Biochimica et Biophysica Acta, 1189 (1994) 38–44

BBAMEM 76157

The solute permeability of thylakoid membranes is reduced by low concentrations of trehalose as a co-solute

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(Received 5 May 1993)
(Revised manuscript received 27 August 1993)

Key words: Galactolipid; Non-electrolyte permeability; Osmotic membrane damage; Thylakoid; Trehalose; Cryoprotection; (Spinach)

The different efficiencies of sucrose and trehalose in protecting isolated spinach (*Spinacia oleracea* L.) thylakoids against freeze-thaw damage is quantitatively related to their ability to reduce the solute loading of the vesicles during freezing. In the present paper we show that this effect is based on a reduction of the solute permeability of the membranes. Permeability was measured with ^{14}C-labeled glucose at temperatures between 0 and 10°C. Glucose permeability was reduced by both sucrose and trehalose, with trehalose effective at much lower concentrations than sucrose. An analysis of the temperature dependence of glucose permeability in the presence and absence of trehalose revealed that a 50% reduction in permeability resulted from a 10% increase in activation energy and a 30% decrease in activation entropy. Using the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH), we found that the reduced permeability of the membranes in the presence of trehalose was unaccompanied by a reduction in lipid fluidity. This also excluded the possibility of a solute-induced liquid crystalline to gel phase transition. A reduced partitioning of the hydrophobicity-sensitive dye merocyanine 540 into thylakoids and into membranes containing 50% digalactosyldiacylglycerol in the presence of trehalose as compared to sucrose and glucose showed that the lipid headgroup region of these membranes became less accessible for solutes. No significant difference in merocyanine partitioning in the presence of trehalose as compared to sucrose or glucose was apparent when monogalactosyldiacylglycerol dispersions or phosphatidylcholine vesicles were investigated.

Introduction

Freeze-thaw damage to higher plant chloroplast thylakoids in vivo is characterized by the loss of the soluble electron transport protein plastocyanin from the intramembrane spaces. This indicates that rupture of the membrane vesicles occurs during a freeze-thaw cycle. Plastocyanin release is also observed when isolated thylakoids are frozen and thawed under appropriate conditions. This leads to an inactivation of light-driven ATP synthesis. Damage in vitro is a complex function of freezing temperature and time, and solute concentration and composition of the suspending medium. We have shown in previous publications that diffusion-driven solute loading of the thylakoid vesicles during freezing leads to osmotic swelling during thawing, which results in rupture when swelling exceeds a critical limit (see Ref. 1 for a review).

Several sugars have been shown to protect thylakoids against loss of plastocyanin during a freeze-thaw cycle [2,3]. The different effectiveness of the sugars could be quantitatively related to their ability to reduce the time dependent solute loading of thylakoids in the frozen state [4,5].

A slow, linearly time dependent release of plastocyanin is observed when thylakoids are incubated in an artificial stroma medium at 0°C in the absence of ice formation [6]. We have also attributed this to the effects of solute diffusion and the resultant excessive vesicle swelling [1]. Trehalose was the only sugar tested so far that reduced vesicle rupture in unfrozen samples [4]. This opens the possibility of investigating the mechanisms of membrane protection afforded by trehalose, and possibly other sugars, without the confounding additional complexities introduced by freezing.

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Abbreviations: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; DPH, 1,6-diphenyl-1,3,5,-hexatriene; MC540, merocyanine 540; PA, phosphatidic acid; PC, phosphatidylcholine; DMPC, dimyristoyl-PC; DPPC, dipalmitoyl-PC; PG, phosphatidylglycerol; PS, phosphatidylserine; SQDG, sulfoquinovosyldiacylglycerol.

SSDI 0005-2736(93)E0265-C

Our previous hypothesis that protective sugars reduce solute loading of thylakoids by reducing the solute permeability of the membranes was based on indirect evidence from thylakoid volume measurements. Here we use permeability measurements to show directly that low concentrations of trehalose lead to a reduction in the permeability of thylakoids for glucose. Spectroscopic evidence suggests that this may be due to an interaction of trehalose with thylakoid lipid headgroups.

Materials and Methods

Thylakoid isolation and incubation. Chloroplasts were isolated from the leaves of non-hardy spinach plants (*Spinacia oleracea* L. cv. Monnopa) as described recently [7]. Thylakoids were washed three times in 5 mM NaCl. This concentration was chosen to allow a direct comparison with previously published data. We have shown before that this hypotonic treatment had no significant influence on the permeability properties of the membranes [8]. For measurements of glucose permeability, samples (0.2 ml) containing approximately 0.4 mg chlorophyll per ml were suspended in 2.5 mM NaCl and sugars as indicated in the figure legends. They were incubated for up to 30 min in a cooling bath containing 1,2-ethanediol preequilibrated at the desired temperature.

Preparation of multilamellar lipid vesicles. Spinach thylakoid lipids were isolated as described [9]. Briefly, lipids were extracted from washed thylakoids in chloroform/methanol (1:2, v/v). The concentrated chloroform phase was applied to a column of silicic acid (SIL-LC, Sigma), activated with petroleum ether and equilibrated with chloroform. Pigments were eluted with chloroform, MGDG with acetone/chloroform (1:1, v/v), and a mixture of DGDG, sulfolipid and phospholipid was eluted with methanol/chloroform (1:1, v/v). The two lipid fractions were concentrated by rotary evaporation. PC and PA (from egg yolk) were obtained from Sigma. Aliquots from the lipids were dried under a stream of nitrogen as a thin-film in glass tubes. They were hydrated in distilled water by shaking in the presence of glass beads (thin-layer technique).

Glucose permeability measurements. D-[U-^{14}C]Glucose (specific activity 304 mCi/mmol (37 MBq/mmol); Amersham) was used as a radioactive tracer to measure the time-dependent flux of glucose into thylakoids. The ratio of labeled to unlabeled glucose was kept constant at $3.98 \cdot 10^{-2}$ μCi/mmol glucose.

After different incubation times, thylakoids were separated from the solution by centrifugation (8 min at $16000 \times g$). The pellets were resuspended in 200 μl of unlabeled glucose at the same concentrations used during incubation and mixed with 4 ml of scintillation cocktail (Rotiscint 2200, Roth). Radioactivity was quantified by liquid scintillation counting using a Beckman Instruments LS 3801 Liquid Scintillation System. A standard quench-curve was determined with different concentrations of thylakoid membranes with a fixed amount of radioactivity. The chlorophyll content [10] in every sample was determined after resuspending the pellets and the measured cpm were transformed into dpm using the quench-curve. The amount of radioactivity remaining with the thylakoids at $t = 0$ (addition of radioactive glucose to the thylakoids and immediate centrifugation) was subtracted from the values obtained at subsequent time points. Over the observed time period (30 min) radioactivity in the thylakoids increased linearly with time (regression coefficients were between $r = 0.92$ and 0.97).

Permeability coefficients (P) were calculated from these data using

$$P = J/(A \cdot \Delta C)$$

with J the influx of glucose in mmol/s, ΔC the concentration gradient of glucose across the thylakoid membranes in mM, and A the membrane area (16.7 cm^2/μg chlorophyll) [11]. Assuming that the internal glucose concentration is zero at the start of the experiment, the gradient is the same as the applied external concentration. The linear increase in radioactivity with time suggests that ΔC remained effectively constant throughout the measurement period.

Merocyanine binding to thylakoid membranes and multilamellar lipid vesicles. To isolated thylakoids at a final concentration of 0.1 mg chlorophyll per ml, or to liposomes at a final concentration of 1 mg lipid per ml, sugars were added from concentrated stock solutions to a final concentration of 50 mM. After incubation for 1 h at 0°C, merocyanine 540 (MC 540; Sigma) was added from a concentrated stock solution to a final concentration of 10^{-5} M. After 5 min the absorbance was measured at 570 nm and 530 nm in an Ultrospec II spectrophotometer (LKB) at room temperature. The values were corrected for absorbance by the thylakoids or liposomes. The absorbance ratio A_{570}/A_{530} can be used as a measure for the degree of binding of merocyanine to hydrophobic sites on the membranes [12]. In order to characterize this hydrophobicity dependence, we measured the absorbance of merocyanine at a final concentration of 10^{-5} M in ethanol/water mixtures of different dielectric constant [13], and determined the absorbance ratios as above.

Fluorescence depolarization measurements. Liposomes were prepared from MGDG or DGDG (Sigma) in distilled water or in 50 mM trehalose using the thin-layer technique as described above. The lipid concentration in the samples was 1 mg/ml. Chloroplasts were isolated from pea leaves as described [14] and thylakoids were washed three times in 50 mM Hepes

(pH 7.6), 5 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA. Liposomes and thylakoids were labeled with DPH at a lipid to probe molar ratio of 100:1. Thylakoids were mixed with a concentrated trehalose solution to a final sugar concentration of 50 mM at a chlorophyll concentration of 0.01 mg/ml. The degree of polarization of fluorescence of DPH was measured in a Perkin–Elmer MPF fluorescence spectrophotometer at room temperature.

Results

When the disaccharides sucrose and trehalose were compared for their ability to protect thylakoids against freeze-thaw damage, it was found that trehalose was a much better protectant [4]. Protection in the presence of trehalose was correlated with a decreased packed volume of thylakoids. A lower packed volume was taken as indirect evidence for a reduced amount of solute loading in the presence of trehalose.

We have now directly measured the glucose permeability of thylakoids at 0°C using ^{14}C-labeled glucose as a tracer. As would be expected for a hydrophilic molecule of this size, permeation was relatively slow ($P = 2.22 \cdot 10^{-9}$ cm/s; Table I). It was, however, sufficiently fast to measure it accurately using the sensitive radiotracer technique described in Materials and Methods.

When different concentrations of either trehalose or sucrose were added, the permeation of glucose was reduced (Fig. 1). This effect was much more pronounced with trehalose, especially at low concentrations of co-solute.

The relative effectiveness of trehalose was strongly dependent on the glucose concentration used to measure permeability. A 50% reduction of glucose permeability was effected by 25 mM trehalose with 25 mM glucose (Fig. 1A), 50 mM trehalose with 50 mM glucose (Fig. 1B), and 80 mM trehalose with 100 mM glucose (Fig. 1C). This difference was much less pro-

TABLE I

Permeability coefficient (P) of thylakoid membranes for glucose at 0°C, activation energy (E_a) and activation entropy (ΔS) of glucose permeation for the temperature range of 0 to 10°C

Trehalose (mM)	P (cm/s)	Activation energy [c] (kJ/mol)	Activation entropy [d] (J/mol per K)
0	$2.22 \pm 0.18 \cdot 10^{-9}$ [a]	84.75 ± 11.27	-83.41 ± 12.18
25	$1.11 \pm 0.35 \cdot 10^{-9}$ [b]	92.98 ± 12.36	-57.64 ± 13.06

[a] Mean ± S.D. from 14 independent experiments with glucose concentrations between 25 mM and 100 mM. The standard deviations for the different concentrations were between 0.2 and $1.4 \cdot 10^{-9}$ cm/s. The S.D. given refers to the mean of the permeability coefficients derived from the different glucose concentrations. There were no significant differences in P for the different concentrations.

[b] The P-value in the presence of 25 mM trehalose refers to experiments performed with 25 mM glucose (compare Fig. 1A). Mean ± S.D. for three independent experiments is shown.

[c] Calculated from the Arrhenius plot shown in Fig. 2. Samples contained 25 mM glucose. E_a in the absence and presence of 25 mM trehalose was significantly different at a 5% level as determined by a t-test for homogeneity of the slopes of the straight lines in the Arrhenius plot.

[d] Calculated from the y-intercept of the extrapolated lines of the Arrhenius plot in Fig. 2 as described in Ref. 15.

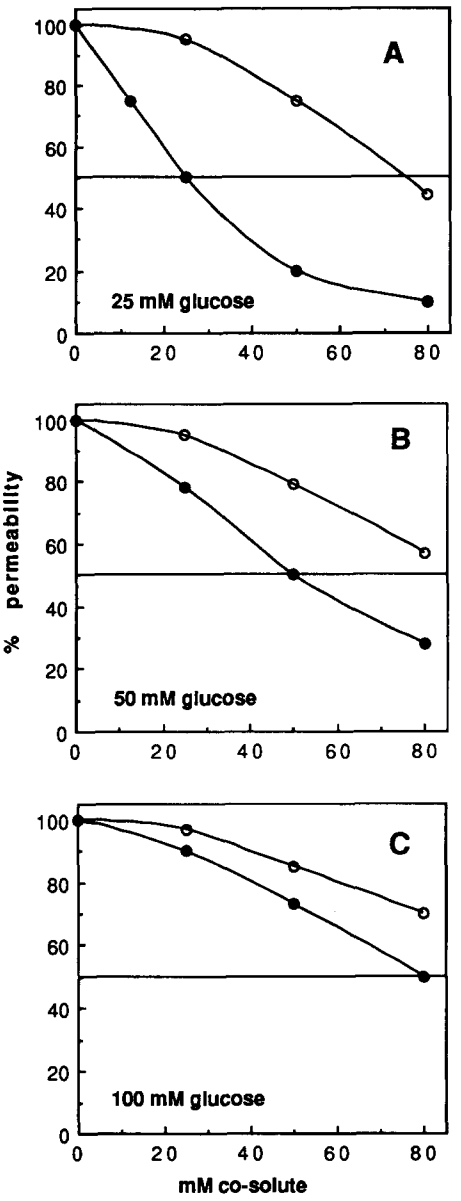

Fig. 1. Effects of the co-solutes trehalose (solid symbols) and sucrose (open symbols) on the glucose permeability of thylakoid membranes. Radioactively labeled glucose was used as a tracer to measure permeation at 0°C in the presence of 25 mM (A), 50 mM (B) or 100 mM (C) glucose, and trehalose or sucrose as indicated. Permeability of glucose in the absence of a co-solute was taken as 100% (see Table I). Each point is the mean of three independent experiments. Standard deviations were between 9.1 and 40.9%.

nounced with sucrose (the sucrose-to-glucose ratios in mM for a 50% permeability reduction were 75/25 (Fig. 1A); 90/50 (Fig. 1B); 95/100 (Fig. 1C)). The higher concentrations needed to reduce permeability in the presence of higher glucose concentrations are probably due to a competitive mechanism of action, as will be discussed below. The permeability coefficient in the absence of an additional co-solute was independent of the applied glucose concentration (Table I), as would be expected for passive permeation.

In order to gain insight into the mechanism underlying the efficient reduction of glucose permeability by trehalose, we measured the temperature dependence of P from 0 to 10°C. We chose a concentration of 25 mM glucose and 25 mM trehalose. This yields a 50% reduction in permeability (Table I) while sucrose shows only a negligible effect at this concentration (Fig. 1A).

Fig. 2 shows the Arrhenius plot of ln P vs. $1/T$. The glucose permeability of the membranes was reduced by the addition of trehalose over the whole temperature range investigated. The lines were linear and no break point occurred, indicating a continuous dependence of P on temperature. From the slopes of the lines the apparent activation energy (E_a) for glucose permeation through thylakoid membranes could be calculated (Table I). Since E_a is the amount of energy necessary to drive 1 mol of glucose through the membrane, it might be expected that the reduction of P by trehalose is the result of an increased activation energy.

This is, however, only partly the case. Although the difference in E_a was statistically significant (Table I), an increase by approximately 10% is not sufficient to explain a 50% reduction in P.

The second factor that determines the permeation rate is the activation entropy (ΔS). Since a membrane

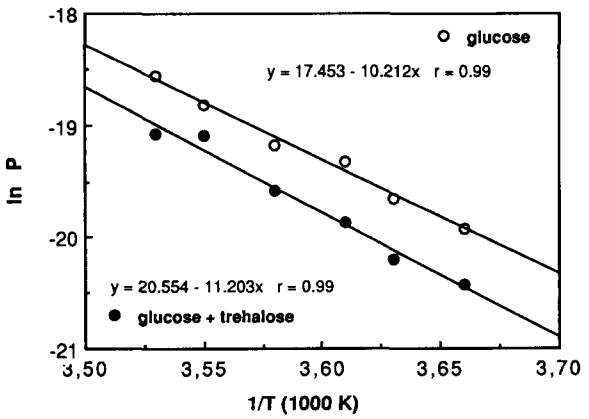

Fig. 2. Arrhenius plot of the logarithm of the permeability coefficient (ln P) versus reciprocal temperature. Permeability was measured as in Fig. 1 in the presence of 25 mM glucose with (●) or without (○) 25 mM trehalose also present at temperatures between 0°C and 10°C. The lines were fitted to the data by linear regression analysis. The regression equations and correlation coefficients (r) are indicated.

TABLE II

Fluidity of isolated galactolipids and of thylakoid membranes in the absence and presence of 50 mM trehalose as estimated by DPH fluorescence depolarization

Means and S.D. of three measurements are shown for the isolated lipids and of 15 measurements for thylakoids.

Sample	−Trehalose	+Trehalose	S.D. (%)	% Difference
MGDG	0.225	0.219	7.2	−2.6
DGDG	0.188	0.192	7.5	2.2
Thylakoids	0.285	0.291	6.0	1.8

is a more ordered structure than a bulk solution, the permeation of a molecule into the bilayer is accompanied by a reduction in entropy. A higher (less negative) value for ΔS indicates a larger difference in entropy. The value of ΔS can be calculated from the intercept of the lines in an Arrhenius plot with the y-axis [15]. The fact that the activation entropy of glucose permeation becomes more positive (by approximately 30%) in the presence of trehalose (Table I) shows that the process becomes thermodynamically less favorable.

Changes in the solute permeability of membranes have been correlated with changes in the fluidity of the membrane lipids, especially the packing order of the lipid hydrocarbon chains [16]. In order to determine if trehalose has an influence on the fluidity of the membrane lipids, we incorporated the fluorescence probe DPH into MGDG and DGDG multilayers and into isolated thylakoid membranes in the absence and presence of trehalose. When DPH is excited by polarized light, the degree of depolarization of the emitted fluorescence is a measure of the fluidity of the lipid phase. The measurements showed that there was no significant difference in lipid fluidity in samples in the absence or presence of 50 mM trehalose (Table II). This was true for both the isolated galactolipids and the thylakoid membranes.

Because of the size and hydrophilicity of the disaccharide trehalose, the most likely interaction with thylakoid membranes would be via hydrogen bonding with the sugar headgroups of the galactolipids. To assess this possibility, we used the dye merocyanine 540. It has been shown that changes in the spectral properties of the dye in the presence of membranes are due to binding to hydrophobic sites in the lipid head group region of the membranes. The chromophore is thought to be oriented parallel to the membrane surface slightly above the domain of the glycerol backbone of the lipids. It is oriented relative to the membrane plane by two butyl groups on one side of the molecule anchoring it in the hydrophobic core region, and a sulfopropyl group protruding into the more hydrophilic part of the headgroup region [17]. The authors could show that changes in the spectral properties of MC540 in the

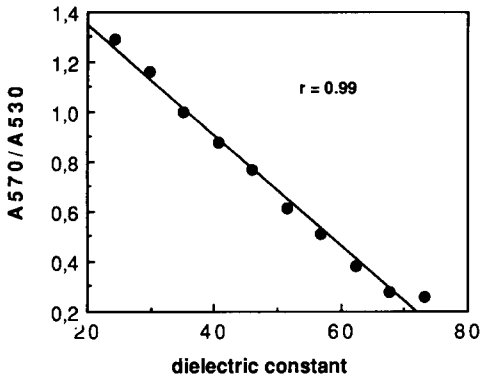

Fig. 3. Changes in the spectral properties of merocyanine 540 as a function of the hydrophobicity of the solvent. The dye was added to different ethanol/water mixtures at a final concentration of 10^{-5} M. The ratio of the absorbance at 570 and 530 nm is plotted as a function of the dielectric constant of the samples which serves as a measure for the hydrophobicity of the solvents.

TABLE III

Accessibility of the membrane headgroup region for hydrophilic solutes in the presence of 50 mM sugar as estimated by the absorbance ratio A_{570}/A_{530} of MC540

A decrease in the absorbance ratio indicates a decrease in the hydrophobic sites on the membrane accessible to merocyanine. The values shown are the means of 10 measurements each.

Sugar	Thylakoids [a]	DGDG [b]	MGDG	PC+4% PA
Glucose	1.07	0.87	0.89	1.64
Sucrose	1.02	0.86	0.88	1.65
Trehalose	0.91	0.77	0.83	1.63

[a] The values for trehalose and sucrose are significantly different at a 1% level as determined with a *t*-test.

[b] The values for trehalose and sucrose are significantly different at a 5% level. All other values are not significantly different as determined with a *t*-test. The 'DGDG' membranes were formed from the MGDG-depleted thylakoid lipid fraction and contained approximately 50% DGDG, 25% SQDG and 25% PG [9].

presence of membranes were related to different fractions of the dye bound to the membranes or free in solution. The dye was shown to be a sensitive probe for changes in lipid packing density that influenced the accessibility of the headgroup region for MC540 [18]. For a given membrane in the presence of different solutes, MC540 would therefore be expected to reflect the relative accessibility of the lipid headgroup region for other solutes.

The absorbance maximum of MC540 is shifted from 540 nm to 570 nm when the dye is transferred from a hydrophilic to a hydrophobic environment. No quantitative data on the correlation between solvent hydrophobicity and the absorbance ratio A_{570}/A_{530} of the dye were available from the literature. Fig. 3 shows that this ratio can be used as a direct measure for the hydrophobicity of the solvent expressed as the dielectric constant of different ethanol/water mixtures. MC540 can therefore be used as a hydrophobicity probe in the presence of membranes.

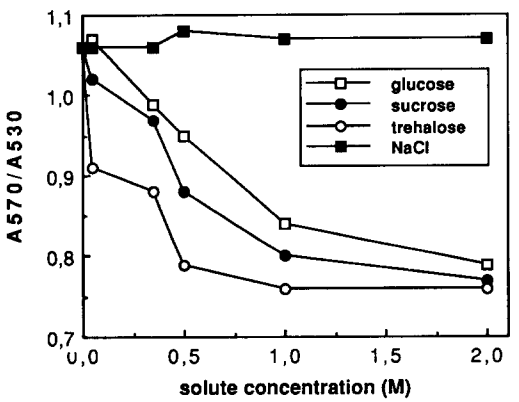

Fig. 4. Absorbance ratio A_{570}/A_{530} of MC540 in the presence of isolated thylakoid membranes as a function of the concentration of different solutes. A reduction in the absorbance ratio indicates reduced partitioning of the dye into the lipid headgroup region of the membranes.

Fig. 4 shows that the penetration of MC540 into the lipid headgroup region of thylakoid membranes was inhibited by increasing concentrations of sugars. All three investigated sugars showed a reduction in the merocyanine absorbance ratio. This was not due to an osmotic dehydration of the membranes, since NaCl showed no measurable effect despite the fact that it exerts a much higher osmotic pressure than a sugar at the same molarity. At concentrations below 1 M, trehalose was much more effective than sucrose or glucose. Control experiments indicated that the observed effects could not be accounted for by direct interactions of the sugars with the dye (data not shown).

Since the differences between the sugars were most pronounced at low concentrations (Fig. 4), where also their differences in effectiveness on permeability are strongest (Fig. 1), we used a concentration of 50 mM to investigate a possible lipid specificity of the sugar–membrane interaction (Table III). Significant differences between sucrose and trehalose were only found with thylakoids (compare Fig. 4) and with lipid vesicles containing a high percentage of DGDG. In the presence pure MGDG or phospholipid (PC + 4% PA) the A_{570}/A_{530} ratio of merocyanine was not significantly different.

Discussion

Solute loading during freezing is an important factor in the freeze-thaw damage to thylakoids [8,19]. All sugars tested so far as cryoprotectants acted by reducing the solute influx into the vesicles and thereby osmotic rupture. However, until now only indirect evi-

dence from hematocrit centrifugation experiments has been presented to show that the sugars reduce the solute permeability of the membranes [4,5].

In the present paper we have for the first time shown directly that a cryoprotective sugar as a co-solute reduces the solute permeability of thylakoid membranes (Fig. 1). Trehalose was much more efficient, especially at low concentrations, than sucrose. A direct comparison of our permeability data (Table I) with values from the literature is not possible, since to the best of our knowledge the glucose permeability of thylakoids has never been reported. Values for P have been reported for glucose with unilamellar vesicles of different lipid composition at temperatures between 30 and 36°C. If we use the E_a from the Arrhenius plot (Fig. 2) to extrapolate our data to this higher temperature range, a comparison can be made. P is higher for thylakoids by 3 orders of magnitude when compared to DGDG or soy bean PC vesicles [20] or DMPC vesicles [21] and by 4 orders of magnitude in comparison to PS vesicles [15].

These differences could be either due to the fact that in all cases cited above glucose efflux has been measured as opposed to the influx measurements in this study, or to the different membrane composition. Thylakoids contain a high amount of protein (approx. 50% by weight) [22], and it is generally assumed that proteins increase the solute permeability of membranes due to packing defects at the interface between lipids and proteins (see Ref. 23 for a review). Phase separations in thylakoid lipids are not expected to play a role in the observed high permeability, since there is no evidence to suggest that phase separations occur in the investigated temperature range in chloroplasts from chilling resistant plants such as spinach and pea [23]. Also, the absence of a break point in the Arrhenius plot (Fig. 2) argues against the occurrence of a low temperature phase separation close to 0°C.

The activation energy for glucose permeation that we determined with 85 kJ/mol (Table I) for a temperature range from 0°C to 10°C is similar to values reported for phospholipid vesicles at temperatures between 20°C and 49°C with 105 kJ/mol [21] and 82 kJ/mol [15]. The addition of trehalose sufficient to decrease P by 50% only increased E_a by 10% (Table I). A similar situation has been described for the addition of cholesterol to phospholipid membranes. The inclusion of cholesterol strongly reduced P for glucose with only a slight change in E_a [15]. Also, when the permeability of phospholipid membranes was reduced by the addition of lipids containing long-chain fatty acids, no change in E_a was evident [21].

In a membrane system, E_a corresponds closely to the activation enthalpy [15] and P is the product of enthalpy and entropy (ΔS) of the permeation process. We have calculated ΔS from the Arrhenius plot (Fig. 2) and found that trehalose changes the activation entropy of solute permeation (Table I).

The mechanism by which trehalose reduces the solute permeability of thylakoid membranes could involve a reduced fluidity of the membrane lipids due to an interaction of the sugar molecules with the membranes. A strong correlation between lipid fluidity and solute permeability of phospholipid membranes has been shown [16], and a reduction of fluidity by binding of a solute to lipid headgroups has also been reported [24].

Our measurements of the fluidity of isolated galactolipids which make up the majority of the thylakoid membrane lipids [23], and of total thylakoids gave no evidence for an influence of trehalose on this parameter (Table II).

We therefore investigated the accessibility of the lipid headgroup region of thylakoids for hydrophilic molecules. For this purpose the hydrophobicity-sensitive dye MC540 (Fig. 3) was used. Our data show that the dye partitions less readily into the membranes in the presence of trehalose than in the presence of sucrose or glucose (Fig. 4). However, all three sugars showed a concentration-dependent effect which was not seen with NaCl. From this we conclude that a specific interaction of the sugars with the membranes changes the properties of the lipid headgroup region.

It has been shown [12] that similar changes in the A_{570}/A_{530} absorbance ratio of MC540 are also observed when membranes go through a liquid crystalline to gel phase transition. The data in Table II, however, clearly show that neither thylakoids nor the isolated thylakoid lipids go through a phase transition, as there is no difference in DPH fluorescence polarization that would indicate differences in the phase state of the lipids in the presence or absence of trehalose.

The fact that glucose also interacts with the membranes explains the glucose concentration dependence of the permeability reduction achieved by trehalose and sucrose (Fig. 1). The data suggest that glucose competes for binding sites on the membrane surface with the other sugars, so that an increase in glucose concentration will necessitate an increase in trehalose or sucrose concentration to achieve the same degree of permeability reduction.

A significant effect of trehalose on the merocyanine absorbance ratio is only seen with thylakoids or with vesicles containing a high proportion of DGDG (Table III). There is only a small (statistically non-significant) effect on MGDG and none on PC/PA vesicles. Since pure MGDG will not form bilayers [23], the results for this lipid are not directly comparable to those for the bilayer-forming lipids. However, the data provide strong evidence that the interactions of the sugars with thylakoids involve glycolipids and not phospholipids (Table III).

Based on these data we can forward the hypothesis that trehalose interacts with the lipid headgroups of thylakoid membranes, presumably by hydrogen bonding to the headgroups of galactolipids. This could explain the decreased entropy of permeation because of a more ordered structure of the solute–membrane interface. It has been shown for other experimental systems that the transition of a hydrophilic molecule from the bulk solution to the membrane rather than the diffusion rate through the hydrocarbon core is the rate limiting step in the permeation process (see Ref. 25 for a review).

Additional support for our hypothesis is provided by recent studies which show that the incorporation of synthetic lipids with covalently attached carbohydrates into liposomes results in changes in the physical behavior of other lipids during freezing and drying [26,27]. Also, the effectiveness of trehalose as a cryoprotectant for PC vesicles could be increased by the incorporation of a synthetic triglucosyl-lipid into the membranes [28]. In this case spectroscopic evidence for direct interactions of trehalose with a triglyco- but not with a diglyco-lipid was presented for the dehydrated membranes.

Trehalose binding to phospholipid membranes has been suggested on the basis of several studies (see Ref. 29 for a review). Direct evidence, however, has only been obtained in the case of dry phospholipid vesicles [30,31]. Our own data (Table III) show no indication for sugar–phospholipid interactions in hydrated membranes.

Acknowledgements

I. Bakaltcheva is on leave from the Institute of Cryobiology and Lyophilization, Sofia, Bulgaria, with financial support from the Hochschulsonderprogramm 2, Freie Universität Berlin. Financial support was provided by BMFT through Genzentrum Berlin.

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