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LOSS OF MEMBRANE PROTEINS FROM THYLAKOIDS DURING FREEZING

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Slow freezing of thylakoids (about 0.5 K/min) in media containing an excess of cryotoxic solutes results in the release of membrane proteins and in the rapid inactivation of photophosphorylation and other processes dependent on structural intactness of membranes. Although loss of photophosphorylation was observed before protein release became extensive, both processes were dependent on the ratio of cryotoxic to cryoprotective solutes in the membrane suspensions. Moreover, inactivation of photophosphorylation and protein release during freezing were similarly influenced by different cryotoxic solutes. Cryotoxicity of different anions increased with increasing Stoke's law hydrated anion radius (I > Br > Cl > F). Divalent cations were more cryotoxic than monovalent alkali cations. Cryotoxicity thus followed a Hofmeister lyotropic power series. Two opposing effects of low temperature influenced protein release. Lowering the temperature increased accumulation of cryotoxic solutes in the membrane vicinity which promoted protein dissociation. However, rates of protein dissociation increased with increasing temperature. While the freezing temperature unequivocally determined the concentration of solutes in a solution coexisting with ice, both the extent of membrane inactivation and protein release depended on the initial concentration of solutes and on membrane concentration. Apparently, the volumes of the unfrozen hydrophilic phase and of the membranes are also important factors in freezing injury. The polypeptide pattern of released proteins differed drastically from that of thylakoids, but the same main proteins were released during freezing of membranes in the presence of different cryotoxic solutes. More than 35 polypeptide bands were observed in SDS-polyacrylamide gel electrophoretograms of released proteins. While the amount of protein released differed depending on freezing conditions and the composition of the suspending medium, more than 10% of the total membrane protein could be solubilized during freezing in the presence of sodium bromide or sodium iodide. Protein release during freezing is thought to be caused primarily by a suppression of intramembrane electrostatic interactions and ion competition although solute effects on water structure may also play a role.

Introduction

Cell damage by freezing is thought to be caused primarily by damage to cellular membranes [1]. Initially, formation of ice crystals which during slow physiological freezing occurs extracellularly was assumed to be mechanically disruptive [2,3]. It is now recognized that the increase in the concentration of intra- or extracellular solutes that is brought about by ice formation is a major factor in freezing injury [4,5]. There is the question of how solutes act on membranes and what the nature of the damage is. In our laboratory we have used thylakoid membranes to investigate these problems in vitro [5–7]. Thylakoids are particularly suitable for such studies, as they are biochemically very active; and their composition is known in some detail [8,9]. During freezing in the presence of a salt such as NaCl, the membranes are inactivated. Inactivation is indicated by the loss of

photophosphorylation, by alterations in the rate of electron transport (both stimulation and inactivation, depending on stress conditions and the type of electron transport reaction), by an increase in membrane permeability and by release of membrane proteins [5,6,10–13,21,29,31]. The most sensitive parameter of membrane inactivation in vitro appeared to be the loss of photophosphorylation and, related to it, an increase in H^+ permeability. Biological membranes are composed of lipids and proteins. Electron spin resonance investigations with spin-labelled thylakoids, while revealing an increase in lipid mobility during membrane inactivation, failed to show significant differences in the spectra of intact and inactivated membranes when measurements were performed under hypotonic conditions at room temperature [7]. Morris [14] concluded from studies with liposomes subjected to a freeze-thaw stress that lipid bilayers while rupturing during the stress cycle were capable of resealing on returning to normal conditions. Freeze-induced alterations of the lipid phase of membranes thus appear to be reversible, while loss of membrane activity brought about by freezing is irreversible. This focuses attention on the protein moiety of biomembranes. In previous work it was observed that membrane inactivation as indicated by the loss of photophosphorylation occurred before much release of protein from the membranes could be detected [6]. At first sight, this might suggest that loss of proteins is a consequence, not a cause of membrane inactivation. However, the possibility remains that crucial proteins are lost before the main part is released. Alternatively, interactions between membrane components might be altered disturbing membrane structure and leading to membrane inactivation before protein release becomes significant. In this work, we further investigate protein release during freezing and its relationship to membrane inactivation.

Materials and Methods

Plant material. Spinacia oleracea cv. Yates or Monatol was grown in the greenhouse (10 h light, 14 h darkness) or in the garden.

Isolation of thylakoids. The washed leaves were blended in the cold in a medium containing 260 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 1 mM KH_2PO_4, 50 mM Tris, 1 mM sodium ascorbate, 1 mM cysteine, pH 7.8. The homogenate was filtered through 20 μm nylon mesh and centrifuged for 1 min at $2000 \times g$. The chloroplasts were resuspended and washed three times in a solution containing 50 mM sorbitol, 5 mM NaCl, 5 mM $MgCl_2$, 2.5 mM KH_2PO_4, adjusted to pH 7.8, at $47\,000 \times g$ for 5 min. The final pellet was resuspended in washing medium. The final chlorophyll concentration was about 3 mg/ml.

Freezing of thylakoids. Salts or sorbitol were added to the thylakoids from concentrated stock solutions. The samples were frozen to $-22°C$. The initial cooling rate was approx. 0.5 K/min. After 3 h (other times are noted in the figures) the samples were rapidly thawed in a waterbath at room temperature. For the analysis of protein release, the samples were centrifuged for 30 min at $47\,000 \times g$. This proved to be sufficient for membrane sedimentation. Sedimentation for 3 h at $100\,000 \times g$ produced identical results. The colorless supernatant was used for protein determination and gel electrophoresis.

Cyclic photophosphorylation. Thylakoids were illuminated with red light ($700\ W \cdot m^{-2}$) in a reaction medium consisting of 25 mM NaCl, 5 mM $MgCl_2$, 2.5 mM KH_2PO_4, 0.4 mM ADP and 15 μM phenazine methosulfate, pH 7.8. The irreversible, light-dependent alkalization of the medium, a consequence of the reaction $ADP^{3-} + HPO_4^{2-} \rightarrow ATP^{4-} + OH^-$, was recorded by a pH electrode [6]. Small aliquots of 0.01 M HCl were used to calibrate the observed pH change.

Electron transport. Oxygen evolution in saturating red light ($400\ W \cdot m^{-2}$) was measured polarographically with ferricyanide as electron acceptor. Basal rates were obtained in the absence of ADP and uncoupled rates in the presence of 10 mM NH_4Cl. The reaction medium contained 50 mM KCl, 5 mM HPO_4^{2-}, 5 mM $MgCl_2$, 20 mM Tricine/KOH, pH 7.6, 2 mM ferricyanide.

Protein determination. Protein was determined spectrophotometrically according to Warburg and Christian [17] or by the procedure of Lowry et al. [18]. Both methods yielded comparable results.

Gel electrophresis. Gel electrophoresis was performed as described previously [16]. In some experiments linear pore gradient gels from 10–18% monomer concentration with reduced catalyst con-

centrations were used. Thylakoid pellets were resuspended in sample buffer, supernatants were dialyzed against sample buffer overnight.

Chlorophyll determination. Chlorophyll was determined according to Arnon [15].

Results

Fig. 1 shows that photophosphorylation of thylakoid membranes decreased rapidly during freezing. The membranes were severely damaged after only 20 min of freezing. Damage occurred faster and was more extensive when the ratio of salt to sorbitol was increased. In Fig. 2 protein loss from the membranes is shown as a function of freezing time at −14 and −16°C. The solute composition of the thylakoid suspension and initial NaCl concentration were chosen so as to give comparable NaCl concentrations in the unfrozen suspension coexisting with ice at the different temperatures. The expectation that protein loss was comparable under these conditions was not fulfilled. Rather, it was decreased at the lower temperature. After 3 h freezing time, about 3.5% of the total membrane protein was released at −16.5°C and about 5% at −14°C.

The data of Fig. 2 may be interpreted to show either the temperature-dependence of protein release from the membranes at the very high ionic strength produced by the removal of water as ice,

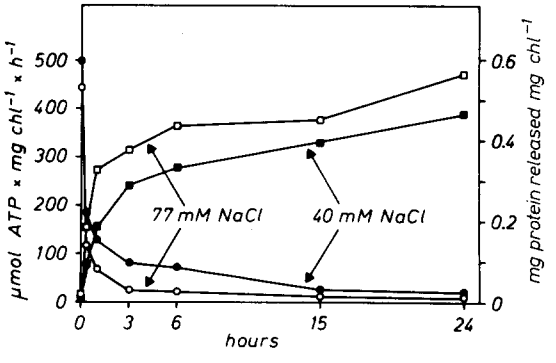


Fig. 1. Time course of protein release from thylakoid membranes and of inactivation of cyclic photophosphorylation during freezing at −22°C. The samples contained 50 mM sorbitol which is a cryoprotectant, 5 mM $MgCl_2$, 2.5 mM KH_2PO_4, pH 7.8 and NaCl as shown in the figure. Circles indicate photophosphorylation, squares protein release.

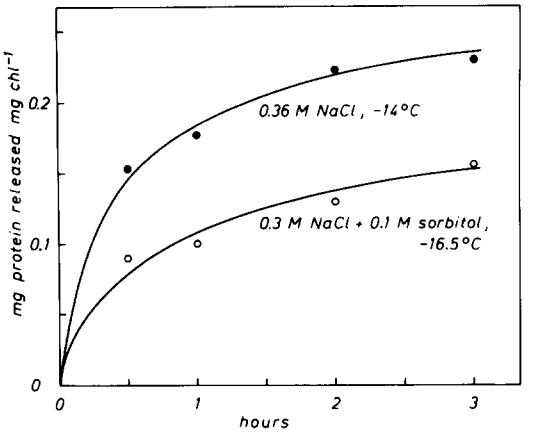


Fig. 2. Protein release from thylakoids during freezing at −14°C and −16.5°C. Solute concentrations were adjusted so as to yield comparable NaCl concentrations in the unfrozen thylakoid suspension coexisting with ice at the different freezing temperatures.

or partial suppression of protein release by the cryoprotectant sorbitol, or both. It was therefore of interest to see how protein release was affected by temperature in the absence of ice formation. Thylakoids were incubated at 0, 15 and 30°C in a medium containing 0.5 M NaCl, 50 mM Tris/HCl and 2 mM $MgCl_2$, pH 7. After sedimentation of

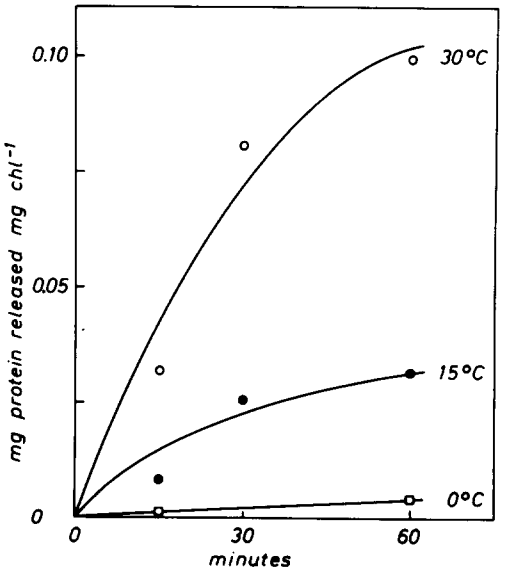


Fig. 3. Protein release as a function of time in the absence of freezing. The thylakoid suspension contained 0.5 M NaCl, 50 mM Tris and 2 mM $MgCl_2$, pH 7.6.

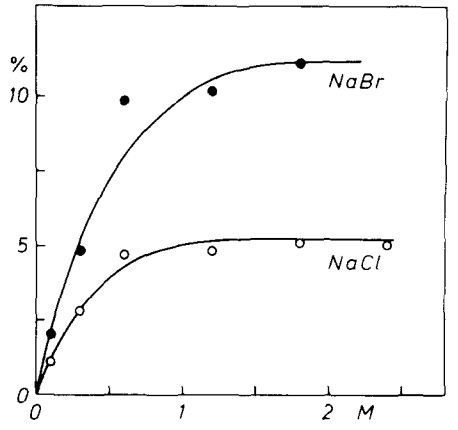
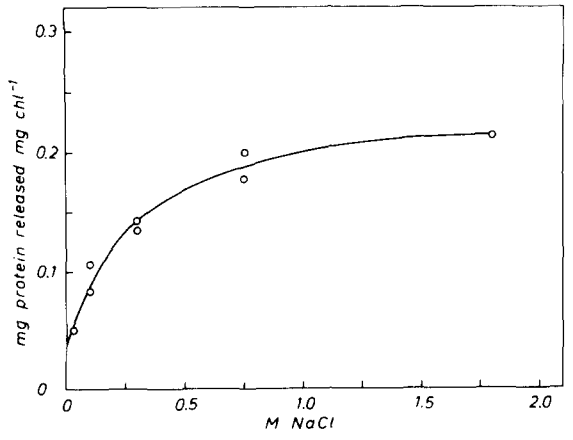


Fig. 4. Percentage of total thylakoid protein released at 30°C after 30 min in the presence of different concentrations of NaCl and NaBr.

the membranes the supernatant was analyzed for soluble protein. Fig. 3 shows that at the comparatively low ionic strength of this medium little protein was released at 0°C, more at 15°C and most at 30°C. Still, even at 30°C, only a low percentage of the total membrane protein dissociated from the membranes. 0.5 M sorbitol had no significant effect on protein release under these conditions (data not shown). Surprisingly, at 30°C loss of protein was largely saturated at a concentration of 0.5 M NaCl. It did not much increase as the NaCl concentration was increased to 2.5 M (Fig. 4). However, when NaCl was replaced by NaBr, much more protein dissociated from the membranes. More than 10% of the total protein was solubilized compared with about 4% in the presence of NaCl. Bromide is a more chaotropic anion than chloride [19].

In freezing experiments, it would at first sight be expected that solute damage to membranes is only a function of temperature and of the molar ratios of cryotoxic to cryoprotective solutes in the membrane suspensions, but not of initial solute concentrations. The final freezing temperature, not the initial solute concentration determines unequivocally the concentration of solutes in the unfrozen part of the system coexisting with ice. However, in contrast to expectation, the extent of membrane damage upon freezing was found to increase as the salt concentration of the medium used for suspending the membranes was increased. It decreased as the concentration of membranes was increased at constant solute concentration [20]. These experimental observations can be understood if membrane dissociation is taken into consideration. While initial solute concentrations do not influence the final solute concentration reached at any one freezing temperature, they de-



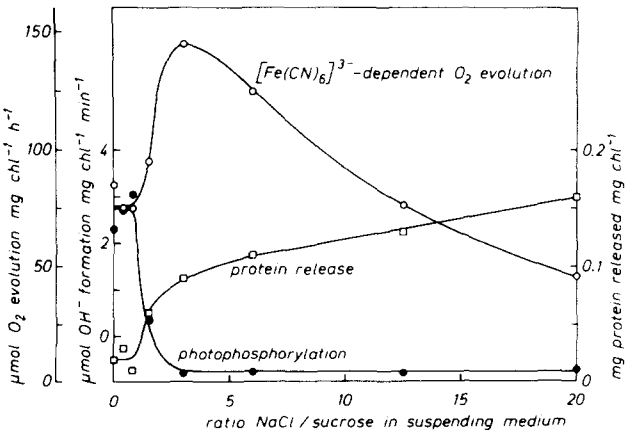


Fig. 5. Protein released from thylakoids (1 mg chlorophyll per ml) during freezing for 3 h at −18°C as a function of different initial concentrations of NaCl. Final NaCl concentrations reached in the thylakoid suspension coexisting with ice at −18°C were comparable in the different samples.

Fig. 6. Inactivation of cyclic photophosphorylation and electron transport from water to ferricyanide and protein release from thylakoids after freezing for 3 h at −22°C at different ratios of the cryoprotectant sucrose to NaCl. (In the suspending medium, sucrose was kept constant at 100 mM and NaCl was varied from 0 to 2 M).

termine the volume of the unfrozen phase in equilibrium with ice. Fig. 5 shows release of membrane proteins upon freezing of membranes for 3 h at −18°C as a function of the initial concentration of NaCl in the membrane suspensions. Protein release increased as the initial NaCl concentration of the suspending medium (or, rather, the volume of the unfrozen phase coexisting with ice at −18°C) was increased. Obviously, the volume of solution capable of dissolving hydrophobic membrane protein is an important factor in determining the extent of membrane dissociation at the high ionic strength reached during freezing. Interestingly, after thawing frozen samples and sedimenting the membranes, supernatant solutions tended to become slowly turbid particularly when NaCl concentrations were high (more than 0.1 M). Sodium dodecyl sulfate (SDS), but not NaCl, dissolved precipitates. Because these were colorless, they could not contain much lipid. Carotenoids and chlorophyll are reliable markers of lipid vesicles derived from thylakoids. Apparently, the combination of low temperature and high ionic strength favored solubilization of membrane proteins even when little solvent phase was available at subzero temperatures.

In Fig. 6, photophosphorylation, electron transport to ferricyanide and loss of membrane protein during a 3 h, −18°C freeze/thaw cycle was measured at different ratios of NaCl to sucrose. At low ratios (< 1), there was little effect on membrane function. As the ratio was increased, photophosphorylation was rapidly inactivated (between salt/sugar ratios of 1–3) together with a large increase in electron transport presumably due to the uncoupling effect (loss of the transmembrane Δ pH). Further increase in the salt/sugar ratio (5–20) led to a progressive decline in the rate of linear electron flow. Protein release followed an inverse relationship to the inactivation of photophosphorylation. At low salt/sugar ratios (< 1) little protein was released. Between ratios 1–3 there was a dramatic increase in the amount of protein released. Further increases in the salt/sugar ratio then led to further, but less significant removal of protein from the membranes. The released proteins were analyzed by gradient gel electrophoresis in the presence of SDS. Fig. 7 compares the patterns obtained at different salt to sorbitol ratios with those of non-frozen samples and a supernatant from the last washing step. The supernatant from the washing step contained mainly traces of the large and the small subunit of ribulose-1,5-bisphosphate carboxylase. The low salt control at 0°C showed increased amounts of the two carboxylase subunits as well as at least three additional bands. The high salt control at 0°C contained more than 20 bands, the alpha and beta subunits of the chloroplast coupling factor (slightly slower in migration than the large subunit of the carboxylase) being the most prominent ones. The thylakoids protected during freezing by 460 mM sorbitol had lost less protein than the thylakoids suspended at 0°C in 270 mM NaCl. Particularly the large coupling factor subunits were barely perceptible in the sorbitol-protected sample. With increasing NaCl to sorbitol ratios the polypeptide bands increased in intensity and new bands showed up. At the highest salt concentration more than 35 bands were observed. No salt dependent changes could however be seen in the thylakoid membrane pattern. This is not surprising when one considers that under high NaCl to sorbitol ratios only about 4–5% of the total membrane proteins were released. Polypeptides released by freezing which have no direct counterpart in the thylakoid membrane pattern are considered to be minor thylakoid proteins which cannot be found in the normal thylakoid patterns because of loading limitations of the gels. It is, however, not excluded that some of them represent proteolytic artifacts, although this possibility seems less likely than the first explanation because of the overall sharpness of the bands.

In Fig. 4 it has been shown that more protein is released at 30°C from the membranes in the presence of NaBr than in the presence of NaCl. In freezing experiments, membrane damage was also critically dependent on the nature of the solutes present. When the cryotoxicity of different anions was compared using photophosphorylation as a measure of membrane intactness, fluoride was the least toxic of the halogenides, and membrane damage increased in that order: fluoride, chloride, bromide, iodide [5]. Thus, membranes are damaged by freezing in the presence of different halogenides according to a Hofmeister lyotropic power series [30]. Fig. 8 shows that protein release by freezing

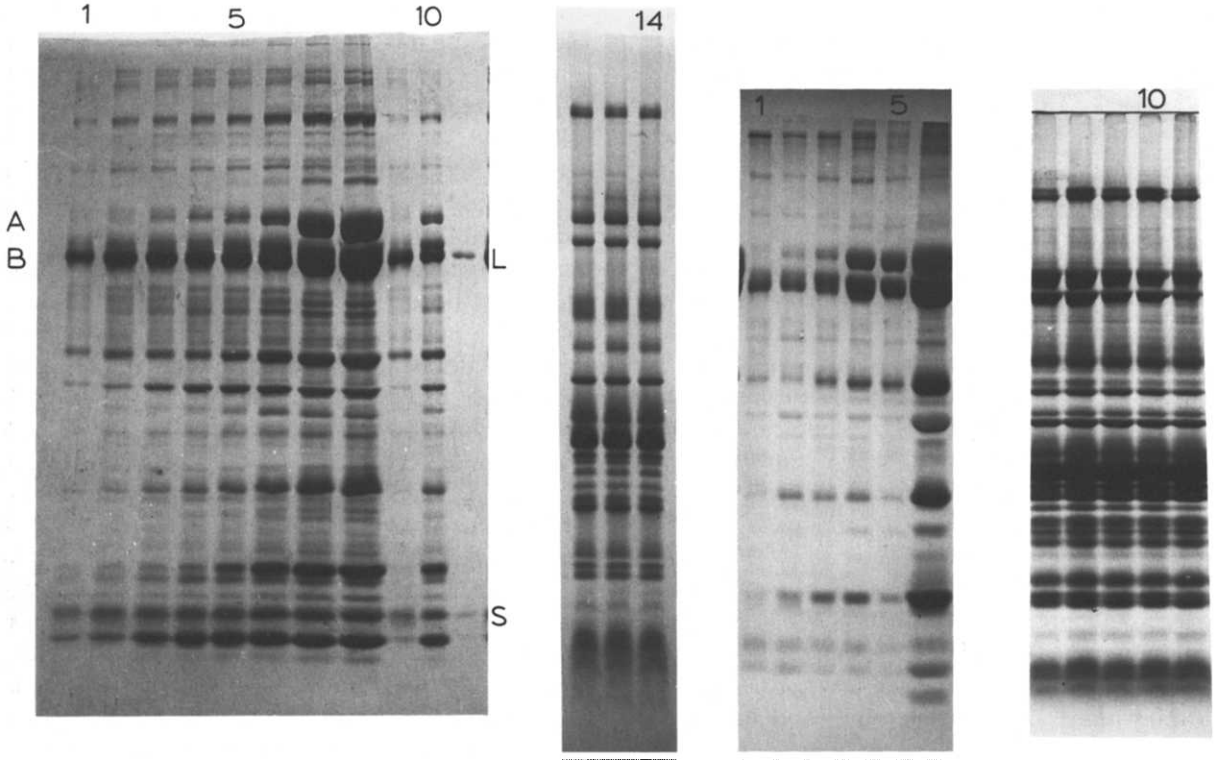


Fig. 7. Gel electrophoretic analysis of the proteins released from thylakoids during freezing at different ratios of cryotoxic to cryoprotective solutes. Lanes 1–10: supernatants; lane 11: supernatant from the last washing of the thylakoids; lanes 12–14: thylakoids.
Conditions for the different lanes:

tLane	1	2	3	4	5	6	7	8	9	10	11	12	13	14
NaCl (mM)	5	5	5	5	5	46	137	273	45	273	–	5	5	273
Sorbitol (mM)	460	280	140	60	50	50	50	50	50	50	–	460	50	50
Temp. (°C)	−20	−20	−20	−20	−20	−20	−20	−20	0	0	–	−20	−20	−20

A, B: Alpha and beta subunits of coupling factor; L, S: Large and small subunits of ribulosebisphosphate carboxylase.

Fig. 8. Gel electrophoretic analysis of the proteins released from thylakoids during freezing in the presence of different anions. Lanes 1–6: supernatants; lanes 7–11: thylakoids. The samples contained 45 mM sodium salt. Lane 1: control containing 180 mM sorbitol; lanes 2 and 7: fluoride; lanes 3 and 8: chloride; lanes 4 and 9: nitrate; lanes 5 and 10: bromide; lanes 6 and 11: iodide.

followed a very similar pattern. After freezing of thylakoids in the presence of iodide protein loss was particularly pronounced. While it was generally difficult to detect protein loss in polypeptide patterns of thylakoids, and supernatants had to be analyzed for the appearance of soluble proteins, in samples frozen in the presence of NaI protein loss was even apparent in the thylakoid pattern. The intensity of bands from supernatants increased with increasing cryotoxicity of the anions, and new bands appeared as the intensity of the more common bands intensified (Fig. 8). Protein release in the presence of nitrate was intermediate between that induced by bromide and iodide.

When cations were varied (with the anion kept constant), loss of photophosphorylation by freezing and release of membrane proteins were also closely related to one another (data not shown). In particular, the monovalent cations of the alkali metals were less cryotoxic than divalent cations

[5]. As should be expected from the anion data, less protein was released from thylakoids during freezing in the presence of monovalent than in the presence of divalent cations.

Discussion

Biomembranes contain as their main components lipids and proteins in variable proportion. After freezing in suitable ionic environments, membrane inactivation is observed. In vivo, it may lead to cell death. In vitro, inactivation can be monitored by changes in membrane permeability characteristics [11]. The question of how freezing alters biomembranes is unresolved. Changes in the lipid and in the protein phase need to be considered. The available evidence [7,14] indicates that damage to the lipid phase cannot account for the observed irreversible thylakoid inactivation during freezing, although osmotic stress during freezing and thawing may result in transient membrane rupture which is followed by resealing. This focuses attention on the protein moiety. Indeed, proteins have been observed to be released from thylakoids on freezing that led to membrane damage [6,21]. The question remains whether protein loss is only a corollary of membrane damage. We have observed in this investigation that protein dissociation induced by freezing in the presence of various anions and cations exhibits a pattern of sensitivity that is practically identical with that of photophosphorylation. It thus appears that freezing damage is brought about by effects on membrane proteins that result first in changes of membrane permeability and finally in protein loss. Permeability changes in turn cause inactivation of photophosphorylation in vitro before electron transport is affected by loss or alteration of proteins engaged in the catalysis of electron transport. Dissociation of coupling factor protein from the membranes also contributes to loss of photophosphorylation, but appears to be less important than other freezing-induced lesions that precede coupling factor release [29]. In contrast to the situation in vitro, inactivation of chloroplast electron transport and photophosphorylation during lethal freezing of leaves appear to be almost simultaneous events [22,23].

The polypeptide pattern of released proteins was very different from the overall thylakoid pattern. This is to be expected since intrinsic membrane proteins are too hydrophobic to enter the hydrophilic phase even if the protein arrangement within the membranes is disturbed by freezing. Polypeptides identified by SDS gel electrophoresis in the supernatants of thylakoids were the coupling factor subunits, the subunits of the ribulosebisphosphate carboxylase (cf. Fig. 7), plastocyanin and NADP reductase [6], all of them extrinsic proteins. While the amount of proteins released during freezing differed dramatically depending on temperature, ratios of cryoprotective to potentially cryotoxic solutes and the chemical nature of the latter, the uniformity of the pattern of released proteins was remarkable (Figs. 7 and 8).

There is the question of how freezing caused protein detachment. The experiments of Figs. 2 and 3 suggest that interference with hydrophobic stabilization of the membranes is less significant than suppression of electrostatic interactions, because protein loss was affected by electrolytes and increased with increasing temperature. Freezing was effective in bringing about membrane disintegration mainly by concentrating ions in the membrane vicinity. By suppressing intramembrane electrostatic interactions, this is thought to cause protein release. As should be expected, anions of a small Stokes law hydrated ion radius were more effective than large hydrated species in releasing protein. Divalent cations were more effective than monovalent cations. Protein release thus followed a Hofmeister lyotropic power series [6]. The same is true for the inactivation of photophosphorylation [5]. Differential effects of cations and anions on membrane inactivation have been discussed previously with the main emphasis on anion action [5]. While we favor the view that protein dissociation is brought about by ion competition, effects of salts on water structure may also play a role in membrane destabilization during freezing [19]. Highly structured water is thought to stabilize membranes by excluding hydrophobic membrane components from the aqueous phase. Chaotropic reagents decrease water structure, particularly when their concentration is increased by freezing. This increases the solubility of hydrophobic solutes and may permit protein release. The

Hofmeister lyotropic power series reflects the order of chaotropicity of salts and is a measure of their capability to disturb water structure.

Freezing acts mainly on a dehydration basis, and low temperatures actually have two opposing effects: The increase in ionic strength in the membrane vicinity destabilizes membranes, and low temperatures decrease the rate of protein dissociation contributing to stabilization. Sensitivity to high concentrations of small hydrated ions seems to be an inbuilt characteristic of biomembranes which are stabilized in part by electrostatic interactions. This raises the question of how such membranes are maintained in a native state during freezing or desiccation of cells. Cryoprotectants are known to stabilize membranes during freezing, and colligative protection by low molecular weight cryoprotectants is understood in principle [24–26]. Protection by high molecular weight compounds is less well understood [5,27,28]. How the biomembrane systems of seeds and desiccation-resistant lichens, mosses and other plants withstand the effects of dramatically increased solute levels at elevated temperatures under drought or under freezing conditions is still a mystery that waits to be resolved.

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