**Communication**

**Membrane Rupture Is the Common Cause of Damage to Chloroplast Membranes in Leaves Injured by Freezing or Excessive Wilting**

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**ABSTRACT**

The effects of freezing and desiccation of spinach leaves (*Spinacia oleracea* L. cv. Yates) on the thylakoid membranes were assessed using antibodies specific for thylakoid membrane proteins. The peripheral part of the chloroplast coupling factor ATPase (CF1) was used as a molecular marker for chemical membrane damage by chaotropic solutes. Plastocyanin, a soluble protein localized inside the closed thylakoid membrane system, was a marker for damage by mechanical membrane rupture. After freezing and wilting of leaves which resulted in damage, very little CF1 was detached from the membranes, whereas almost all plastocyanin was released from the thylakoids. It is suggested that in *vivo* dehybridation both by freezing and desiccation results in membrane rupture rather than in the dissociation of peripheral thylakoid membrane proteins.

Plants may experience different environmental stresses such as frost, drought and salt stress (see Ref. 10 for a review). Common to these stresses is a lowering of cell water potentials. There is general agreement now that cellular membranes are a principal site of susceptibility to environmental stress (20). Thylakoid membranes have been used for many years as an *in vitro* model system to study the effects of membrane dehydration caused by freezing (2), desiccation (14) or high salt concentrations (13). In freezing experiments, it has been shown that two kinds of freezing damage can be distinguished (5, 15, 16). Damage suffered by thylakoids suspended in solutions of low osmolarity (below approximately 0.1 osmolar) has been termed mechanical freezing damage (6) or mechanical membrane breakage (1). It is characterized by the release of the soluble luminal protein plastocyanin from closed thylakoid vesicles (4), indicating membrane rupture (3). Loss of plastocyanin during an *in vitro* freeze-thaw cycle has been inferred from the protective effect of added plastocyanin present during freezing (1). It has been detected in the supernatants of centrifuged frozen-thawed thylakoids with a polyspecific antisemur (21) and quantitated using monospecific antibodies (4).

At high initial solute concentrations during freezing in *vivo* membranes may suffer damage from the effects of chaotropic salts if such damage is not prevented by cryoprotectants. The accumulation of chaotropic agents causes dissociation of peripheral proteins such as the CF1\(^\text{1}\) of the coupling factor ATPase from the membranes (1, 4, 21). Its release during freezing produces uncoupling of phosphorylation from light-dependent electron transport (see Ref. 18 for a recent review). This type of damage has been termed chemical freezing damage (6). Mechanical damage has been observed only at unphysiologically low osmolalities in *vivo*, whereas chemical damage occurs at solute concentrations in the physiological range (6). It has therefore been argued that chemical membrane damage had to be expected during freezing of leaves (18). Here we show that contrary to such expectations mechanical damage predominates when leaves rather than isolated thylakoids are injured by freezing or wilting.

**MATERIALS AND METHODS**

**Plant Material.** For freezing experiments spinach plants (*Spinacia oleracea* L. cv. Yates) were grown in hydroponic culture as described previously (8). Salt treatment of the plants followed the procedure described recently (17). Leaves hardened under salt stress survived freezing to temperatures as low as -10°C, whereas leaves from control plants were killed at temperatures below -5°C (17). Spinach plants for drought stress experiments were grown in soil without added salt under the same conditions. Freezing and Wilting of Leaves. Leaves were frozen and thawed at 4°C/h as described before (17). Detached leaves were wilted at room temperature and ambient air humidity (19).

**Immunochemical Protein Determination.** For protein determination frozen-thawed leaves were homogenized for 5 s in an Ultra-Turrax (IKA-Werke, Staufen, FRG) in a medium comprising 100 mM NaCl, 5 mM MgCl\(_2\), 5 mM cysteine, 1% PVP-40 (soluble polyvinylpyrrolidone; Sigma), 150 mM sucrose, and 50 mM Tris (pH 7.8). Wilted leaves were homogenized in a medium comprising 10 mM NaCl, 5 mM MgCl\(_2\), 1% PVP-40, 2 mM glycine-betaine, and 15 mM Tris (pH 8.0). The homogenates were filtered through nylon gauze and an aliquot was lyzed in 2% Triton X-100 to determine the total amount of a given protein. The remainder of the samples was centrifuged for 20 min at 45,000g. For single radial immunodiffusion (11) pellets were

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3. Abbreviation: CF1, peripheral part of chloroplast coupling factor ATPase.
lysed in 2% Triton X-100, the supernatants were used directly (4). For the experiments reported here the amount of a given protein in the pellet and the supernatant added up to 102.3 ± 5.2% SD of the total homogenates. CF1 and ferredoxin-NADP* reductase were isolated as described in Ref. 22. Purification of plastocyanin and the preparation of monospecific antisera against the three proteins have been described recently (4).

RESULTS AND DISCUSSION

Damage by Freezing. Leaves of hardened and unhardened spinach were subjected to freeze-thaw cycles. Figure 1 shows the release of the two peripheral thylakoid proteins ferredoxin-NADP* reductase (FNR, a) and CF1 (b) and of the soluble luminal protein plastocyanin (c) from the membranes after freezing and thawing in vivo. It shows a distribution for ferredoxin-NADP* reductase of about 40% in the soluble supernatant and 60% in the sedimented thylakoids, regardless of whether homogenates were prepared from unfrozen or frozen leaves and whether frozen leaves had been damaged or not. This is in accordance with the finding that the release of ferredoxin-NADP* reductase was not influenced by any freeze-thaw treatment in vitro (4). A similar distribution between supernatant (20%) and pellet (80%) was also found for CF1 which is a marker protein for chemical freezing damage (see the introduction). The solubilized fraction of these proteins was obviously detached from thylakoids during mechanical grinding of the leaves.

In contrast to the distribution of the peripheral membrane proteins, the distribution of plastocyanin depended on whether the leaves were damaged by freezing or not (Fig. 1c). Whereas most of the plastocyanin was retained inside the thylakoids of unjured leaves whether or not they had been frozen, practically all was lost from thylakoids of frost-injured leaves. The temperature dependence of plastocyanin release followed the course of damage to leaf photosynthesis both in unhardened leaves and in leaves frost hardened by exposure of the plants to NaCl (17).

Whereas during freezing of thylakoids in vitro, phosphorylation may become uncoupled from linear electron transport so that electron transport is stimulated (reviewed in Ref. 18), Klosson and Krause (9) have reported that photosynthetic electron flow and phosphorylation are damaged simultaneously in frozen-

![Fig. 1. Protein release from thylakoid membranes after exposing leaves of frost-sensitive spinach (A) and of spinach hardened by exposure to 300 mM NaCl (B) (17) to freeze-thaw cycles. Immunochemically determined percentages of (a) ferredoxin-NADP* reductase (FNR), (b) peripheral part of chloroplast ATPase (CF1) and (c) plastocyanin (PC) in the supernatant fraction are shown as a function of the minimum temperature during freezing.](image)

thawed leaves. The parallel breakdown of these two reactions can now be explained by loss of plastocyanin from the thylakoid lumen. Damage to the water-splitting side of PSII had been reported to be severe (9), but the photosystems themselves were only mildly affected. We have observed that cyclic photophosphorylation of thylakoids isolated from frost-damaged leaves could be partially reactivated by increasing the concentration of phenazine methosulfate in the assay medium (data not shown), thus bypassing plastocyanin (12). With the same treatment, cyclic photophosphorylation could also be restored in thylakoids injured by mechanical freezing damage in vitro (4). This shows that PSI had remained largely intact in damaged membranes. Moreover, as photophosphorylation requires a low permeability of thylakoids to protons, reactivation of ATP synthesis shows that the vesicles had resealed after rupture. Whereas it has been shown that mechanical freezing damage in vitro occurs during thawing (3), no conclusions can be drawn from the present data on whether membrane destabilization in vivo takes place during freezing or during thawing or both.

Damage by Excessive Wilting. Drought is an even more important stress factor during the life cycle of many plants than frost. We have subjected detached spinach leaves to different degrees of desiccation. Figure 2 shows that release of plastocyanin from thylakoids increased as the leaves lost more than 60% of their water. Thylakoids isolated from dry leaves contained practically no plastocyanin. Irreversible damage to leaf photosynthesis occurred when more than 50% of the cellular water had been lost (7).

The leaf homogenates were prepared in a medium (approximately 2 osmolal) hypotonic to leaves which had lost up to 80% of their water content. Homogenization in hypertonic media did not release more plastocyanin from thylakoids than homogenization in isotonic media (approximately 30% in undamaged leaves, compare Figs. 1 and 2). The osmolarity of severely desiccated leaves with a water content below 20% exceeded that of the grinding medium by a factor of maximally 4 or 5. In these cases, the cellular compartments had to absorb much less water to reach isotonicity with the grinding medium than they had originally contained in turgid leaves. This makes it unlikely that the observed damage is solely attributable to rehydration during homogenization.

Damage to leaves caused by wilting is similar to damage caused by dehydration due to freezing not only in respect to plastocyanin release. Figure 2a shows that little CF1 was detached from thylakoids during wilting of leaves beyond that part which was also solubilized by grinding turgid leaves. When water loss became excessive and resulted in loss of viability, the percentage of

![Fig. 2. Protein release from thylakoids after wilting of leaves. The percentage of (a) chloroplast coupling factor (CF1) and (b) plastocyanin (PC) in the supernatant fraction is shown as a function of the relative water content of the leaves.](image)
solubilized CF1 increased from about 30 to 50%, suggesting that chemical membrane damage may have contributed to injury when leaves lost more than 80% of their water content at room temperature. In conclusion, we have observed damage to thylakoid membranes under conditions of frost stress and drought stress, which injured leaves. Membrane damage was predominantly mechanical. It was similar after freezing and after desiccation of leaves. At least in the case of desiccation down to a relative water content of about 20% damage must have occurred during water loss because rehydration was prevented by the use of a grinding medium of high osmolality.

LITERATURE CITED
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