Biochimica et Biophysica Acta, 987 (1989) 231–234
Elsevier

BBAMEM 74689

Low concentrations of trehalose protect isolated thylakoids against mechanical freeze-thaw damage

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(Received 4 July 1989)

Key words: Cryoprotection; Freeze-thaw damage, mechanical; Trehalose; Membrane permeability; Osmotic stress; (Spinach thylakoid)

The disaccharides sucrose and trehalose were compared in regard to their ability to protect isolated spinach chloroplast membranes against rupture during a freeze-thaw cycle. Thylakoids frozen and thawed in the presence of 2.5 mM $MgCl_2$ or NaCl or in an artificial chloroplast stroma medium suffer osmotic rupture as indicated by the release of the soluble electron transport protein plastocyanin from the intrathylakoid spaces. Low concentrations of trehalose (up to 2 mM) provide much more efficient cryoprotection against thylakoid membrane rupture than sucrose at the same concentrations. Protection by trehalose can be attributed to a reduction in the solute permeability of the membranes which reduces solute influx during freezing and therefore osmotic swelling and rupture during thawing. It is suggested that trehalose affects protection by hydrogen bonding to hydrophilic groups at the surface of the thylakoid membrane.

Introduction

Sugars have been used extensively as cryoprotectants for photosynthetic membranes of plants (see Refs. 1 and 2 for reviews). Differences in the ability of different sugars to protect thylakoid membranes against inactivation during a freeze-thaw cycle have been described by several investigators [3–5].

Recently it has been shown that such differences also exist in the protective efficiency of different sugars during freezing, drying, or freeze-drying of pure phospholipid vesicles (see Refs. 6 and 7 for recent reviews). The disaccharides sucrose and trehalose were the most efficient cryoprotectants [8].

We have routinely used sucrose as a protectant for spinach thylakoids in freezing experiments. Thylakoid membranes contain mainly galactolipids (up to 80 mol% of the total membrane lipid) and only very little phospholipid (see Ref. 9 for a review). Therefore results obtained with phospholipid model membranes can not be directly applied to thylakoids. The high density of uncharged sugar headgroups in the galactolipids could give rise to different interactions of the membranes with free sugars which may result in protective effects not seen with phospholipid membranes. In the present contribution evidence is presented for the specific protection of thylakoids by low concentrations of trehalose against osmotic rupture.

Materials and Methods

Thylakoid isolation and freezing. Chloroplasts were isolated from non-hardy spinach (*Spinacia oleracea* L. cv. Yates) as described before [10]. Thylakoids were washed three times in hypotonic solutions (see figure legends for details). Samples containing approx. 0.5 mg chlorophyll per ml were stored for 3 h at −20°C and were thawed within 2–3 min in a water bath at room temperature.

Assessment of damage. After thawing, the membranes were removed from the samples by centrifugation ($16000 \times g$ for 15 min). The amount of the soluble lumenal protein plastocyanin in the supernatants was determined immunologically [10]. The total amount of plastocyanin was determined from aliquots of unfrozen membranes lysed in 2% Triton X-100.

Thylakoid volume. Packed thylakoid volume was measured after micro-haematocrit centrifugation as described before [11]. The solute composition during the measurements was the same as during the freeze-thaw experiments.

All other methods have been described recently [10].

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Results

During a lethal freeze-thaw cycle the photosynthetic membranes of spinach leaves suffer mechanical membrane rupture which is indicated by the loss of the electron transport protein plastocyanin from the intrathylakoid spaces [12]. In vitro, mechanical damage can be induced by freezing thylakoids in low-osmolality media [13]. It can be quantitated after thawing as the amount of plastocyanin released from the membrane vesicles.

Fig. 1 show the protective effect of the disaccharides sucrose and trehalose on plastocyanin release from isolated thylakoids after a freeze-thaw cycle in the presence of 2.5 mM $MgCl_2$. At concentrations up to 1 mM, trehalose is a much more effective protectant than sucrose, although the relationship between osmotic activity and concentration is the same for both sugars in this concentration range (data not shown).

This is further explored in Fig. 2. Sucrose concentrations up to 100 mM were employed and gave good cryoprotection already in the absence of trehalose (compare Refs. 13 and 14). The addition of 0.5 mM or 1 mM trehalose to the sucrose solutions resulted in a marked decrease of plastocyanin loss (Fig. 2). Samples frozen in the presence of higher concentrations of trehalose fell on the same line as the 1 mM samples when plotted according to their osmolalities.

Two factors contribute to mechanical membrane rupture [11]. Influx of osmotically active solutes during freezing is the result of diffusion, driven by the steep concentration gradients that develop when water crystallizes to ice and the solutes are concentrated in the unfrozen solution. During thawing, this solute uptake leads to an osmotic influx of water and a concomitant increase in thylakoid volume which may result in rupture [15].

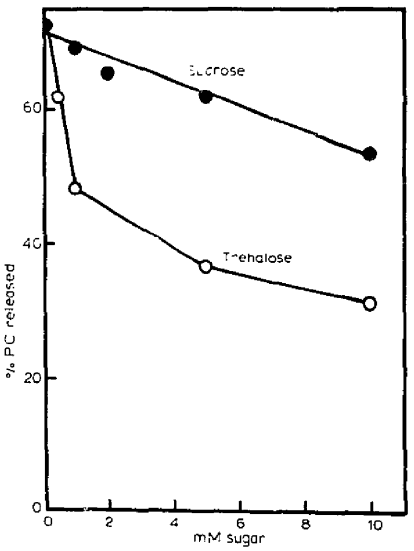

Fig. 1. Protection of thylakoids against freeze-thaw damage by sucrose (●) or trehalose (○). The membranes were washed in 5 mM $MgCl_2$. Samples contained 2.5 mM $MgCl_2$ and additional sugar as indicated. Damage was measured as release of the soluble protein plastocyanin (PC) from the intrathylakoid spaces.

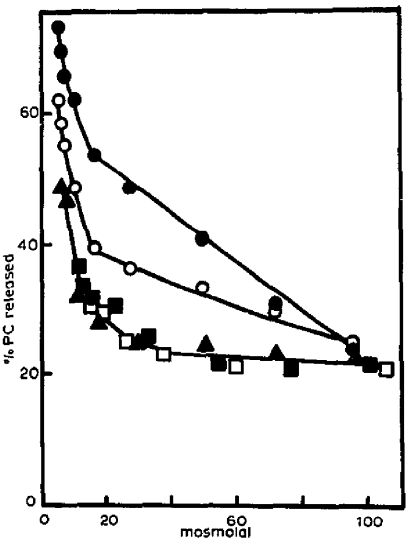

Fig. 2. Protection of thylakoids against loss of plastocyanin (PC) during a freeze-thaw cycle. The membranes were washed in 5 mM $MgCl_2$. The samples contained 2.5 mM $MgCl_2$ and sucrose at concentrations between 1 mM and 100 mM without trehalose (●) or in the presence of 0.5 mM (○), 1 mM (▲), 5 mM (■) or 10 mM (□) trehalose. The resulting total osmolality of the samples is indicated.

The second factor is the apparent inability of thylakoids to reexpand during thawing to the same volume they had occupied before freezing in dilute solutions (Ref. 11; see also Fig. 3).

Fig. 3 shows that trehalose protects thylakoids against rupture by decreasing solute influx during freezing. In the absence of trehalose, freezing in sucrose solutions results in a volume increase at high initial osmolalities and in rupture and a volume reduction at lower concentrations. In the presence of 1 mM trehalose in addition to the sucrose, the volume increase is reduced by about 60% as compared to the 0 °C controls (Fig. 3).

The maximum volume which the thylakoids reached after a freeze-thaw cycle was unaffected by trehalose (Fig. 3). Also, trehalose had no effect on the osmotic behavior of the control membranes, since in unfrozen samples with or without trehalose the data fell on the same regression line.

Mechanical freeze-thaw damage can not only be seen in solutions of low initial osmolality but also in media of a solute composition similar to the composition of the chloroplast stroma (artificial stroma medium [10]).

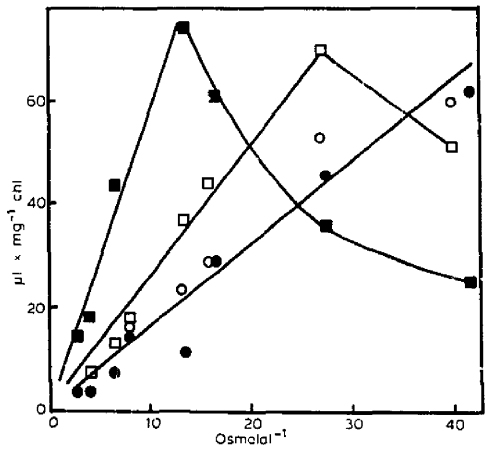

Fig. 3. Thylakoid volume in response to different osmolalities of the suspending medium after 3 h at 0 °C (●; ○) or −20 °C (■; □). The membranes were washed in 5 mM NaCl. Samples contained 2.5 mM NaCl and sucrose between 500 mM and 20 mM with (○; □) or without (●; ■) 1 mM trehalose. Packed thylakoid volume is plotted as a function of the reciprocal osmolality of the suspending medium (Boyle-van't Hoff plot). The straight lines were fitted to the data by linear regression analysis (●/○, $r = 0.97$; ■, $r = 0.98$; □, $r = 0.98$).

Specific cryoprotection of thylakoids by trehalose is also evident under such conditions (Fig. 4). The efficiency of both sucrose and trehalose was lower as compared to the conditions in Fig. 1 and the specific effect of trehalose was saturated at about 2 mM (Fig. 4).

The time course of membrane damage in an artificial stroma medium is biphasic [10]. The rapid phase is only

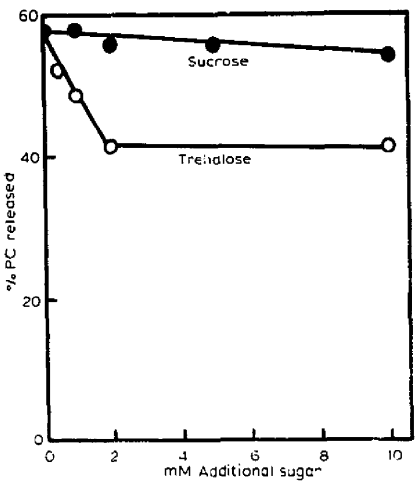

Fig. 4. Protection of thylakoids against freeze-thaw damage by sucrose (●) or trehalose (○). The membranes were washed in 10 mM $MgCl_2$, 20 mM K_2SO_4. Samples contained 5 mM $MgCl_2$, 10 mM K_2SO_4, 150 mM potassium glutamate and 50 mM sucrose and additional sugar as indicated. Damage was measured as release of the lumenal protein plastocyanin (PC) from the vesicles.

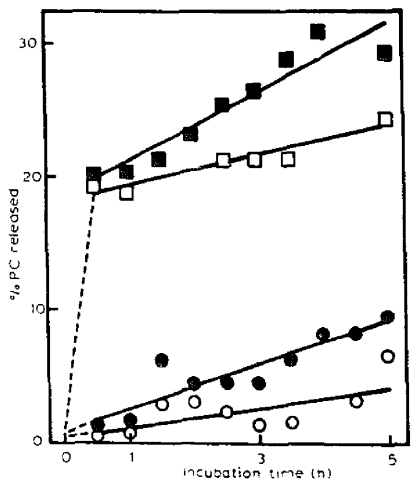

Fig. 5. Time-course of plastocyanin (PC) release from thylakoids at 0 °C (○; ●) or −20 °C (□; ■). Washing medium and sample composition were the same as in Fig. 4. In addition, samples contained 5 mM sucrose (solid symbols) or trehalose (open symbols). At $t = 0$ part of the samples were transferred from an ice bath to a freezer at −20 °C. The lines were fitted to the data by linear regression analysis (○, $r = 0.69$; ●, $r = 0.90$; □, $r = 0.95$; ■, $r = 0.94$). For better comparison the amount of plastocyanin released directly after transfer of the thylakoids from the washing medium to the incubation media was subtracted from the values measured after different incubation times.

seen after freezing and it was not influenced by the presence of trehalose (Fig. 5). The slow phase which has been related to diffusion induced membrane rupture [10,15] is evident both at 0 °C and −20 °C. The rate of plastocyanin release during the slow phase was reduced by about 60% in the presence of 5 mM trehalose both in frozen and unfrozen samples (Fig. 5).

Discussion

Membranes may be stabilized during freezing in different ways. Thylakoids are protected from mechanical freeze-thaw damage by sugars or sugar alcohols such as glucose [16], sorbitol [15] and sucrose [13]. In the presence of an artificial stroma medium even a normally cryotoxic salt such as NaCl can be protective [10]. It has been suggested that protection by these chemically very different substances is mainly achieved by osmotically supporting the vesicles during thawing and thereby reducing rupture [15]. If a solute protects membranes more efficiently, on an osmolal basis, than such unspecific protectants this should be the result of a specific interaction of the protective solute with the membranes.

The ability of carbohydrates to form hydrogen bonds has long been viewed as an important aspect of the membrane stabilizing effect of sugars [17]. However, in addition to the number of hydroxyl groups available for hydrogen bonds, structural properties of the sugars also play a role in membrane protection [6,7].

The ability of trehalose and sucrose to stabilize phospholipid vesicles during freezing has been attributed to the effective hydrogen bonding of the sugars to the phospholipid head groups [8]. The authors showed that sucrose and trehalose were equally effective cryoprotectants for small unilamellar phospholipid vesicles.

In contrast, for thylakoid membranes, trehalose is a much more effective protectant than sucrose at very low sugar concentrations both on a molar (Fig. 1 and 4) and on an osmolal basis (Fig. 2). At higher concentrations both sugars were equally effective (Figs. 1 and 4) indicating mainly osmotic stabilization by the additional trehalose. This may be the result of a saturation of possible sites for hydrogen bonds between membrane and cryoprotectant.

There is no evidence for a competition between sucrose and trehalose for binding sites on the membranes, since the saturation occurs at the same trehalose concentrations with or without sucrose also present (compare Figs. 1 and 2).

The specific protection by trehalose is achieved by reducing the solute permeability of the membranes (Fig. 3 and 5). It has been show before that a reduced permeability of thylakoids during cold acclimation of spinach plants is correlated with a reduced mechanical freeze-thaw damage to the isolated membranes [18].

The solute permeability of a membrane has been shown to be a function of membrane lipid fluidity [19]. Fluidity can not only be influenced by the lipid composition (see Ref. 20 for a review) but also by solutes which bind to the lipid head groups [21]. For phospholipid vesicles it has been shown that disaccharides hydrogen bond to the phosphate head groups and that trehalose has a three-dimensional structure which makes it especially effective in this respect [22].

It is tempting to speculate that trehalose may have a structural disposition to hydrogen bond to hydrophilic groups at the surface of thylakoid membranes. This binding would lead to a reduced membrane fluidity which would result in the observed reduction in solute permeability (Figs. 3 and 5). This effect is not dependent on the freezing process but binding already takes place under non-freezing conditions (Fig. 5). This is in agreement with trehalose binding studies performed with phospholipid monolayers and bilayers under non-freezing conditions [22].

The observed specific protection by trehalose as compared to sucrose which was not evident in phospholipid vesicles [8] could be due to interactions with the galactolipids or to the high protein content of thylakoid membranes (approx. 50% of the dry weight [9]). To resolve this question, additional experiments with model membranes will be necessary to establish whether trehalose binds to specific thylakoid membrane components and how this binding effects membrane stability.

Acknowledgements

I would like to thank Prof. U. Heber and Dr. J.M. Schmitt for critically reading the manuscript, and the Deutsche Forschungsgemeinschaft for financial support through Forschergruppe Ökophysiologie and SFB 251.

References

1 Steponkus, P.L. (1984) Annu. Rev. Plant. Physiol. 35, 543–584.
2 Schmitt, J.M., Schramm, M.J., Pfanz, H., Coughlan, S. and Heber, U. (1985) Cryobiology 22, 93–104.
3 Santarius, K.A. (1973) Planta 113, 105–114.
4 Steponkus, P.L., Garber, M.P., Myers, S.P. and Lineberger, R. D. (1977) Cryobiology 14, 303–321.
5 Lineberger, R.D. and Steponkus, P.L. (1980) Plant Physiol. 65, 298–304.
6 Crowe, J.H., Crowe, L.M., Carpenter, J.F. and Aurell Wistrom, C. (1987) Biochem. J. 242, 1–10.
7 Crowe, J.H.., Crowe, L.M., Carpenter, J.F., Rudolph, A.S., Aurell Wistrom, C., Spargo, B.J. and Anchordoguy, T.J. (1988) Biochim. Biophys. Acta 947, 367–384.
8 Anchordoguy, T.J., Rudolph, A.S., Carpenter, J.F. and Crowe, J.H. (1987) Cryobiology 24, 324–331.
9 Quinn, P.J. and Williams, W.P. (1983) Biochim. Biophys. Acta 737, 223–266.
10 Hincha, D.K. and Schmitt, J.M. (1988) Plant Cell Env. 11, 41–47.
11 Hincha, D.K. (1986) Biochim. Biophys. Acta 861, 152–158.
12 Hincha, D.K., Höfner, R., Schwab, K.B., Heber, U. and Schmitt, J.M. (1987) Plant Physiol. 83, 251–253.
13 Hincha, D.K., Heber, U. and Schmitt, J.M. (1985) Biochim. Biophys. Acta 809, 337–344.
14 Hincha, D.K., Schmidt, J.E., Heber, U. and Schmitt, J.M. (1984) Biochim. Biophys. Acta 769, 8–14.
15 Hincha, D.K., Müller, M., Hillmann, T. and Schmitt, J.M. (1989) in Environmental Stress in Plant (Cherry, J.H., ed.), pp. 303–315, Springer, Berlin, New York.
16 Santarius, K.A. and Giersch, C. (1984) Biophys, J. 46, 129–139.
17 Franks, F. (1983) Cryobiology 20, 335–345.
18 Hincha, D.K. and Schmitt, J.M. (1988) Plant Cell Env. 11, 48–50.
19 Van Zoelen, E.J.J., de Jesus, C.H., De Jonge, E., Mulder, M., Blok, M.C. and De Gier, J. (1978) Biochim. Biophys. Acta 511, 335–347.
20 Shinitzky, M. (1984) in Physiology of Membrane Fluidity (Shinitzky, M., ed.), Vol. I, pp. 1–51, CRC Press, Boca Raton.
21 Roberts, D.R., Dumbroff, E.B. and Thompson, J.E. (1986) Planta 167, 395–401.
22 Rudolph, A.S., Crowe, J.H. and Crowe, L.M. (1986) Arch. Biochem. Biophys. 245, 134–143.