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Antibodies against individual thylakoid membrane proteins as molecular probes to study chemical and mechanical freezing damage in vitro

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The release of proteins and the loss of biochemical activities under mechanical and chemical stresses during freezing of isolated thylakoid membranes were investigated, using polyacrylamide gel electrophoresis, single radial immunodiffusion and the measurement of cyclic photophosphorylation. Antibodies against purified proteins derived from the stromal (coupling factor CF1, ferredoxin-NADP$^+$ reductase) and the lumenal side (plastocyanin) of the membrane vesicles were used as probes. Low initial solute concentrations were employed to generate mechanical stress. Chemical stresses were manipulated by varying the molar ratios of cryotoxic to cryoprotective solutes at high initial solute concentrations. Constant low amounts of ferredoxin-NADP$^+$ reductase were lost from the membranes during freezing, irrespective of the composition of the suspending media. Damage at high initial osmolalities was accompanied by the release of CF1, which was influenced by the ratio of potentially cryotoxic to cryoprotective solutes, as demanded by the colligative theory of membrane cryopreservation. CF1 release and loss of cyclic photophosphorylation were linearly correlated at different ratios of salt to sucrose. However, the correlation data revealed that CF1 release could account for only part of the observed cryoinjury. Plastocyanin release was predominant at low initial osmolalities and was not influenced by the chemical composition of the suspending media. This indicates mechanical damage by membrane rupture. Under these circumstances loss of plastocyanin and loss of cyclic photophosphorylation were linearly correlated. Loss of photophosphorylation could be prevented by the addition of up to 1.2 mg plastocyanin/ml prior to freezing. It could also be ameliorated to a large extent by raising the phenazine methosulfate concentration in the test assay from 30 to 230 μM. This indicates that the membranes are able to reseal after rupture, maintaining a proton gradient upon illumination and that it is the loss of plastocyanin from their lumen that inhibits cyclic photophosphorylation.

Introduction

Over the last years it has been shown that the inactivation of biomembranes during freezing is accompanied, if not caused, by the solubilization of membrane proteins (see Ref. 1 for a recent review). It has been reported [2] that isolated thylakoid membranes lost CF1 particles during a freeze-thaw cycle. In the same report it was shown that thylakoids could be partially protected from freezing damage by the addition of plastocyanin prior to freezing and by the addition of CF1 after thawing. Protein loss during freezing was demonstrated by gel electrophoresis when membrane proteins were found in supernatants of suspensions from which the membranes were removed after freezing by centrifugation [3–6]. Subunits of

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Abbreviations: CF1, peripheral part of chloroplast coupling factor ATPase; DCCD, dicyclohexylcarbodiimide; Chl, chorophyll.

the chloroplast coupling factor could be identified in all four cases but, in addition, more than 25 further polypeptides originating from the membranes were found in the supernatants. Using a polyspecific antiserum raised against the supernatant of frost-treated thylakoids, ferredoxin-$NADP^+$ reductase and plastocyanin, in addition to CF1 have been identified as proteins that are released during freezing [3]. It has also been reported [7] that the electrophoretic pattern of proteins released during freezing was very similar to the pattern observed when the membranes were washed in dilute solutions of EDTA.

Protein release is thought to be due mainly to solute effects. Chaotropic ions are known to be able to solubilize peripheral membrane proteins in the absence of freezing [4,8]. During freezing, water is converted to ice, and solutes are concentrated together with the membranes, subjecting these to high solute concentrations which may affect membrane stability. The degree of membrane toxicity of a solute depends on its chaotropicity [3,4,6,9]. Damage resulting from chaotropic effects may be termed chemical to distinguish it from the physical damaged caused by membrane rupture (for a recent review see Ref. 10). Chemical damage can be prevented by the addition of sufficient amounts of cryoprotectants, such as sugars of sugar alcohols, which are thought to protect the membranes on a colligative basis. According to the colligative theory of membrane cryopreservation [11,12,13] the degree of chemical damage will be determined by the molar ratio of potentially cryotoxic to membrane-compatible solutes.

Recent work has indicated that other mechanisms are also operative in causing cryoinjury to thylakoid membranes in vitro [5,6,14,15]. Damage to membranes that had been suspended in media of low osmolality was not predominantly of chemical nature [6]. Even when the salt to sugar ratio was minimized by washing the membranes in 10 mM sucrose and freezing them without added salt, damage as revealed by the inhibition of cyclic photophosphorylation was severe [5]. Two mechanisms have been discussed to account for freezing damage under these circumstances. Injury was either attributed to the direct effects of ice crystals [14,15] or to membrane rupture due to osmotic contraction or expansion [5].

The electrophoretic patterns of polypeptides liberated during a freeze-thaw cycle exhibit significant differences between chemically and mechanically injured membranes [5,6]. We have measured the release of plastocyanin, ferredoxin-$NADP^+$ reductase and CF1 under conditions of mechanical and chemical freezing stress to assess the suitability of these proteins to serve as markers for the two kinds of injury. Marker proteins would permit a study of the mechanisms of freezing damage at the molecular level. The use of antibodies against such proteins would furthermore open the possibility to probe for the relative contributions of chemical and mechanical stresses in the inactivation of thylakoid membranes in vivo.

Materials and methods

Isolation of thylakoids. Thylakoid membranes were isolated from spinach leaves (*Spinacia oleracea* cv. Yates) as described previously [5]. The washing media are given in the figure legends. Thylakoid suspensions were frozen as described in Ref. 5.

Cyclic photophosphorylation. Thylakoid suspensions were assayed for cyclic photophosphorylation as described recently [6].

Osmometry. The osmolality of solutions was determined by freezing-point depression measurements using a Knauer semi-micro osmometer (Knauer K.G., Oberursel, F.R.G.).

Gel electrophoresis. Gel electrophoresis was performed as described previously [4,5]. The silver nitrate procedure of Ansorge [16] was used for protein staining.

Protein purification. Ferredoxin-$NADP^+$ reductase and CF1 were isolated as described previously [7]. Plastocyanin was extracted from isolated spinach chloroplasts by sonic treatment following the procedure described in Ref. 17. The sonicated suspension was centrifuged for 30 min at 45000 × *g*, and the supernatant was brought to 50% saturation by addition of solid ammonium sulfate. After stirring for 30 min in the cold it was again centrifuged as described above. The clear supernatant was desalted by gel filtration on a Sephadex G-25 column (3 × 90 cm) equilibrated with 100 mM NaCl and 50 mM Tris (pH 7.3) and dialysis against 50 mM Tris (pH 7.3) for 4 h. The dialysis residue

was loaded on a DEAE-cellulose collumn (0.5 × 9 cm, Whatman DE-52) equilibrated with the same buffer.

Plastocyanin was eluted using a linear gradient from 0 to 1 M NaCl in starting buffer. The total volume of the gradient was 100 ml at a flow rate of 12 ml/h. Fractions containing plastocyanin were identified by their dark blue color after addition of small amounts of $K_3Fe(CN)_6$. The combined fractions were loaded on a Sephadex G-50F column (1.5 × 50 cm) equilibrated with 100 mM NaCl, 50 mM Tris (pH 7.3) and eluted with the same buffer at a flow rate of 6 ml/h. The plastocyanin peak was collected. The protein was pure as determined by gel electrophoresis.

Protein determination. Plastocyanin was determined using an absorption coefficient of 4.9 $mM^{-1} \cdot cm^{-1}$ and a molecular weight of 10.5 kDa [18]. Ferredoxin-NADP$^+$ reductase and CF1 were determined according to Warburg and Christian [19]

Immunization. For immunization of a rabbit, 1 mg of plastocyanin was emulsified in complete Freund's adjuvant and injected subcutaneously. After 3 weeks, a second injection of 1.7 mg plastocyanin was applied without the addition of adjuvant. After another 3 weeks, blood was drawn from an ear vein.

For the immunization of rabbits against CF1 and ferredoxin-NADP$^+$ reductase, 3.8 and 2 mg, respectively, in complete Freund's adjuvant were used for the first injection. The second injection in both cases contained 2 mg protein without adjuvant.

Sera prepared from rabbits prior to the first immunization did not show any reaction with crude preparations from spinach leaves. All antisera showed only one precipitate in immunodiffusion against such preparations and were thus considered to be monospecific.

Immunodiffusion. The release of CF1, ferredoxin-NADP$^+$ reductase and plastocyanin was measured using single radial immunodiffusion [20]. The gels contained 1% agar, 1% Triton X-100, 0.1% NaN_3, 25 mM Tris and 190 mM glycine (pH 8.4). Samples were allowed to diffuse for 40 to 50 h in a moist chamber at room temperature. Precipitates were stained with Serva Blue.

Before freezing, an aliquot of the thylakoid suspension was lysed in 2% Triton X-100 to determine the total amount of a given protein. After freezing, samples were centrifuged for 30 min at 10000 × *g*. The pellet was lysed in 2% Triton X-100 before subjecting it to immunodiffusion. The supernatant was used directly without further processing. For all experiments reported here the amount of a given protein in the pellet and the supernatant after freezing added up to 99.8% (± 7.1% S.D.) of the pre-freezing control.

Results

Fig. 1 shows that the modes of release of coupling factor CF1, ferredoxin-NADP$^+$ reductase and plastocyanin from thylakoids suspended and then frozen in media of different initial osmolality and composition differed from one another. Very little CF1 dissociated from the membranes during freezing when membrane suspensions contained only low concentrations of sucrose and NaCl at a fixed molar ratio. Dissociation increased as the osmolality of the media used for suspending the

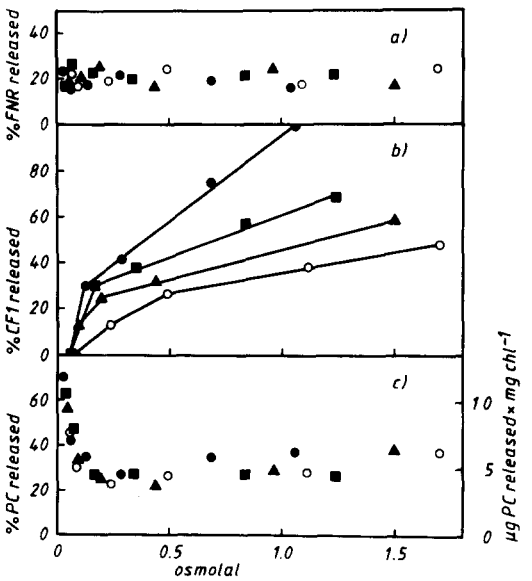

Fig. 1. Percentage of (a) ferredoxin-NADP$^+$-reductase (FNR), (b) coupling factor (CF1) and (c) plastocyanin (PC) released during a freeze-thaw cycle as a function of the osmolality of the suspending medium prior to freezing. The samples contained sodium chloride and sucrose at constant molar ratios of 1 : 1.5 (●), 1 : 2 (■), 1 : 2.5 (▲), 1 : 3 (○). The membranes were washed in media containing 10 mM NaCl and the appropriate sucrose concentrations yielding the NaCl to the sucrose ratios listed above.

membranes was increased (Fig. 1b). Dissociation also increased when, at constant osmolality, the ratio of the cryoprotective solute sucrose to the potentially cryotoxic solute NaCl was decreased. Such behavior is predictable on the basis of the colligative theory of cryoprotection [11–13]. Not predictable in this context is the increase in dissociation with increasing solute concentration in the suspending media. It can be explained on the basis of a dissociation/association equilibrium as has been discussed before [5].

In contrast to the behavior of CF1, the release of ferredoxin-NADP$^+$ reductase was independent of concentration and composition of the suspending media (Fig. 1a). Both CF1 and ferredoxin-NADP$^+$ reductase are peripheral membrane proteins situated on the outside of the membrane vesicles facing the medium. Apparently, different rules govern the release of CF1 and ferredoxin-NADP$^+$ reductase.

When release of CF1 was negligible, release of plastocyanin was maximal (Fig. 1c). It decreased with increasing osmolality of the suspending media and appeared to be practically independent of their composition. Plastocyanin is a soluble protein component of the inner phase of the thylakoid vesicles [21,22]. It can be released only after removal of permeability barriers.

Since the total amount of protein released during freezing and loss of photophosphorylation are linearly correlated [5,6], it was of interest to see whether the loss of a specific protein is correlated with the loss of biochemical activities thylakoid membranes suffer during a freeze-thaw cycle. Cyclic photophosphorylation has been shown by many workers to be a frost-sensitive process, in which CF1 is involved. Fig. 2 shows that loss of CF1 and loss of photophosphorylation are linearly correlated in samples that did not suffer heavy loss of plastocyanin. However, the slopes were different for different ratios of NaCl to sucrose. When the ratios were high (and initial osmolalities were comparatively low) only low rates of photophosphorylation were measured after freezing, even in the absence of much CF1 release. This shows that loss of CF1 is only one of the parameters causing inactivation of photophosphorylation during freezing. It should be noted that loss of plastocyanin from the thylakoids cannot explain the different

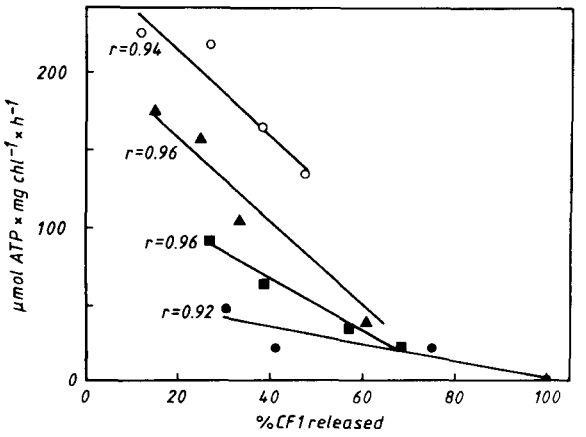

Fig. 2. Correlation of the rate of cyclic photophosphorylation and the amount of coupling factor (CF1) released during freezing. The data were taken from Fig. 1. Only samples that had lost less than 40% plastocyanin were used for this graph. The lines were fitted to the data using linear regression analysis, the respective r values are shown . For symbols, see legend to Fig. 1.

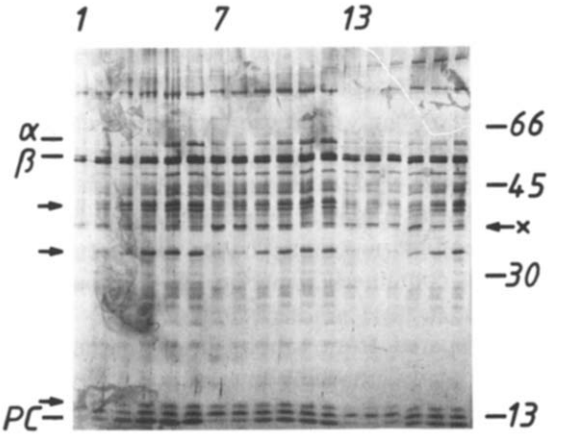

Fig. 3. Gel electrophoretic analysis of proteins released from the membranes after freezing to −20°C. The washing medium contained 10 mM NaCl. The positions of molecular weight standards are indicated. Molecular mass is given in kDa. PC indicates the position of plastocyanin and α and β those of subunits of coupling factor CF1. All samples contained 5 mM NaCl. The arrows mark bands that increase with decreasing osmolality of the suspending media. X is discussed in the text. Lanes 1–6, dimethyl sulfoxide; lanes 7–12, glycerol; lanes 13–18, sucrose. Concentrations: lanes 1, 7 and 13, 500 mM; lanes 2, 8 and 14, 250 mM; lanes 3, 9 and 15, 100 mM; lanes 4, 10 and 16, 50 mM; lanes 5, 11 and 17, 20 mM; lanes 6, 12 and 18, 10 mM.

slopes in Fig. 2, since it was comparable in the different samples of which the assay produced the data in Fig. 2.

To gain more insight into the process of freezing damage at low osmolalities, thylakoid membranes were frozen in the presence of 5 mM NaCl and increasing concentrations of the cryoprotectants sucrose, glycerol and dimethyl sulfoxide. The gel electrophoretic analysis of the proteins released during freezing (Fig. 3) shows that the pattern of protein release was very similar in all three cases. A prominent band was the β subunit of the coupling factor CF1. However, loss of CF1 from the membranes was comparable at different osmolalities of the suspending medium. It could not account for the differences in photosynthetic phosphorylation measured after freezing. There was an increase in the intensity of bands of several polypeptides as initial osmolalities decreased. One of these polypeptides had the same electrophoretic mobility as plastocyanin, the others have not been identified. Only one of the unidentified bands increased with increasing osmolalities, as has been described before [5]. This effect was particularly marked when glycerol was used as a cryoprotectant. Apparently, glycerol is capable of solubilizing a membrane-bound protein, which had no detectable effects on cyclic photophosphorylation.

Under the conditions of this experiment, chemical damage to peripheral membrane proteins is negligible [5,6]. But a comparison of the liberation of plastocyanin during freezing with the phosphorylation activity of the membranes after freezing revealed a linear correlation between loss of plastocyanin and loss of cyclic photophosphorylation (Fig. 4). Release of plastocyanin from the intrathylakoid space requires an opening of the membranes. Thus the results may be explained in different ways. Either loss of plastocyanin (or other polypeptides) inhibits cyclic electron flow, a prerequisite of light-dependent phosphorylation, or the membranes become leaky during freezing and can no longer maintain the proton gradient and electrical potential difference which are built up during electron transport and provide energy for photophosphorylation.

To distinguish between these two possibilities, we froze thylakoid membranes in the presence of varying concentrations of purified plastocyanin

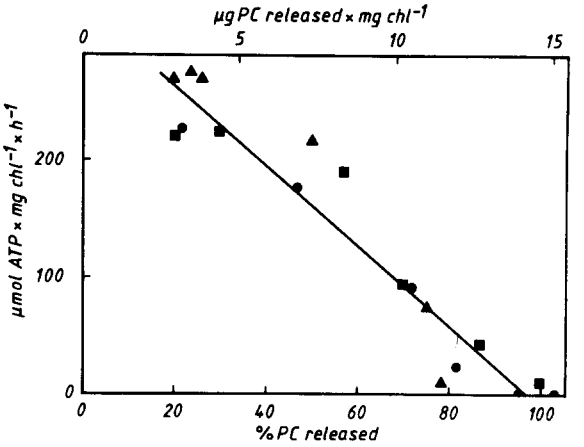

Fig. 4. Correlation of the rate of cyclic photophosphorylation with the amount of plastocyanin released from the membranes during a freeze-thaw cycle. The samples were the same as in Fig. 3 containing sucrose (▲), glycerol (■), dimethyl sulfoxide (●).

(Fig. 5). It has been shown [21] that the addition of plastocyanin to the suspending medium prevents damage to cyclic photophosphorylation by sonic treatment and thylakoid membranes could be partially protected from damage when they

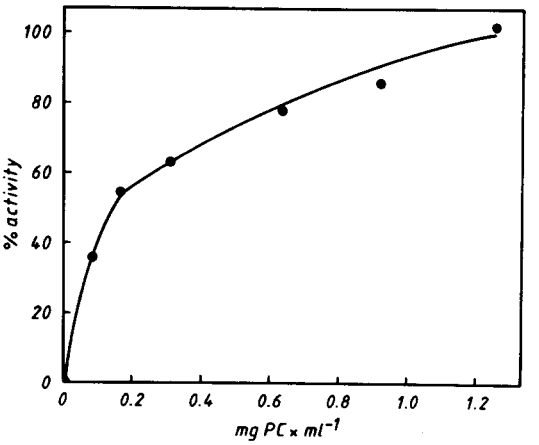

Fig. 5. The retention of cyclic photophosphorylation during a freeze-thaw cycle as a function of the amount of plastocyanin (PC) added to the samples prior to freezing. The washing medium contained 10 mM sucrose. Sample size was 0.4 ml. All samples contained 10 mM NaCl and 20 mM sucrose. For better comparison, the activity of the membranes is given as percentage of a sample frozen in the presence of 500 mM sucrose which yielded maximum cryoprotection (110 μmol ATP/mg chl per h). Plastocyanin was taken from a peak fraction of the G-50F column (see Materials and Methods) and was dialysed for 20 h against 40 mM NaCl prior to use.

were frozen under conditions which were similar to those applied here [2,23]. Fig. 5 shows that it is possible to protect thylakoid membranes from mechanical freezing damage by adding sufficient amounts of plastocyanin. About 1.2 mg plastocyanin/ml (approx. 120 μM) protect cyclic photophosphorylation to the same extent as the addition of 500 mM sucrose. This indicates that it is the loss of plastocyanin by which photophosphorylation in inactivated by a freeze-thaw cycle at low initial osmolalities and that the membrane vesicles reseal after they have lost the protein from their lumen, regaining a low permeability to protons. The same concentration of plastocyanin had no effect on the photophosphorylation rates of thylakoids stored for the same time at ice-bath temperature.

It has been shown [24] that plastocyanin is necessary as an electron carrier in cyclic photophosphorylation with phenazine methosulfate as a cofactor only as long as the concentration of phenazine methosulfate is low. At high concentrations, phenazine methosulfate can donate electrons directly into the Photosystem I reaction center P700. The conclusion that freezing of thylakoids suspended in media of low osmolality inactivates photophosphorylation by causing transient membrane rupture which permits the escape of plastocyanin is supported by the experiment shown in Fig. 6. Photophosphorylation of thylakoids inactivated by freezing in a medium containing 5 mM NaCl and 10 mM sucrose can be reestablished by increasing the concentration of phenazine methosulfate in the test assay.

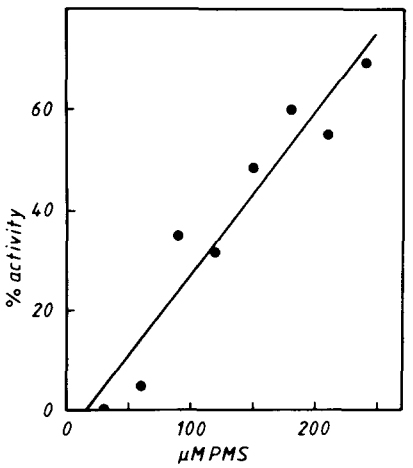

Fig. 6. Dependence of the rate of cyclic photophosphorylation on the concentration of phenazine methosulfate (PMS) present during illumination. Samples were frozen for 3 h to −20°C in the presence of 5 mM NaCl and 10 mM sucrose or stored for the same time at ice-bath temperature. The washing medium contained 10 mM NaCl. Activity is given as the percentage of photophosphorylation reached by frozen samples as compared to controls at the same phenazine methosulfate concentration. The line was fitted to the data using linear regression analysis ($r = 0.96$).

Discussion

When thylakoids were frozen in media containing sucrose and NaCl at a constant molar ratio but in different concentrations, plastocyanin is preferentially released at low initial osmolalities and CF1 at high initial osmolalities (Fig 1b,c). This should be expected if high initial solute concentrations favor chemical membrane damage while low concentrations result in mechanical damage. Different ratios of cryoprotective (sucrose) to cryotoxic solute (NaCl) influenced the amount of released CF1, but not that of plastocyanin (Fig. 1b,c).

Not all peripheral membrane proteins responded to freezing stress in a comparable manner. This is illustrated by the reaction of ferredoxin-$NADP^+$ reductase (Fig. 1a) of which about 20% were solubilized during freezing, irrespective of the osmolality or chemical composition of the suspending media.

As the concentrations of the cryoprotectants glycerol, dimethyl sulfoxide and sucrose are decreased (Fig. 3), freezing of thylakoid suspensions liberates increasing amounts of several membrane proteins, among them the soluble electron carrier protein plastocyanin. Quantification of released protein after electrophoresis is inaccurate with the silver staining procedure used, since the staining intensity is not linearly dependent on the protein concentration. In addition, some protein (for example, the α-subunit of CF1) are only weakly stained under our condition [7]. The pattern of membrane damage revealed in this experiment and in the experiment of Fig. 1 at osmolalities up to 100 mosmolal is different from the pattern produced by the accumulation of chaotropic solutes

during freezing [5,6]. The latter promotes the dissociation of peripheral membrane proteins. Dissociation is favored by high initial solute concentrations (see Fig. 1) which increase the solvent space available for protein dissociation during freezing.

The complexity of solute damage is illustrated in Fig. 2, which shows the relationship between loss of CF1 from the membranes and loss of membrane function (i.e., photophosphorylation) at different ratios of sucrose to NaCl and elevated solute levels. Solute damage during freezing is accompanied, but not fully characterized by the appearance of the coupling factor CF1 in the membrane supernatants. The concentration of other membrane components (more than 25 additional polypeptides) in the membrane supernatants is increased with increasing solute damage. Is should be noted that removal of only a fraction of CF1 from the membranes by EDTA can result in the complete inactivation of photophosphorylation owing to the opening of proton channels (see Ref. 25 for a review). It has been shown [2,23] that the light-dependent proton uptake of thylakoids can be partly reestablished after freeze-induced loss of CF1 by the addition of DCCD to the membranes after freezing. DCCD has been shown to specifically close the proton channels opened by the loss of CF1 [25]. CF1, which is attached to the thylakoids and protrudes into the suspending medium, has long been known to be cold-labile [26], and cold lability is enhanced in the presence of chaotropic agents [27].

Plastocyanin is an intermediate electron carrier which donates electrons to the reaction center of Photosystem I (P700) and is localized in the thylakoid lumen [22]. Its liberation into the suspending medium (Fig. 4) during freezing requires thylakoid rupture. The experiments of Figs. 5 and 6 show that rupture is transient and followed by resealing which reestablishes the permeability characteristics of thylakoids necessary for photophosphorylation. In contrast, chaotropic membrane damage is characterized by irreversible alterations of the permeability properties of the thylakoids so that ion gradients can no longer be maintained [28] and photophosphorylation is permanently lost.

Transient membrane rupture resulting in the liberation of soluble proteins of the intrathylakoid space may be occasioned by the hypertonic stress produced during freezing as has been suggested before [29], or by the subsequent solute dilution during thawing. Dilution could lead to membrane rupture if osmotically active particles were trapped in the intrathylakoid space and would then lead to excessive swelling of the vesicles upon thawing. It has been shown recently [30] that thylakoids take up normally non-penetrating substances under the extremely hypertonic conditions during freezing. After thawing, these vesicles regained their full osmotic responsiveness. Two of the cryoprotectants used in the experiment of Figs. 3 and 4 (glycerol, dimethyl sulfoxide) are known to penetrate biomembranes, whereas the permeability of the thylakoids to sucrose and NaCl is low under non-freezing conditions [31].

In consequence, the concentration of dimethyl sulfoxide and glycerol will, during slow freezing, be comparable on both sides of the membranes, whereas sucrose will accumulate together with NaCl outside the thylakoids. Osmotic stress on the membrane vesicles will therefore be larger with sucrose than with the other cryoprotectants used. However, liberation of plastocyanin was less significant at comparable osmolalities with sucrose than with glycerol or dimethyl sulfoxide (Fig. 4). This may be due to the fact that more of the penetrating agents than of the normally non-penetrating sucrose had entered the intrathylakoid space. Then the membranes would have ruptured during thawing when glycerol and dimethyl sulfoxide which had accumulated in the thylakoid lumen were not released rapidly enough to avoid excessive thylakoid swelling.

When loss of plastocyanin was responsible for thylakoid inactivation during freezing, the membranes could be protected against loss of function by the addition of plastocyanin to the suspending medium prior to freezing (Fig. 5). The extent of protection given by 120 μM plastocyanin was comparable to that afforded by 500 mM sucrose. Obviously, protection by plastocyanin is not of a colligative nature. Rather, transient opening of the membranes during freezing or thawing no longer resulted in the net loss of plastocyanin from the intrathylakoid space.

It has previously been reported that several low

molecular weight proteins isolated from frost-hardy leaf material provided non-colligative protection to thylakoids during freezing in vitro [32,33].

The results presented here provide a model system to monitor freezing damage and to correlate physiological parameters with events on the molecular level, using specific antibodies as probes. In vitro, two different mechanisms of freeze-thaw injury could be distinguished by the release of the marker proteins CF1 and plastocyanin. It is now possible to study the release of these proteins and thus the relative contributions of mechanical and chemical freezing stress on thylakoid membranes in vivo. These experiments are currently under way in our laboratory.

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