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LOSS OF FUNCTION OF BIOMEMBRANES AND SOLUBILIZATION OF MEMBRANE PROTEINS DURING FREEZING

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Summary

Isolated thylakoid membranes are damaged during freezing in dilute salt solutions, as shown by the inactivation of photochemical thylakoid reactions. After freezing, a number of membrane proteins were found in the particle-free supernatant. Up to 5% of the total membrane protein was solubilized by freezing, and the pattern of released proteins as seen in sodium dodecyl sulfate gel electrophoretograms was influenced by the nature of the solutes present. Membranes protected by sucrose did not release much protein during freezing. Concentrated salt solutions caused protein release also in the absence of freezing. Among the proteins released were ferredoxin—$NADP^+$ reductase, plastocyanin and coupling factor CF_1. Subunits of CF_1 were found in different proportions in the supernatants of thylakoid suspensions after freezing in the presence of different salts. Cyclic photophosphorylation was largely inactivated before significant protein release could be detected.

It is suggested that protein release is the final consequence of the non-specific suppression of intramembrane ionic interactions by the high ionic strength created in the vicinity of the membranes by the accumulation of salts during slow freezing. Salt effects on water structure and alterations of nonpolar membrane interactions by the incorporation of (protonated) lipophilic anions from organic salts into the membrane phase during freezing may also be involved.

Introduction

Thylakoid membranes isolated from frost-killed leaves of spinach no longer exhibit normal membrane functions [1]. In contrast, thylaskoids from unfrozen tissue or from frozen hardy leaves show, among other activities, high rates of cyclic photophosphorylation. During illumination, ion gradients are formed

across the membranes [2].However, when the thylakoids are frozen in vitro in the absence of a cryoprotectant such as sucrose, photophosphorylation is inactivated [3] and ion gradients can no longer be set up across the membranes [4,5].Osmotic properties are also lost; before freezing, solutes such as sucrose or NaCl cause osmotic shrinkage of membrane vesicles, but this is no longer observed after freezing [4]. All membrane functions survive freezing undamaged in the presence of a sufficient concentration of a cryoprotective solute such as sucrose. However, protection by sucrose can be abolished by many salts, which thus act as cryotoxic solutes [3,5—7].The extent of membrane damage during freezing is a function of the ratio between cryotoxic and cryoprotective solutes, the freezing time and the freezing temperature [8—10]. Little is known of the cause of the breakdown of membrane functions during freezing. Garber and Steponkus [5] have reported that the coupling factor CF_1 is lost from thylakoids during freezing. However, this alone cannot be responsible for freezing injury, since membranes depleted of coupling factor were inactivated by freezing and could not be reconstituted by native coupling factor [4]. It has been suggested that SH-group oxidation causes protein denaturation during freezing [11]. However, no SH-group oxidation was found during inactivation of thylakoids and thiols cannot prevent membrane inactivation during freezing [3]. In this communication, we report on the release of membrane proteins during freezing.

Material and Methods

1. Isolation of thylakoids

Chloroplasts were isolated from field or greenhouse spinach in a medium containing 50 mM Tris, 350 mM NaCl, 10 mM P_i (potassium salt), 10 mM sodium ascorbate, 3.3 mM cysteine and 40 mM mercaptoethanol, pH 8 [12]. Thylakoids were released from the chloroplasts by osmotic shock and were washed twice in water by sedimenting them at 25 000 and 40 000 $\times g$ and resuspending the pellet in water. This removed most stroma proteins and reduced the NaCl concentration to approx. 10 mM. The chlorophyll content was determined according to Arnon [13].

2. Freezing

Aliquots of a suspension of thylakoids containing approx. 500 μg chlorophyll/ml were added to equal volumes of solutions containing NaCl, KBr, sodium phenylpyruvate, sodium caprylate or isoleucine. In some experiments sucrose was added in different concentrations as a cryoprotectant. The samples were frozen for 3 h at $-20°C$. The rate of freezing was 4°C per min or slower. Mechanical membrane destruction by the formation of intravesicular ice does not occur under these conditions as long as the final freezing temperature remains above the eutectic temperature. Controls were kept at 0°C.

3. Measurements

a. Cyclic photophosphorylation. After rapid thawing, photophosphorylation was measured as phosphate uptake in the presence of phenazine methosulfate

as described previously [3]. Phosphate was determined according to Fiske and SubbaRow [14].

b. Electron transport. Rates of light-dependent ferricyanide and NADP reduction were recorded photometrically at 400 and 340 nm using a cross-beam illumination system. Saturating red actinic light was produced by a water filter, a red cutoff filter RG 630 from Schott, Mainz, and a Calflex C heat absorbing filter from Balzers, Liechtenstein. The photomultiplier was protected against actinic light by Corning filters 9782 and 5030 or a 340 nm interference filter. The reaction medium for ferricyanide reduction contained 50 mM Tris, 1.5 mM potassium phosphate, 1.8 mM ADP, 3.3 mM $MgCl_2$ and 0.5 mM $K_3Fe(CN)_6$, pH 7.8. NADP reduction was measured in a medium containing, at pH 7.6, 20 mM Tris, 40 mM NaCl, 2.6 mM potassium phosphate, 2.6 mM ADP, 4.5 mM $MgCl_2$, 1 mM NADP and ferredoxin (70 μg protein/ml of a preparation isolated according to Buchanan and Arnon [15]). Rates of photosystem-I-dependent electron flow from ascorbate to methyl viologen were determined polarographically. The reaction medium contained 50 mM Tricine (*N*-tris(hydroxymethyl)methylglycine), 5 mM $MgCl_2$, 3 mM $NaHCO_3$, 5 mM sodium ascorbate, 40 μM dichlorophenolindophenol, 0.4 mM methyl viologen and 1 mM NaN_3, pH 7.6.

4. Gel electrophoresis

To separate proteins which were released from the thylakoids during freezing, thylakoid suspensions were centrifuged after thawing at 60 000 $\times g$ for 2 or 3 h. The supernatants were heated to 90°C for 2 min in the presence of 1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol. The electrophoretic separation of the resulting polypeptides was performed in SDS gel (7.5%) according to Weber and Osborn [16]. The electrode buffer was continuously exchanged. It contained 0.025 M phosphate, pH 7.1, and 0.1% SDS. Coomassie Brilliant Blue R 250 was used to stain the gels [17]. Occasionally the proteins in the supernatants were separated directly without denaturing them in the presence of SDS and mercaptoethanol. In such cases the Tris · HCl/Tricine buffer and the gel system 1 of Maurer [18] were used for separation. Molecular weights were determined according to Weber and Osborn [16]. Reference proteins were aldolase (EC 4.1.2.13,), glyceraldehydephosphate dehydrogenase (EC 1.2.1.12), glutamate dehydrogenase (EC 1.4.1.3) hemoglobin after Anson, pyruvate kinase (EC 2.7.1.40) and trypsin inhibitor from soybeans, all from Boehringer, Mannheim. Cytochrome *c*, ovalbumin and bovine albumin were obtained from Serva, Heidelberg. Protein was determined according to Lowry et al. [19] as described by Layne [20].

5. Immunization of rabbits and immunoassays

As described in detail elsewhere [21], 4.6 mg protein, which was released from thylakoids during freezing in the presence of NaCl, was mixed thoroughly with 3 ml Freund's complete adjuvant (Difco). Total volume was 5 ml. Equal portions of the mixture were injected intradermally at multiple sites into rabbits B-71 and B-72. Rabbits B-73 and B-74 were similarly treated with soluble protein obtained by freezing thylakoids in the presence of sodium caprylate. After 4 weeks, blood was withdrawn and tested for the presence of

antibodies. The obtained antisera were termed B-71-1, B-72-1, B-73-1 and B-74-1, respectively. The antigen-antibody reaction was weak enough to permit the intraveneous injection of further antigen (1.2 mg protein from the NaCl extract into the rabbits B-71 and -72, 2.9 mg protein from the caprylate extract into the rabbits B-73 and -74; the proteins were dissolved in 0.15 M NaCl). Antisera were taken 7, 9 and 12 days later. They are termed B-71-2, B-71-3, B-71-4; B-72-2 etc.

The booster injection was repeated 4 weeks later and further bleedings yielded the sera B-71-6, B-71-7, B-71-8 etc. Immunoelectrophoretic analysis with the LKB/Gelman/Camag equipment, agglutination and absorption experiments were carried out according to standard procedures (see ref. 21).

Antisera were prepared in a similar procedure against the δ-subunit of CF_1 in collaboration with G. Bonnenkamp, against ribulose bisphosphate carboxylase from rye in collaboration with J. Feierabend and G. Wildner, and against ferredoxin—$NADP^+$ reductase in collaboration with N. Nelson. The antiserum against plastocyanin was a gift by G. Hauska.

Results

Fig. 1 shows polypeptide patterns of proteins which were released from thylakoid membranes during freezing or standing at 0°C in the presence of different compounds. When the membranes were frozen in the presence of 0.2 M NaCl, eight polypeptide bands were clearly visible. Seven further bands were faint and became visible only after longer staining. They are not shown in Fig. 1. In the presence of KBr, more protein was released from the membranes

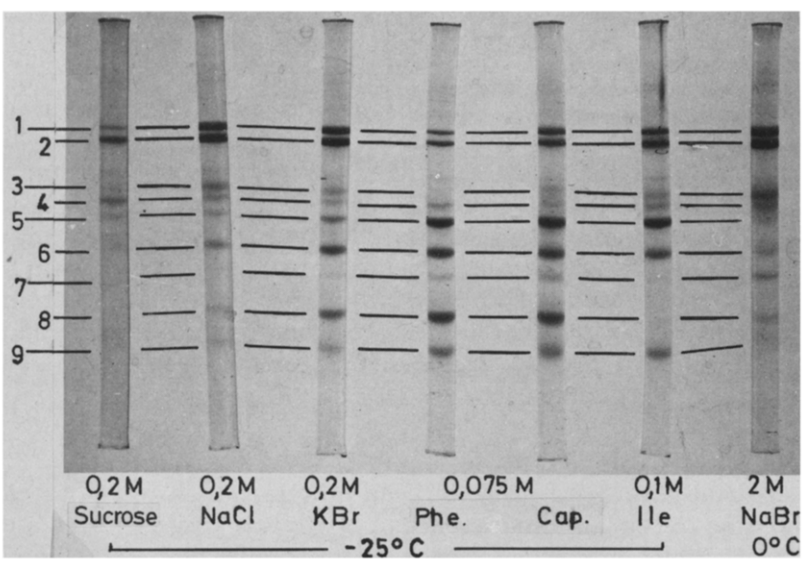

Fig. 1. SDS gel electrophoretic pattern of polypeptides from proteins which were released from thylakoids during freezing. The membranes were suspended in solutions of the composition shown in the figure and either frozen for 3 h at —25° C or kept at 0° C. After thawing and sedimentation of the membranes, the supernatants were treated with SDS and mercaptoethanol and subjected to electrophoresis. Phe, sodium phenylpyruvate; Cap, sodium caprylate; Ile, isoleucine.

during freezing than in the presence of KCl (not shown) or of NaCl. In experiments on the sensitivity of photophosphorylation to different anions it was found that membrane damage increased from fluorides to chlorides and from there to bromides and iodides [7]. The quantitative distribution of released polypeptides was different in the experiments with chloride and bromide.

During slow freezing, ice crystallizes and the concentration of the remaining solution, which contains the membranes, increases until the eutectic temperature is reached. Membrane damage occurs both above and below the eutectic temperature [10]. The observation that protein release from the membranes does not require freezing and occurs already at 0°C, if the salt concentration is sufficiently high (Fig. 1), suggests that the concentration of certain solutes rather than the temperature is the critical factor in the release of proteins from the thylakoids. Photophosphorylation has also been observed to be inactivated by high concentrations of salts at 0°C [7,10]. Freezing of thylakoids in 0.1 M NaCl for 3 h produced a pattern of released proteins which was very similar to the pattern obtained when the thylakoids were exposed to 2 M NaCl at 0°C for the same length of time (not shown). In Fig. 1, the protein release from thylakoids produced by 2 M NaBr at 0°C was more pronounced than the protein release caused by freezing in 0.2 M NaCl, while freezing in 0.2 M KBr appeared to lead to somewhat more membrane damage than simple exposure to 2 M NaBr at 0°C. According to Kamenietzky and Nelson [22], treatment with a high concentration of NaBr at 0°C results in the release of the coupling factor CF_1 from the thylakoid membranes. Due to its cold lability, the factor dissociates into subunits [23].

In the presence of a low concentration of sodium phenylpyruvate, freezing liberated more proteins from the membranes than in the presence of a higher NaCl concentration. In the phenylpyruvate experiment of Fig. 1, ten polypeptide bands are clearly visible. Minor bands were also present, but required longer staining times. The pattern of polypeptides produced by freezing with phenylpyruvate showed rather drastic quantitative and even some qualitative differences to the pattern obtained when the membranes were frozen in NaCl solution. A new band not seen in the presence of NaCl appeared between bands

TABLE I

MOLECULAR WEIGHTS OF POLYPEPTIDES FROM PROTEINS WHICH WERE RELEASED FROM THYLAKOIDS DURING FREEZING

The polypeptide numbers refer to the number in the polypeptide patterns in Fig. 1.

No.	Molecular weight	σ
1	57 100	±1100
2	53 700	±1200
3	38 300	±600
4	36 500	±200
5	33 400	±300
6	26 900	±600
7	23 000	±500
8	18 500	±300
9	15 200	±100

2 and 3. It had a molecular weight of 43 700. Another new band, M_r 20 800 is faintly visible between bands 7 and 8. The polypeptides 1 (M_r = 57 100, see Table I) and 2 (53 700) were much less prominent in the samples frozen with phenylpyruvate than in those frozen with NaCl. On the other hand, bands 5 (33 400), 6 (26 900), 8 (18 500) and 9 (15 200) were much more pronounced when the membranes were frozen in the presence of phenylpyruvate instead of NaCl.

When sodium caprylate was present during freezing, the pattern of released proteins was rather similar to the pattern seen with phenylpyruvate. However, more of the polypeptides 1 and 2 appeared in solution. Moreover, even after prolonged sedimentation of the membranes at 65 000 × g the supernatant remained slightly green, apparently because a soluble chlorophyll · protein complex was released from the membranes.

During freezing in the presence of isoleucine, less polypeptide 8 was released from the membranes than in the presence of the salts.

A comparison of the polypeptide patterns of Fig. 1 reveals that cryotoxic compounds possessing apolar side chains (isoleucine, caprylate and phenylpyruvate) released much more of the polypeptides 5 and 6 from the membranes than the inorganic salts. Effects of these compounds on apolar membrane proteins are also likely, but difficult to see in protein release experiments, since these proteins remain insoluble.

In contrast to membranes frozen in the presence of the salts and of isoleucine, membranes frozen in sucrose solution remain functional. Fig. 1 shows that little protein is released, when sucrose is present during freezing. A similar small release is seen in controls kept at 0°C in the presence of salts (0.2 M or

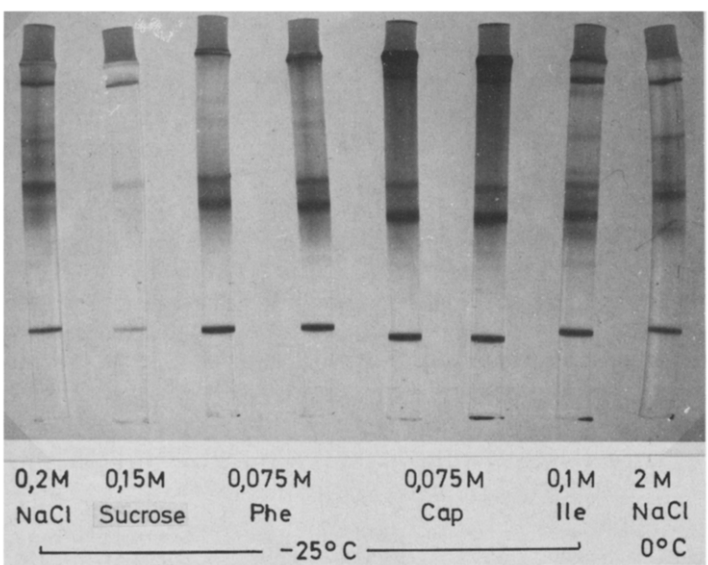

Fig. 2. Electrophoretic pattern of proteins released from thylakoids during freezing. The membranes were suspended in salt solutions as shown and either frozen at −25°C for 3 h or kept at 0°C. It should be noticed that some protein material did not enter the separation gel in the experiments with phenylpyruvate (Phe), caprylate (Cap) and isoleucine (Ile) but remained at the top of the gels.

less) or sucrose (not shown). Bands 1 and 2 constituted a considerable part of the polypeptides released in the presence of sucrose. Occasionally band 2 was seen to have two components.

The differences in the action of organic and inorganic cryotoxic compounds on thylakoid membranes during freezing are more pronounced than shown in Fig. 1, when the patterns of undenatured membrane proteins found after freezing in the supernatants are compared (Fig. 2). The total number of bands of undenatured proteins was reduced compared with the number of bands in the SDS gels, when caprylate and phenylpyruvate were used as cryotoxic compounds. Apparently due to their high molecular weight or charge (see below), part of the proteins released by freezing in the presence of the organic salts and of isoleucine did no move into the separation gel. In contrast, no, or only a little, retention of protein was found when inorganic salts or sucrose were present during freezing. Freezing in the presence of sucrose did not lead to much protein release from the membranes. In general, the reproducibility of protein patterns as shown in Figs. 1 and 2 was good. Occasionally, however, differences in the protein distribution both in SDS gels and immunoelectrophoretic analyses were observed in different experiments even if freezing conditions were comparable. Physiological differences in the membrane material used for the investigations may be the reason.

The maximum amount of protein released from frost-damaged thylakoids was approx. 5% of their total protein. The released proportion of individual proteins is unknown. More protein was usually found in the supernatants after freezing with phenylpyruvate or caprylate than after freezing in the presence of inorganic salts.

Identification of released proteins and their localization in the membrane

We wanted to know whether the soluble proteins released during freezing are detached from the outer or the inner surface of the thylakoids.

Supernatants containing membrane proteins, which were released from thylakoids by freezing, were used to produce rabbit antisera. When released proteins were electrophoretically separated and then exposed to the antisera, complex precipitation patterns appeared (Fig. 3). In several assays, a total of seven antigens was discernible in the extract obtained after freezing in the presence of NaCl, and nine antigens in the caprylate extract. Most of the antigens in both extracts were crossreacting and appear to be the same antigens. They differed in their relative proportions (Table II, a and b). Two antigens which could not be found in the protein extract obtained by freezing thylakoids in the presence of NaCl were present in the caprylate extract. Interestingly, antigen 6′ (Fig. 3) was positively charged at pH 8.8. It therefore could not enter the polyacrylamide gel at the usual polarity in the absence of SDS.

All antisera agglutinated suspensions of freshly isolated photochemically active thylakoids. Therefore, at least some of the released proteins had retained their native conformation during freezing and had given rise to antibodies which were capable of recognizing native antigens in the membrane. The latter were obviously accessible to the large antibody molecules. This is possible only if they are located on the membrane surface, that is, on the matrix side of the thylakoids. An absorption experiment confirmed this conclusion. After

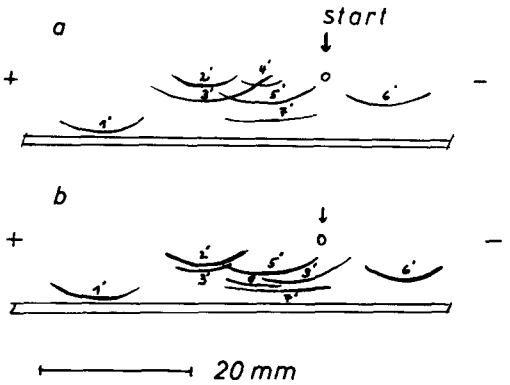

Fig. 3. Scheme of antigen-antibody precipitation arcs obtained during the immunoelectrophoretic analysis of proteins released from thylakoids which were frozen in the presence of NaCl (a) and sodium caprylate (b). The trough contained antiserum. Experimental details: 5—8 μg protein were added into the well. Electrophoretic separation was performed in 0.8% agarose (Sigma), Camag high resolution buffer, 0.05 M Tris barbiturate at pH 8.8. Running time was 30 min at 300 V and 4—10°C. 40 μl antiserum (a: B-71-7 or B-72-7; b: B-73-7 or B-74-7) were filled into the trough. The diffusion time was 20 h at 20°C in a moisture chamber.

preincubation of the antiserum B-74-7 (which was obtained by immunization of rabbit B-74 with caprylate extract) with increasing amounts of freshly isolated thylakoids, both the strength and number of precipitation arcs in an immunoelectrophoretic analysis with chloride and caprylate extracts decreased (Fig. 4). Antibodies which produced band 5′ (see Fig. 3) required more membranes to be removed from the antisera than did other bands, and band 2′ did

TABLE II

STRENGTH OF ANTIGEN-ANTIBODY PRECIPITATION IN THE IMMUNOELECTROPHORETIC ANALYSIS OF PROTEINS WHICH WERE RELEASED FORM THYLAKOIDS BY FREEZING IN THE PRESENCE OF NaCl (CHLORIDE EXTRACT) OR SODIUM CAPRYLATE (CAPRYLATE EXTRACT)

For methods, numbering and identification of antigens, see text and Fig. 3. Symbols: +++, very strong reaction; ++, strong reaction; +, significant reaction; ±, weak reaction; —, no reaction detectable. ?, no decision due to overlap of arcs.

Antiserum No.	Immunogen	(a) Antigens in chloride extracts								
		1′	2′	3′	4′	5′	6′	7′		
B-71-7	chloride extract	—	++	++	—	++	—	—		
B-72-7	chloride extract	++	++	++	—	—	+	—		
B-73-7	caprylate extract	—	++	++	+	++	±	±		
B-74-7	caprylate extract	+	—	++	?	++	±	±		
		(b) Antigens in caprylate extracts								
		1′	2′	3′	4′	5′	6′	7′	8′	9′
B-71-1	chloride extract	—	+++	?	?	+	±	—	?	±
B-72-7	chloride extract	++	+++	?	?	—	±	++	?	±
B-73-7	caprylate extract	—	+++	?	?	++	+	++	+	+
B-74-7	caprylate extract	++	+++	?	?	++	+	++	—	+

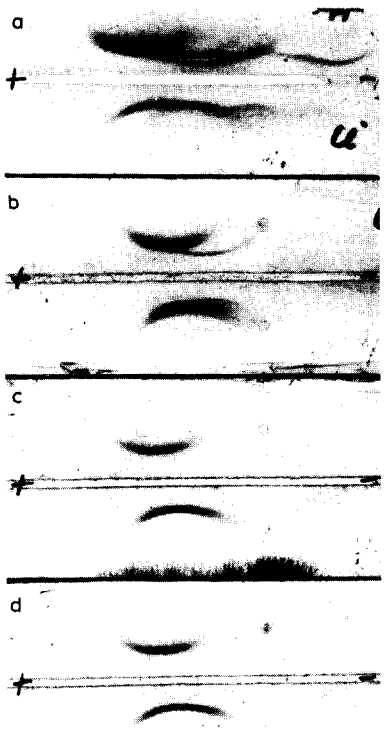

Fig. 4. Immunoelectrophoretic analysis of antibodies after absorption of antiserum B-74-7 with increasing amounts of freshly isolated thylakoids.
Upper part of each slide: 7 μg protein released from thylakoids during freezing in the presence of caprylate were electrophoretically separated.
Lower part of each slide: 6 μg protein released from thylakoids during freezing in the presence of NaCl were electrophoretically separated.
Antisera in the troughs: a. 40 μl serum B-74-7. 1 : 2 diluted; b. serum B-74-7 was incubated for 15 min at 0° C with an equal volume of a suspension of freshly isolated thylakoids (0.25 mg chlorophyll/ml). After centrifugation, 40 μl of the supernatant were added; c. treatment of the serum as in b but with 0.5 mg chlorophyll/ml; d. as in b, but with 1 mg chlorophyll/ml.

not disappear at all. The corresponding antigen is either inacessible or is denatured during the release by freezing. Antigen 1′ was not detected in this particular experiment and so we are unable to localize this protein in the membrane.

Control sera obtained from rabbits before immunization did not agglutinate thylakoids. Furthermore, no agglutination of freshly isolated thylakoids was observed when antisera against released proteins were absorbed with the supernatants of frost-damaged thylakoids. On the other hand, agglutination of thylakoids did occur after the addition of antisera which had been preincubated with the supernatants of functional thylakoids. Obviously, antigens were not present in these supernatants in concentrations sufficient for absorption (neutralization) of the agglutinating antibodies. It is concluded that there is no significant release of the peripheral proteins investigated without freezing.

Since the precipitation arcs in Figs. 3a and b show some similarity to patterns of CF_1 dissociation products produced in the cold or by urea and

TABLE III

PRECIPITATION OF SEVERAL CHLOROPLAST PROTEINS FROM SPINACH BY ANTISERA AGAINST PROTEINS WHICH WERE RELEASED FROM THYLAKOIDS BY FREEZING IN THE PRESENCE OF CHLORIDE (CHLORIDE EXTRACT) AND CAPRYLATE (CAPRYLATE EXTRACT), RESPECTIVELY

Antiserum No.	Immunogen	Precipitation with antigens *							
		Chloride extract	Caprylate extract	CF_1	δ	Ribulose bisphosphate carboxylase	NADP reductase	Plastocyanin	Ferredoxin
B 71-7	chloride extract	⩾4 ⩾	⩾3	++	n.t.	+	+	—	—
B 72-7	chloride extract	⩾5	⩾4	++	n.t.	+	+	±	—
B 73-7	caprylate extract	⩾3	⩾5	++	+	±	+	—	—
B 74-7	caprylate extract	⩾4	⩾5	++	n.t.	—	+	±	—

* Number of arcs and/or strength of reaction in immunoelectrophoretic analysis in agarose gels; ++, very strong; +, strong; ± weak; —, absent; n.t., not tested.

precipitated by specific antisera [23], and since the SDS electrophoretograms of Fig. 1 show some resemblance to the electrophoretic pattern of pure CF_1 subunits, the reaction of antisera against released proteins with partially denatured CF_1 preparations was tested (Table III). Precipitation occurred. The antisera reacted also with ribulose bisphosphate carboxylase, ferredoxin—$NADP^+$ reductase and plastocyanin (in some cases), but not with ferredoxin (Table III). On the other hand, antisera against several chloroplast proteins precipitated

TABLE IV

ANTIGEN PRECIPITATION BY DIFFERENT ANTISERA IN EXTRACTS CONTAINING MEMBRANE PROTEINS WHICH WERE RELEASED FROM THYLAKOIDS DURING FREEZING IN THE PRESENCE OF NaCl (CHLORIDE EXTRACT) OR SODIUM CAPRYLATE (CAPRYLATE EXTRACT)

Symbols as in Table III; n.t., not tested. RuDP, ribulose bisphosphate.

Antiserum No.	Immunogen	Precipitation with	
		Chloride extract	Caprylate extract
B-71-7	chloride extract	++++	+++
B-72-7	chloride extract	+++++	++++
B-73-7	caprylate extract	+++	+++++
B-74-7	caprylate extract	++++	+++++
B-80-8	crude CF_1	+++	++
C-56-11	homogenous, native CF_1	—	—
C-116-3	enriched δ-peptide of CF_1	—	+
B-120-4	pure δ-peptide of CF_1	n.t.	+
C-112-3	β-peptide of CF_1	n.t.	+
C-114-4	α-peptide of CF_1	+	+
B-97-5	RuDP-carboxylase (rye)	—	—
C-1-9	CF_1 and RuDP-carboxylase	±	—
C-41-8	ferrodoxin—$NADP^+$ reductase	++	++
B-2-2	plastomycin	++	++

specifically some of the released proteins (Table IV). From the data it is concluded that the mixture of released proteins contained ferredoxin—$NADP^+$ reductase, which is localized in situ on the matrix side of the thylakoids [24], some ribulose bisphosphate carboxylase and CF_1. The latter is known to be attached to the outside of the thylakoids. Since serum C_{56} which was shown to precipitate native CF_1 did not react, released CF_1 was no longer native. Indeed, no ATPase activity could be elicited in the supernatants of damaged membranes. Plastocyanin, which usually is not accessible to antibodies in thylakoid preparations [25], was also found in the membrane supernatants after freezing, although is was not immunogenic in rabbits B-71 and B-73 and yielded only a very low antibody titer in rabbits B-72 and B-74 (Table III and Figs. 5a and b).

From experiments such as shown in Fig. 5 and Tables III and IV, and from the comparison of the SDS gels (Fig. 1) with electropherograms of CF_1, we conclude that band 1 of Fig. 1 and Table I contains the α subunit of CF_1, band 2 the β subunit, band 8 the δ subunit, band 4 the γ subunit and band 9 the ϵ subunit. Occasionally, band 2 had two components. The second one is probably the large subunit of ribulose bisphosphate carboxylase. The small subunit of the carboxylase and plastocyanin are located in the area of band 9. Other bands may also contain more than one component. From its molecular

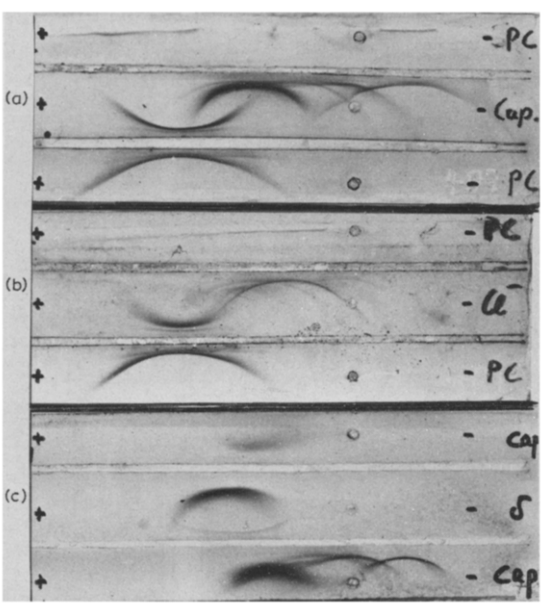

Fig. 5. Identification by immunoelectrophoretic analysis of plastocyanin and the δ-subunit of CF_1 among the membrane proteins released by freezing thylakoids in the presence of caprylate and NaCl. a. Additions to upper and lower antigen reservoir before electrophoresis: 1.2 μg plastocyanin (PC); to the middle reservoir: 7 μg protein from a caprylate extract (cap); upper trough: 40 μl antiserum B-73-7 against caprylate extract; lower trough: 40 μl antiserum B-2-2 against plastocyanin. b. Upper and lower antigen reservoirs: 1.2 μg plastocyanin; middle antigen reservoir: 5 μg protein from a chloride extract (Cl^-); upper trough: 40 μl antiserum B-2-2 against plastocyanin. c. Upper and lower antigen reservoirs: 7 μg protein from a caprylate extract; middle reservoir: 0.5 μg δ-subunit of CF_1 (δ); upper trough: 40 μl antiserum B-120-4 against the δ-subunit; lower trough: 40 μl antiserum B-73-7 against caprylate extract. Other conditions were as described in the legend to Fig. 3.

weight [26], the protein of band 3 of Fig. 1 and Table I is probably ferredoxin—$NADP^+$ reductase.

The δ subunit of CF_1 was shown by specific antisera (Fig. 5c) to be present in high amounts in caprylate extracts. From Fig. 1 it can be seen that in relation to bands 1 and 2 (α and β subunits) the protein of band 8 (δ subunit) was extracted from thylakoids during freezing in the presence of phenylpyruvate or caprylate in far larger amounts than during freezing in the presence of isoleucine. The caprylate extract can therefore be used as an enriched source for the isolation of the δ subunit (Berzborn, R.J. and Bonnenkamp, G., unpublished).

In Fig. 3 and Table II a and b, antigen 1′ is plastocyanin, antigen 2′ a CF_1 dissociation product (α, β and/or aggregates), antigen 3′ the δ subunit of CF_1 or ribulose bisphosphate carboxylase, which is precipitated in this area, if present, and antigen 5′ the NADP reductase. The antigens 6′, 7′, 8′ and 9′ are not identified. The discrepencies in the reaction of antisera against the ribulose bisphosphate carboxylase seen in Table III and IV may be due to immunological differences in the enzymes from spinach and rye or to different titers.

Correlation of protein release with loss of membrane function

Fig. 6 a shows the progressive loss of photophosphorylation as thylakoids were frozen in media containing sucrose (as cryoprotectant) and NaCl (as a cryotoxic salt) in decreasing ratios. Uncoupling of photophosphorylation from electron transport was indicated by the increase in the rates of ferricyanide or NADP photoreduction, as the molar ratio of sucrose to NaCl fell from higher

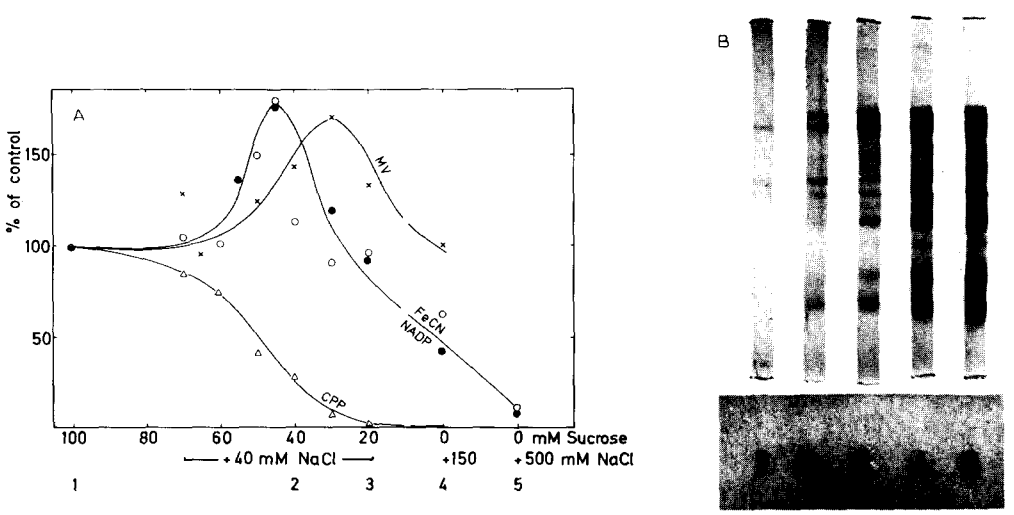

Fig. 6. a. Photochemical activities of thylakoids, which were suspended in solutions of the composition shown in the abscissa, after 3 h freezing at −25°C. The activities of thylakoids frozen in 100 mM sucrose served as controls. These rates were similar to the rates shown by unfrozen thylakoids. The activities of the controls were in µmol/mg chlorophyll per h: photophosphorylation (CPP), 485: ferricyanide reduction, 392; NADP reduction, 83: methylviologen reduction, 180. The numbers 1 to 5 in the abscissa refer to the patterns of released proteins shown in b and indicate the conditions of freezing, under which protein release was observed. b. SDS gel electrophoretic pattern of polypeptides from proteins, which were released from thylakoids during freezing. The conditions of freezing are indicated in the abscissa of a.

values to approx. unity. At low ratios and in the absence of sucrose, even electron transport from water to the acceptors was inactivated by freezing. Photosystem I-dependent electron transport from ascorbate to methyl viologen which increased as photophosphorylation was lost, was remarkably resistant to freezing in the absence of a cryoprotectant.

The numbers 1 to 5 below the abscissa of Fig. 6a refer to the electrophoretograms of released proteins shown in Fig. 6b. They reveal the extent of protein release occurring under the experimental conditions indicated in the abscissa of Fig. 6a. At a sucrose/NaCl ratio of 2.5, very little protein was released during freezing from the thylakoids. Even after 50% of photophosphorylation was inactivated by freezing, not much protein had left the membrane phase (not shown). There was a significant, but still small, increase in the amount of released protein after approx. 80% of photophosphorylation was lost at a sucrose/NaCl ratio of approx. 1 (electrophoretogram 2 in Fig. 6b). Protein loss increased dramatically after photophosphorylation was completely inactivated and electron transport significantly reduced (electropherograms 4 and 5 of Fig. 6b).

From the protein patterns it appears that the coupling factor CF_1, whose α and β subunits are located in the upper two bands of the electropherograms, is among the first proteins detached from the membranes. This might suggest a causal relation between loss of photophosphorylation and loss of the coupling factor, which is needed for ATP synthesis. However, the small loss of coupling factor in Expt. 2 of Fig. 6 is quite insufficient to explain the extensive loss of photophosphorylation. Under saturating light intensities, 20% loss of CF_1 is required to produce approx. 50% loss of the photophosphorylation capacity [27]. Even in the presence of sucrose some small loss of coupling factor was often observed (see bands 1 and 2 in the sucrose experiment of Fig. 1). It did not result in much loss of photophosphorylation, which was protected by sucrose against freeze inactivation. Indeed, reconstitution of freeze-inactivated thylakoids with coupling factor did not reconstitute photophosphorylation [4]. Obviously, loss of CF_1 was just one of the factors causing inactivation of photophosphorylation during freezing (see ref. 5).

Discussion

Garber and Steponkus [5] have reported that CF_1 and plastocyanin are lost from thylakoids during freezing. Our results show that a considerable number of other proteins, amounting to up to 5% of the total membrane protein, are also released, when thylakoids are damaged by freezing. There was little protein release from membranes during freezing in sucrose, which preserves membrane activities. Membranes contain so-called intrinsic and extrinsic proteins [28]. The former are supposed to be integrated into the membrane phase by hydrophobic interactions and association with lipids, the latter are attached to the membranes. Ionic interactions are considered to be important, especially for the binding of extrinsic proteins. During freezing, water is removed from the membrane suspension whose ionic strength increases owing to the concentrating effect freezing has on the solutes of the system. At elevated concentrations, solute ions will compete with membrane ions for counterionic binding

sites. Successful competition will finally cause the release of extrinsic proteins. In the case of cryotoxic anions, the extent of membrane inactivation was shown to be a function of anion size [7]. Poorly solvated anions were particularly destructive. As in the case of anion binding to cationic micelles [29], binding of inorganic salt anions appears to depend on how closely the anions can approach cationic binding sites of the membrane. This can explain why bromide, for instance, produces more membrane damage than chloride.

As an alternative to the binding concept, salt effects on water structure have been proposed to lead to the release of membrane proteins [30]. Hydrophobic interactions within the membranes are assumed to be weakened, with a consequent loss of sufficiently water-soluble proteins, when disordering of water by high concentrations particularly of poorly solvated ions facilitates increased interaction between membrane constitutents and water.

Salts of weak organic acids such as phenylpyruvates of caprylates are not completely ionized. Depending on their pK, a more-or-less significant proportion of the anions occurs in the protonated neutral form even at physiological pH values. If sufficiently apolar, the protonated species are lipid-soluble. On freezing, their concentration increases together with the concentration of the ions. Lipid-soluble material will enter the lipid part of the membrane phase and disturb hydrophobic membrane interactions. Such effects may explain why the pattern of released proteins is different, when membranes are frozen in the presence of inorganic salts and of organic solutes having a partially apolar structure. Only part of the membrane damage will become apparent in protein release experiments, since only water-soluble proteins will dissociate from the membranes. Owing to their hydrophobic membrane interactions, intrinsic proteins cannot be expected to leave the membrane phase even if they are affected by freezing.

Is protein release the cause or a consequence of membrane damage during freezing? Our data leave little doubt that the latter is the case. Garber and Steponkus [5] have drawn similar conclusions. Serious membrane damage is apparent from the loss of photophosphorylation, an essential thylakoid function, before much protein is released. It appears that changes in membrane permeability, loss of photophosphorylation and protein release are just manifestations of membrane damage. The cause of damage is seen in the disturbance of membrane structure, which occurs on freezing of membrane system in the absence of cryoprotectants. It is very likely that the solute effects described above are the causes of membrane damage. Relatively slight structural changes caused by solute interaction will be sufficient for changes in permeability characteristics and loss of photophosphorylation, while a more drastic disturbance of membrane structure is required for significant protein release.

Do model experiments such as those performed in this investigation bear any relation to the situation of membranes in situ, when living cells are subjected to freezing conditions? A large proportion of the total protein of cells of higher organisms is organized in membranes. The membranes are embedded into solute phases of different composition. Among their components are potentially membrane-toxic inorganic and organic solutes and cryoprotectants such as soluble sugars. Depending on the relative ratios of different solutes,

freezing may or may not concentrate potentially toxic solutes to the point of membrane damage. In the former case, cell death or damage will occur, in the latter the cell system is resistant against freezing. We conclude that model experiments with isolated biomembranes give useful information on freezing damage in vivo.

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