Freezing Injury and Phospholipid Degradation in Vivo in Woody Plant Cells

III. EFFECTS OF FREEZING ON ACTIVITY OF MEMBRANE-BOUND PHOSPHOLIPASE D IN MICROSOME-ENRICHED MEMBRANES

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ABSTRACT

Freeze-thawing of microsome-enriched membranes from living bark tissues of black locust trees, especially those from less hardy tissues, caused a drastic increase in sensitivity to Ca\(^{2+}\) and a complete loss of the regulatory action of Mg\(^{2+}\) in membrane-bound phospholipase D activity with endogenous (membrane-bound) substrates. Also, the freeze-thaw cycle made phospholipase D in these membranes more resistant to digestion by proteases. Thus, the regulatory properties of the membrane-bound phospholipase D seem to be dependent on the nature of the membranes and on the interaction between the enzyme and membranes as well. The alteration of regulatory properties by freezing was protected by sucrose, at lower concentrations, and more effectively for membranes from hardy tissues than for membranes from less hardy tissue. Addition of partially purified soluble phospholipase D to the reaction system containing membranes caused only a slight stimulation of the degradation of endogenous phospholipids. Phospholipid degradation in vivo during freezing of less hardy tissue may be catalyzed mainly by the bound enzyme. Disintegration of the tonoplast, however, besides releasing soluble phospholipase D into the cytosol, would release organic acids (lowering the pH) and free Ca\(^{2+}\). Both factors would stimulate drastically the membrane-bound phospholipase D, causing degradation of membrane phospholipids.

In the present study, special attention is focused on the possible involvement of an alteration of the regulatory properties of the membrane-bound phospholipase D due to freezing. A preliminary account of portions of this study has been presented elsewhere (17).

MATERIALS AND METHODS

Preparation of EGTA-washed Microsome-enriched Membranes. Living bark tissue of black locust trees (Robinia pseudoacacia L.) was used as the experimental material. The EW-Ms were prepared from the tissues as previously reported (18, 19). The membranes contained no detectable amount of soluble phospholipase D.

Freezing of EW-Ms. Five-tenths ml of the EW-Ms suspension (0.6-0.8 mg protein) in a test tube (13 × 100 mm) was frozen in air at temperatures ranging from -5 to -30°C in cold boxes. After thawing of the frozen specimens in air at room temperature, they were then incubated with 60 mM buffer solutions varied in pH, and divalent cations as required in a final volume of 1 ml at 25°C. Buffer systems were glycine-HCl (pH 3.5), Na-acetate (pH 4.0-6.5), and Tris-HCl (pH 7.0-8.0). The reaction was terminated by the addition of 0.2 ml of 1 N HClO₄. Assay of the liberated choline in the supernatant was done as described before (18, 19). Enzymic degradation of phosphatidylcholine during freezing of EW-Ms was followed by determining the liberated choline. Two-tenths ml of 1 N HClO₄ was added to frozen specimens immediately before thawing and brought to 1.2 ml by the addition of distilled H₂O. The liberated choline in the supernatant was assayed as described before (18, 19).

Determination of in Vivo Degradation of Phospholipids during Freezing. Small pieces of tissues were frozen in a test tube (1.3 × 18 cm) at -5°C. The frozen tissues in the test tubes were then cooled in 5°C steps at hourly intervals to successively lower temperatures. After keeping at desired temperatures for 18 h, the frozen tissues were ground in cooled isopropanol alcohol (at -10°C) with sea sand. Grinding was done at -10°C in a cold room. Lipids were extracted successively with chloroform-methanol (1:1, v/v) and then chloroform-methanol (2:1, v/v). The combined lipid extracts were subjected to Folch's procedure to remove nonlipid contaminants. Aliquots of the purified lipid samples were loaded on silica gel plates and were developed with chloroform-methanol-acetic acid (65:25:8, v/v/v). The area corresponding to each standard phospholipid was scraped into test tubes and directly heated with 0.5 ml of 70% HClO₄ for 45 min at 180°C. The lipid phosphorus was determined according to Fiske-SubbaRow method (3).

One of the most remarkable characteristics of certain perennial plants growing in cold climates is the ability to survive a deep freeze in midwinter. They may be severely injured by freezing at relatively higher temperatures when they are not in a fully hardened state. In less hardy poplar, it has been reported that a drastic degradation of phosphatidylcholine occurs in the cortical tissues frozen at a critical temperature (15, 20). The same phenomenon has also been observed in frozen seedlings of bean plants (14). These facts may indicate that extracellular freezing at a certain critical temperature causes deleterious changes in membrane systems, especially related to activation of phospholipase D in the cells.

In previous studies (18, 19), it was demonstrated that phospholipase D is located in both soluble and particulate cell fractions in living bark tissues of the black locust tree. The soluble enzyme was suggested to be compartmentalized in a lysosomal apparatus, such as vacuoles. A considerable amount of phospholipase D, however, was found to be tightly bound to membranes. The membrane-bound phospholipase D was observed to be regulated by binding of divalent cations and also by pH (19).

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Determination of Divalent Cations in Cell Fractions. Cell fractions were obtained by a differential centrifugation procedure as described in this text. Particulate cell fractions were washed once with 1 mM EGTA buffered with 5 mM Tris-HCl (pH 7.5). The content of divalent cations in each cell fraction was determined with an atomic absorption spectrophotometer.

Enzyme Assays. Antimycin A-insensitive NADH-Cyt c reductase was assayed at 25 C by following the reduction of Cyt c at 550 nm in the reaction mixture containing 100 μmol of K-phosphate (pH 7.2), 0.5 mg of oxidized form of Cyt c, 5 μmol of NaN₃, 1 nmol of antimycin A, and 50 μl of an enzyme preparation in a final volume of 2 ml (16). The reaction was started by the addition of 0.5 μmol of NADH. ATPase was assayed by the method described by Hodges and Leonard (5). The reaction mixture contained 3 μmol of ATP, 1.5 μmol of MgCl₂, 50 μmol of KCl, 100 μmol of Tris-Mes (pH 6.1) or Tris-HCl (pH 7.8), and 0.2 ml of an enzyme preparation. The reaction was performed at 30 C for 30 min and then stopped by the addition of 1.0 ml of 10% trichloroacetic acid. One drop of 1% BSA was added immediately before the termination of the reaction to make the supernatant clear. The released Pi was determined by the Fiske-SubbaRow method (3).

Assay of phospholipase D was done as described before (18, 19).

RESULTS

In tissues of black locust trees collected in the middle of June, no injury was observed after freezing at −10 C for 18 h, whereas they suffered serious injury by freezing below −10 C. In the tissues frozen below −10 C for 18 hr, remarkable degradation of phosphatidylcholine and phosphatidylethanolamine were observed (Fig. 1).

Questions now arise as to how freezing alters membrane properties, and whether the altered membrane properties activated phospholipase D in situ. To give some insights into these questions, experiments were focused on the effect of freezing on properties of membrane-bound phospholipase D in EW-Ms. Freeze-thawing of EW-Ms from hardy tissues to −30 C resulted in no significant change in the pH activity profile with endogenous substrate (Fig. 2A). However, freeze-thawing of less hardy membranes resulted in both shifting of the optimum pH toward neutral and much increase in the activity at the neutral pH (Fig. 2B). Thus, it is likely that microsome-enriched membranes from tissues varying in hardiness respond to freezing in a different manner.

Freeze-thawing of EW-Ms at −30 C increased the sensitivity to Ca²⁺ in both samples at pH 7.0 (Fig. 3). The increase in the Ca sensitivity after freeze-thawing, however, was quite remarkable in EW-Ms from less hardy tissues. In freeze-thawed EW-Ms from hardy tissues, the activity of phospholipase D increased proportionally as Ca²⁺ concentration increased up to 10 mM. However, in freeze-thawed EW-Ms from less hardy tissues the activity reached plateau within 3 mM of Ca concentration.

Freeze-thawing of EW-Ms at −30 C also resulted in decrease or loss of the inhibitory effect of Mg²⁺ upon the activity of membrane-bound phospholipase D on endogenous substrate depending on the hardiness of the samples. Freeze-thawing of EW-Ms from less hardy tissues resulted in a drastic loss of the inhibitory effect of Mg²⁺ (Fig. 4A), independently of pH, whereas relatively small changes were observed in freeze-thawed EW-Ms from hardy tissues (Fig. 4B). Thus, it now appears that the regulatory system operating in membrane-bound phospholipase D is susceptible to freezing, especially in membranes from less hardy tissues.

To check the effect of freezing temperatures on the reaction of phospholipase D on endogenous substrate, EW-Ms suspensions prepared from hardy and less hardy tissues were frozen at various temperatures for 16 h. Choline liberation was followed both immediately before thawing, and after incubation at 25 C for 10 min in a buffer solution (pH 5.5) following thawing. Unexpectedly, when EW-Ms suspensions in 5 mM Tris-HCl buffer (pH 7.6) were frozen at −5 or −10 C for 16 h, marked degradation of endogenous phosphatidylcholine occurred during freezing regardless of the hardiness of the materials (Fig. 5). Below −20 C or at 0 C, however, little or no degradation was observed in either sample. When frozen specimens were then incubated at 25 C in a buffer...
solution (pH 5.5), following thawing, an additional degradation of phosphatidylcholine was observed in less hardy EW-Ms, independently of the freezing temperatures. On the other hand, in hardy EW-Ms little or no additional degradation was observed during the postincubation following thawing below -15 C. The reaction of phospholipase D using endogenous substrate during freezing at -10 C was relatively unaffected by pH between 4.5 and 7.8 (data not shown). Subcellular distributions of Mg²⁺ and Ca²⁺ are summarized in Table I. There was no significant difference in the total amount of divalent cations between hardy and less hardy tissues. The highest concentration of Ca and Mg ions was distributed in the supernatant. If these are distributed uniformly in the cytosol and vacuoles, the cellular concentration will be calculated as 30 mm in Ca²⁺ and 8 mm in Mg²⁺. In particulate fractions, the highest amount of Ca²⁺ was distributed in the mitochondrial fraction and less in the microsomes. In particulate cell fractions, there was no significant difference in the specific amount of divalent cations per mg protein between hardy and less hardy tissues.

EW-Ms suspensions were frozen at -10 C for 16 h with possible protective substances, and then incubated at 25 C in a buffer solution (pH 5.5) for 10 min after thawing. Sucrose as well as MgCl₂ prevented degradation of phosphatidylcholine during the freeze-thawing cycle to -10 C (Fig. 6). In EW-Ms from hardy tissues, less than 10 mm of sucrose protected completely against the degradation of phosphatidylcholine. DMSO, however, was less effective than sucrose. In EW-Ms from less hardy tissues, sucrose protected only 50% and DMSO showed little or no protection in the whole range of concentrations tested. The inhibitory effect of Mg²⁺ upon the degradation of phospholipids during freeze-thawing cycle was also reduced in EW-Ms from less hardy tissues.

These protective substances could also minimize the change in the regulatory properties of membrane-bound phospholipase D caused by freezing. Addition of Mg²⁺ or sucrose to EW-Ms before freezing at -10 C restored the sensitivity to Ca²⁺ to the level of the unfrozen control (Fig. 7).

To give some insights into the binding mode or the locus of phospholipase D on membranes, effects of pronase treatments were examined. Unfrozen and freeze-thawed (at -30 C) EW-Ms suspensions from hardy tissues were preincubated with pronase (1 mg/ml) at pH 7.5 for various lengths of time at 10 C and then incubated at 25 C in buffer solution (pH 4.5) to follow the activity of phospholipase D on endogenous substrate. In unfrozen EW-Ms, the activity was decreased to nearly 50% of the original activity by treatment with pronase for 10 min (Fig. 8). On the other hand, after freeze-thawing of EW-Ms at -30 C, the same concentration of pronase was less inhibitory and took 30 to 40 min to cause a 50% inhibition. Experiments utilizing trypsin gave also nearly the same results (data not shown).

**DISCUSSION**

An intimate relation between phospholipid degradation in vivo during freezing at sublethal temperatures and freezing injury of plant cells was confirmed with less hardy living bark tissues of black locust trees as reported with cortical cells of poplar (15, 20). As mentioned before (19), the activity of membrane-bound phospholipase D on endogenous substrate is regulated by binding of divalent cations to the enzyme and also by pH. The results of
supporting this speculation, phospholipase D in microsomes-enriched membranes from less hardy tissues showed more resistance against digestion by proteases (unpublished data).

Freezing of microsomes without addition of exogenous Ca\(^{2+}\) at relatively higher temperatures (−5 to −10 °C) caused a drastic degradation of endogenous phosphatidylcholine during freezing, irrespective of the tissue hardiness. This reaction was less dependent on the pH. Freezing of membranes in vitro at these temperatures seems to produce nearly the same conformational changes of phospholipase D as lowering in pH and/or increasing in Ca\(^{2+}\) concentration.

The alteration in the regulatory properties of membrane-bound phospholipase D as affected by freezing was more effectively restored by the presence of lower concentration of sucrose in hardy membranes than in less hardy membranes. Accordingly, some qualitative differences may exist between hardy and less hardy cellular membranes, especially in terms of effectiveness of sucrose, as demonstrated by Garber and Steponkus (4) with thylakoid membranes from differentially hardened spinach leaves. According to Matile (7–9) and others (2, 10, 11, 13), vacuoles in plant cells are quite similar in their functions to the lysosomal apparatus in animal cells. Vacuoles contain many kinds of acid hydrolases and the pH in cell sap is much lower than the cytosol (12). As reported in a previous paper (10), soluble phospholipase D may be compartmentalized in vacuoles. Vacuoles in plant cells may also regulate the concentration of Ca\(^{2+}\) in the cytosol (1) as do mitochondria in vertebrate cells (6). Thus, a loss of the integrity of the tonoplast during freezing could bring about leakages of soluble phospholipase D as well as the other hydrolases, Ca\(^{2+}\), and organic acids into the cytosol. When microsome-enriched membranes were incubated at pH 4.5 in the presence of 5 mM Ca with partially purified soluble phospholipase D from bark tissues of black locust tree, the degradation of endogenous phosphatidylcholine was stimulated only by 8% (unpublished data). Thus, in microsome-enriched membranes, the degradation of endogenous phospholipids may have been dominantly catalyzed by the endogenous enzyme rather than exogenous soluble enzyme.

An intimate relation was observed in the present study between the initiation of the degradation of phospholipids and the loss of activities in membrane-associated enzymes such as Cyt c reductase and ATPase (data not shown). From this fact it may be stated that loss of the regulatory system of membrane-bound phospholipase D by itself or resultant degradation of phospholipids in membranes causes an irreversible dysfunction of membranes and cell death.

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