14). Depending on the type of freezing stress experienced, this loss of membrane integrity may be a consequence of the physical tearing of the plasmalemma by ice crystals, or a consequence of altered physical properties of the membrane induced by cellular dehydration during the freezing process. However, reports concerning freezing mediated changes in the physical properties of the biological membranes are scarce and contradictory; in one study (13), ESR measurements using 5NS indicate that freezing reorganizes bilayer lipids into a less ordered (i.e., more fluid) amorphous state. In contrast, x-ray diffraction of microsomal membranes, isolated from frozen wheat tissue, showed a substantial increase in the lipid phase transition temperature (5). Both studies indicate that cell membranes are reversibly altered following a lethal freezing stress. In contrast to the previous results, another ESR study using the probe 5NS did not detect any change in the physical properties of the lipid bilayer in microsomal membranes of wheat crown and root tissue following a lethal freezing stress at -6°C for 35 d (9). However, ESR and x-ray diffraction do not necessarily examine the same lipid domains within the bilayer structure and have provided quite different perspectives even in the same experimental system (8).

The objective of the present study was to examine the physical and compositional properties of cellular membranes of winter wheat crowns after exposure to a lethal freeze-thaw stress. To supplement the previous studies using wide angle x-ray diffraction (5) and ESR (9, 13), microviscosity measurements were made using fluorescence polarization as an alternative technique.

MATERIALS AND METHODS

Plant Material and Environmental Conditions. Seeds of winter wheat (Triticum aestivum L. cv Frederick) were planted and grown as before (16). One-week-old plants (nonacclimated), or plants grown at 2°C for a subsequent period of 42 d (acclimated), were used for the different experiments. Freezing was carried out in a programmable freezer. Seedlings were trimmed to give 2 cm crown sections, which were wrapped in wet paper towels, and held in a plastic beaker. The tissue was held at -2°C until all the water was frozen, cooled at a rate of 2°C/h to -12°C, held at that temperature for 6 h, and then thawed at 2°C for 4 h. Viability of the tissue was evaluated on the basis of regrowth after 24 h and also on the basis of leakage of electrolytes from the treated tissue (17).

Membrane Preparation. Membranes were isolated from control crown tissue and tissue subjected to the freeze/thaw cycle immediately after termination of the freezing stress or after the 4 h thawing period. Using a chilled mortar and pestle 5 g of crown tissue were ground in 25 ml buffer composed as described by Yoshida et al. (20), but without BSA. The homogenate was centrifuged at 10,000g for 20 min. Microsomal membranes were

1 Supported by the Natural Science and Engineering Research Council of Canada (grant and international scientific exchange grants) and the Ontario Ministry of Agriculture and Food.

2 On leave from the Faculty of Agriculture, the Hebrew University of Jerusalem, Rehovot 76100, Israel.

3 Abbreviations: ESR, electron spin resonance; DA, N-dansylaziridine; DPH, 1,6-diphenyl-1,3,5-hexatriene; 5NS, the N-oxyl-4,4-dimethyloxazolidine derivative of 5-ketostearic acid.
prepared from the supernatant by centrifugation at 156,000g, and the membrane pellet was resuspended in 4 ml of 10 mM Hepes buffer (pH 7.5) for further measurements. All the different steps of the membrane isolation and handling were done at 0 to 4°C unless stated otherwise.

**Lipid Extraction and Analysis.** Lipids were extracted from 1 ml aliquots of membrane suspensions using chloroform/methanol (1). Phospholipids in the extract were measured as PI after perchlorate oxidation using a Fiske-Subbarow reagent (Sigma). Fatty acids in the lipid extract were methylated for 50 min at 90°C with 14% BF₃ in methanol (Pierce) and analyzed by GC. Fatty acids were quantified using heptadecanoic acid (Sigma) as an internal standard.

**Protein Measurements.** The content of proteins in the membranes was measured according to Bradford (3) using BSA (Sigma) as a standard.

**Fluorescence Measurements.** Microsomal membranes and liposomes, prepared from total microsomal membrane lipid extracts were labeled with the fluorescent probe, DPH (Sigma). Membranes at a concentration of 25 µg protein per ml, and multimammal liposomes, prepared by bath sonication of lipid extracts into 10 mM Hepes (pH 7.5) buffer at a concentration of 50 nmol Pi/ml, were incubated for 60 min with DPH, at a final concentration of 1 µM (1, 2, 12). Fluorescence polarization measurements of DPH labeled membranes were conducted using SLM spectrofluorometer (model 4800) at 21°C as before (1, 2, 15) and from the polarization values obtained, anisotropy parameters and apparent microviscosity values were calculated (12).

The thiol groups of membrane proteins were labeled with DA by incubating membranes at 100 µg protein per ml with 10 µM DA (Sigma), solubilized in ethanol, for 60 min at 21°C in the dark (2, 10). Fluorescence emission spectra of the DPH and DA labeled membranes were obtained using a Perkin-Elmer Fluorescence Spectrophotometer, model LS-5, at room temperature with excitation at 350 nm, excitation and emission slits at 3 nm.

**RESULTS AND DISCUSSION**

Freezing tolerance of cells within the crown of winter wheat is heterogeneous (16). Therefore, exposure of seedlings to a freezing stress which prevents regrowth does not necessarily injure all cells within the crown to the same degree. In the first experiments a freeze-thaw cycle to −12°C was chosen which caused lethal injury to all cells within the crown of nonacclimated seedlings of Dreadnought wheat and only minimal injury to the crowns of acclimated seedlings (16). Accordingly, when 7-d-old, nonacclimated wheat seedlings were exposed to a −12°C freeze-thaw cycle, the crown lost its capacity to regrow, and exhibited enhanced lipid leakage (Table I). In contrast, when seedlings, which had been acclimated at −2°C for a further 6 weeks, were exposed to the same freeze-thaw cycle survival was not reduced although there was a notable increase in electrolyte leakage, suggesting that this stress caused some sublethal injury.

The properties of microsomal membranes from the crown tissue of both nonacclimated and acclimated wheat seedlings were contrasted before and after this freeze-thaw cycle. Levels of protein and phospholipid in the microsomal fraction extracted from a given weight of nonacclimated crown tissue declined by 50 and 70%, respectively, as a result of the freezing treatment (Table II). This can be equated with a decrease in the phospholipid:protein ratio from 0.5 to 0.3 (nmol/µg). The same freezing treatment applied to acclimated seedlings had a negligible effect on protein content but caused a 30% decrease in the amount of neutral phospholipid extracted from the membranes. It is clear, therefore, that the freezing process induced quantitative changes in microsomal membrane content which may have been lost from the microsomal fraction as a result of membrane vesicle aggregation, fragmentation into smaller vesicles, or solubilization. These possibilities were examined by subjecting the supernatant of the 156,000g centrifugation used to isolate microsomal membranes, to a further centrifugation at 230,000g for 150 min. Only a small, additional (about 5% of total) amount of protein was recovered as a pellet by this procedure, independent of the prior treatment given to the tissue, indicating that freezing did not significantly decrease microsomal particles. The protein content of the 230,000g supernatant was greater in the freeze-thaw treatments and quantitatively accounted for all of the protein lost from the microsomal pellet suggesting that the freezing treatment led to a solubilization of microsomal protein.

A lethal freeze-thaw cycle has been previously shown to increase the lipid phase transition temperature of microsomal membranes by approximately 40°C as detected by wide angle x-ray diffraction (5). These observations were expanded by labeling the isolated microsomal membranes with the lipophilic fluorescence probe, DPH, and measuring the fluorescence anisotropy after exposure with polarized light. The anisotropy values can be equated to membrane microviscosity (1, 12). Exposure of nonacclimated seedlings to the −12°C freeze-thaw cycle, which was lethal, nearly doubled membrane microviscosity compared to the nonfrozen control (Table III). In contrast, this treatment did not alter the microviscosity of microsomal membranes isolated from acclimated seedlings exposed to the same stress. When the lipids extracted from the microsomal fraction were formed into liposomes, very similar microviscosities were obtained, which suggests that the changes in this parameter following a lethal freeze-thaw cycle can be attributed primarily to alterations in the lipid composition of the membrane.

Qualitative changes in the lipid composition of the membrane lipids also accompanied the quantitative loss of phospholipid induced by freezing. The ratio of total fatty acids to phospholipid in nonacclimated tissue nearly doubled as a result of freezing (Table II). One possible interpretation of these results is that they reflect an increase in free fatty acids in the membranes, as previously observed (5), or the selective loss of phospholipid relative to other lipid classes. This latter possibility was investigated using the fluorescence intensity of DPH which, because the probe is lipid soluble and has a much higher quantum yield in lipophylic than in hydrophobic environments (12), quantifies those lipophylic sites on a relative scale. In addition, the partitioning of the probe is independent of the lipid class or phase and thus, the measurements of DPH fluorescence intensity of membranes reflect the relative total lipid content of these membranes (12, 15) and can avoid the problem of incomplete extraction of different lipid classes. The intensity of DPH fluorescence in membranes of frozen, nonacclimated tissue was less than 40% of the values obtained for membranes from the nonfrozen tissue (Table II). In contrast, fluorescence of membranes from acclimated tissue changed marginally following the freezing treatment. The similarity between the changes in DPH fluorescence

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**Table I. Effect of a Freeze-Thaw Cycle to −12°C on Ion Leakage and Survival of Winter Wheat Crown Tissue**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Frozen</th>
<th>Ion Leakage</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonacclimated</td>
<td>−</td>
<td>3.30 ± 0.08</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>31.60 ± 0.82</td>
<td>0</td>
</tr>
<tr>
<td>Acclimated</td>
<td>−</td>
<td>1.80 ± 0.10</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>11.50 ± 0.71</td>
<td>100</td>
</tr>
</tbody>
</table>
FREEZE-THAW EFFECTS ON WHEAT MEMBRANES

Table II. Effect of a Freeze-Thaw Cycle to \(-12^\circ C\) on the Protein and Phospholipid Content and DPH Fluorescence of Membranes Isolated from Winter Wheat Crown Tissue

Fluorescence intensity of DPH labeled membranes was measured at 428 nm with excitation at 350 nm and slits at 3 nm. The results for protein and phospholipid content are means \(\pm SE\) \((n = 3)\).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Frozen</th>
<th>Protein (\mu g/g) fresh wt</th>
<th>Phospholipid nmol/g fresh wt</th>
<th>DPH Fluorescence relative units/25 (\mu g) protein</th>
<th>TFA/PL* mol/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonacclimated</td>
<td>–</td>
<td>1037 ± 52</td>
<td>527 ± 42</td>
<td>95</td>
<td>2.30 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>445 ± 22</td>
<td>132 ± 15</td>
<td>37</td>
<td>3.75 ± 0.25</td>
</tr>
<tr>
<td>Acclimated</td>
<td>–</td>
<td>1339 ± 67</td>
<td>571 ± 53</td>
<td>74</td>
<td>2.15 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1379 ± 97</td>
<td>392 ± 31</td>
<td>64</td>
<td>2.75 ± 0.12</td>
</tr>
</tbody>
</table>

* Ratio of total fatty acids to phospholipid content \((n = 4)\).

Table III. Effect of a Freeze-Thaw Cycle to \(-12^\circ C\) on Anisotropy and Microviscosity of Microsomal Membranes Isolated from Winter Wheat Crown Tissue and Liposomes Prepared from Total Lipid Extracts of the Microsomal Membranes

Values were calculated from fluorescence polarization values obtained at \(21^\circ C\) for DPH labeled membranes. Results are means \(\pm SE\) \((n = 3)\).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Frozen</th>
<th>Membrane Anisotropy</th>
<th>Microviscosity poise</th>
<th>Liposome Anisotropy</th>
<th>Microviscosity poise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonacclimated</td>
<td>–</td>
<td>0.89 ± 0.03</td>
<td>2.1 ± 0.1</td>
<td>0.71 ± 0.01</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.52 ± 0.05</td>
<td>3.8 ± 0.1</td>
<td>1.11 ± 0.04</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Acclimated</td>
<td>–</td>
<td>0.93 ± 0.02</td>
<td>2.2 ± 0.1</td>
<td>0.75 ± 0.01</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.97 ± 0.04</td>
<td>2.3 ± 0.1</td>
<td>0.79 ± 0.01</td>
<td>1.9 ± 0.1</td>
</tr>
</tbody>
</table>

Fig. 1. Emission spectrum of DA labeled microsomal membranes, obtained from nonacclimated winter wheat crown tissue before and after a lethal freeze-thaw stress to \(-12^\circ C\). Excitation was at 350 nm and slits a 3 nm. The spectrum was corrected by subtracting the spectra obtained from controls containing DA or membranes alone.

intensities and phospholipid:protein ratio (Table II) suggests that a general decline in total lipid classes in the membrane occurred after the freeze-thaw stress.

Membrane proteins also exhibited qualitative, as well as the previously detailed quantitative, modifications in response to the freeze-thaw treatment. The thiol levels in membrane proteins were measured using the thiol specific fluorescent probe, DA \((2, 10)\). The fluorescence intensity was much lower for membranes isolated from nonacclimated crown tissue exposed to a lethal freezing stress than for membranes isolated from control tissue (Fig. 1). Since the spectral characteristics of the fluorescence emitted from the labeled membrane preparation were similar, the drop in fluorescence can be attributed to a reduction in the number of reacting thiol groups and not from quantum yield differences \((2)\). Contrary to the effects observed for nonacclimated tissue, a similar freezing stress imposed on acclimated tissue had virtually no effect on DA fluorescence and, therefore, on thiol group content (Fig. 2).

The increase in membrane lipid microviscosity (Table III) and the increase in the liquid-crystalline to gel phase transition temperature \((5)\) observed in isolated microsomal membranes from lethally freeze-stressed tissue appear to be related to specific compositional changes in the membrane lipid fraction in particular: a decrease in the relative amount of phospholipid in the membrane (Table II) and an increase in the level of free fatty acids in the membrane \((5)\). These compositional changes might be expected to increase molecular order \((12)\). There are also
Table IV. Effects of the Freezing and Thawing Stages in the Freeze-Thaw Cycle on the Composition and Microviscosity of Microsomal Membranes Isolated from Nonacclimated Winter Wheat Crown Tissue

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>Frozen to −12°C</th>
<th>Frozen/Thawed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal protein</td>
<td>1976</td>
<td>1361</td>
<td>1242</td>
</tr>
<tr>
<td>(µg/g fresh weight)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipid (nmol/g fresh weight)</td>
<td>412</td>
<td>292</td>
<td>261</td>
</tr>
<tr>
<td>Microviscosity (poise)</td>
<td>1.83</td>
<td>2.35</td>
<td>2.34</td>
</tr>
<tr>
<td>TFA/PL* (mol/ml)</td>
<td>2.20</td>
<td>3.83</td>
<td>4.38</td>
</tr>
</tbody>
</table>

* Ratio of total fatty acids to phospholipid content.

quantitative and qualitative changes in the membrane proteins, possibly indicating loss of specific types of proteins, which are rich in thiol groups, from the membrane.

To determine whether the composition and structural changes, described above, occur during the freezing or thawing stages of the freeze/thaw cycle, several comparisons were made between membranes isolated from crown tissue frozen at −12°C and not thawed, and membranes obtained from frozen tissue that was thawed at 2°C for 4 h. The freezing step alone decreased the amount of microsomal membrane protein and lipid by 24 and 29%, respectively, and increased membrane microviscosity by 28% (Table IV). Similarly, the ratio of total fatty acids to phospholipid increased in the membranes isolated from the tissue frozen at −12°C, indicating a loss of phospholipid head groups from the membrane. Relatively little change in these parameters occurred during the subsequent thawing period, although the degradative processes continued as shown by the lower values for protein and phospholipid. The results suggest that most of the phospholipid and protein degradation in the cellular membranes occurs during freezing and relatively little during thawing.

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LITERATURE CITED
