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Cryotoxicity of antifreeze proteins and glycoproteins to spinach thylakoid membranes – comparison with cryotoxic sugar acids

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We have used thylakoids from spinach (*Spinacia oleracea* L.) chloroplasts to test the effects of antifreeze proteins (AFP) from the starry flounder (*Platichthys stellatus*; AFP-SF) and from the antarctic eel pout (*Austrolycichthys brachycephalus*; AFP-AB), and antifreeze glycoproteins (AFGP) from the antarctic fish *Dissostichus mawsoni* on biological membranes during freezing. Freeze-thaw damage, measured as the release of the lumenal protein plastocyanin from the thylakoid vesicles, was strongly increased in the presence of all proteins tested. Measurements of the time dependence of plastocyanin release in a simplified artificial chloroplast stroma medium showed that all the fish proteins increased damage during the initial rapid phase while only AFGP increased plastocyanin release during the linearly time dependent slow phase. A slow plastocyanin release is also seen in the absence of freezing. It is increased by the presence of AFGP and AFP-AB, but not by AFP-SF. In order to distinguish between the contribution of the polypeptide and the carbohydrate part of AFGP on freeze-thaw damage we investigated the effects of galactose and *N*-acetylgalactosamine. While galactose was protective, *N*-acetylgalactosamine increased the rate of plastocyanin release in an artificial stroma medium at −20°C. It had no effect on the rapid phase of damage and was also ineffective at 0°C. The same was found for several other sugar derivatives (*N*-acetylglucosamine, gluconic acid, glucuronic acid, galacturonic acid). From these data we conclude that the increased plastocyanin release during the rapid phase of freeze-thaw damage is a function of the polypeptide part of AFGP. The increased rate of plastocyanin loss at longer incubation times both at 0°C and at −20°C may be mediated by the *N*-acetylgalactosamine moiety of the AFGP, but is strongly amplified by the polypeptide.

Introduction

Many arctic and antarctic fishes live at temperatures below the equilibrium freezing point of their body fluids in water containing high concentrations of ice crystals. They avoid freezing due to the presence of antifreeze proteins (AFP) or antifreeze glycoproteins (AFGP) in their blood [1]. AFGP in antarctic fishes are found in at least eight size classes from 2300 to 34000 Da and consist of repeating units of the tripeptide Ala-Ala-Thr with a disaccharide consisting of galactose and *N*-acetylgalactosamine linked to the Thr residues (see Ref. 2 for a review). AFP are structurally more diverse [3]. Some show a characteristic repeat structure such as the 11 amino acid repeats in the alanine-rich AFP from arctic flounders and sculpins [4]. All antifreeze proteins bind to the ice crystals and are thought to prevent their growth via the Kelvin effect [5,6].

From recent experiments it has been suggested that both AFGP and AFP have the capability to interact with biological membranes. AFGP protected pig oocytes against damage to their plasma membranes in the apparent absence of ice crystal formation during rapid cooling in liquid N_2 and rapid warming from the cryogenic temperature [7], and during hypothermic storage in the absence of freezing [8]. AFGP were also shown to enhance survival of pig oocytes and bovine and mouse embryos after vitrification [9]. AFP from the ocean pout preserved the metabolic integrity of whole rat livers during hypothermic storage [10].

In plants, antifreeze proteins have been implicated in the development of frost hardiness from sequence comparisons with proteins induced in *Arabidopsis thaliana* during cold treatment [11]. It has also been shown that AFP reduce freeze-thaw damage when they

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Abbreviations: AFP, antifreeze proteins; AFP-AB, antifreeze protein from the antarctic eel pout, *Austrolycichtys brachycephalus*; AFP-SF, antifreeze protein from the starry flounder, *Platichthys stellatus*; AFGP, antifreeze glycoproteins; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine.

are infiltrated into plant leaves or when suspension cultured plant cells are exposed to AFP during freezing [12]. A synthetic AFP gene has been expressed in maize protoplasts with the aim of improving plant frost hardiness by the introduction of such genes into the cells of tender plant species [13]. In recent studies it was shown that transgenic tomato plants express a fusion protein between an AFP and a truncated staphylococcal protein A at high levels. This fusion protein has recrystallization inhibition activity both after expression in *Escherichia coli* and tomato leaves [14,15]. Expression of this fusion protein in yeast (*Saccharomyces cerevisiae*) lead to an increased survival of the cells after freezing in liquid N_2 and slow warming, presumably by reducing cellular damage due to recrystallization of ice during thawing [16].

In order to elucidate the mechanism of the cryoprotective effect of antifreezes on biomembranes and to see whether the expression of such proteins may be a feasible way to increase plant frost hardiness, we have determined their effects on the integrity of spinach thylakoids. Thylakoids are a suitable in vitro system for the investigation of freeze-thaw damage (see Ref. 17 for a recent review) and have already been successfully used to identify cryoprotective plant proteins [18–20]. In the present contribution we show that contrary to expectation AFGP and AFP are cryotoxic to thylakoid membranes even at low concentrations.

Materials and Methods

Thylakoid isolation and incubation

Chloroplasts were isolated from non-hardy spinach (*Spinacia oleracea* L. cv Monnopa) as described [21]. Thylakoids were washed three times in 10 mM $MgCl_2$, 20 mM K_2SO_4. Samples (200 μl) contained approx. 0.5 mg chlorophyll per ml in 5 mM $MgCl_2$, 10 mM K_2SO_4, 150 mM potassium glutamate, 50 mM sucrose and proteins as indicated in the figures. If not noted otherwise in the figure legends, they were stored at 0°C or −20°C for up to 6 h. Frozen samples were thawed within 2–3 min in a water-bath at room temperature. Nucleation temperatures for the different samples were not determined.

Assessment of damage

After thawing, the membranes were sedimented by centrifugation (16000 × *g* for 15 min). The amount of the soluble lumenal protein plastocyanin in the supernatants was determined immunologically [21]. The total amount of plastocyanin was determined from aliquots of unfrozen membranes lysed in 2% Triton X-100.

Sugars and proteins

All sugars were purchased from Sigma. Sugar acids were used as their sodium salts. Solutions of uncharged sugars were made up with an appropriate concentration of NaCl to ensure that the osmotic pressure of the solutions was the same for all substances.

Antifreeze glycoproteins were isolated from the blood of the antarctic notothenid fish, *Dissostichus mawsoni* by ion exchange chromatography [22]. A mixture of the different size AFGPs was used in the approximate proportions that they are present in the blood (one part by weight of AFGPs 1 to 5 with a molecular mass between 33700 and 10500 Da, and three parts of AFGPs 7 and 8 with a molecular mass of 3500 and 2600 Da, respectively) and was termed a physiological mixture.

Antifreeze peptides were isolated from the blood serum of the arctic starry flounder (*Platichthys stellatus*) and the antarctic eel pout (*Austrolycichthys*

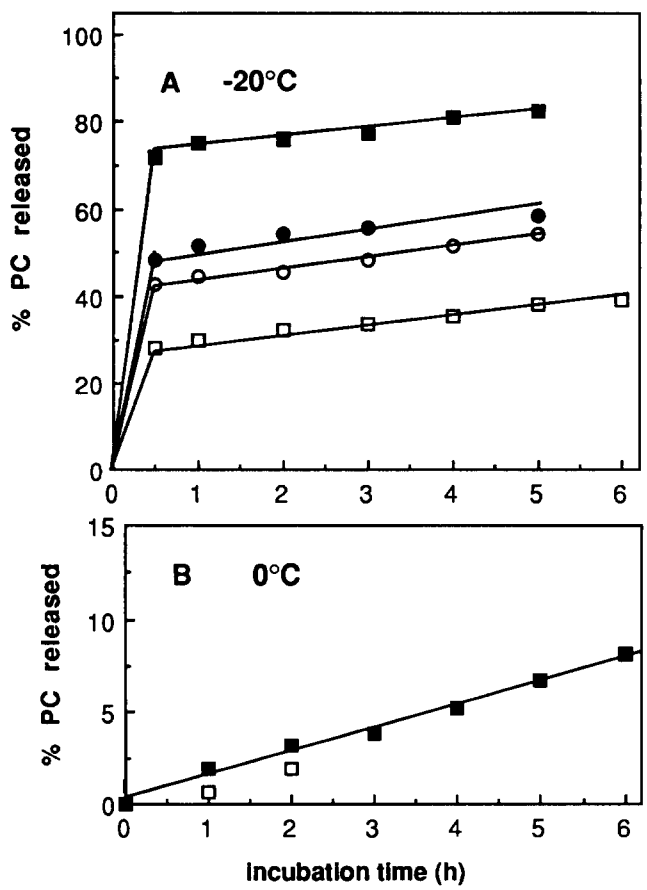

Fig. 1. Effect of the antifreeze protein from the starry flounder (AFP-SF) on time dependent plastocyanin release from thylakoids suspended in an artificial stroma medium (5 mM $MgCl_2$, 10 mM K_2SO_4, 150 mM potassium glutamate and 50 mM sucrose). Samples containing 0 mg/ml (□), 0.166 mg/ml (○), 0.333 mg/ml (●) or 1 mg/ml (■) AFP-SF were incubated at −20°C (A) or 0°C (B) for the times indicated. The amount of plastocyanin released after different incubation times was corrected for the amount released directly after mixing the membranes with the respective incubation solutions (t = 0). The straight lines (in A for incubation times of 0.5 h and longer) were fitted to the data by linear regression analysis (r = 0.99 − 0.96). In B the data from 0 mg/ml and 1 mg/ml samples fell on the same regression line. (PC = plastocyanin).

brachycephalus) by seperating the peptides from blood proteins and solutes on a 2.5 × 150 cm column of Sephadex G-75 (Pharmacia) in 50 mM NH_4HCO_3 according to the method of Cheng and DeVries [23].

All lyophilized antifreeze fractions were 95% pure and readily soluble in water.

Results

Freeze-thaw damage to thylakoid membranes can be quantitated by measuring the release of the soluble lumenal protein plastocyanin which indicates transient rupture of the membrane vesicles [24]. In the following experiments the membranes were suspended in a medium that, in a simplified way, models the conditions in the chloroplast stroma that surrounds the membranes in vivo [21]. The curves derived from samples after a freeze-thaw cycle show the characteristic biphasic time dependence of plastocyanin release (Figs. 1A, 2A, 3A), with a rapid loss during the first 30 min of freezing.

While the physical basis of the rapidly developing damage is not clear, there is good evidence that the slow release of plastocyanin is driven by an influx of solutes into the vesicles during freezing that leads to osmotic rupture during thawing (reviewed in Ref. 17). This type of damage is not limited to freezing conditions, but also occurs in the absence of ice formation (Figs. 1B, 2B, 3B).

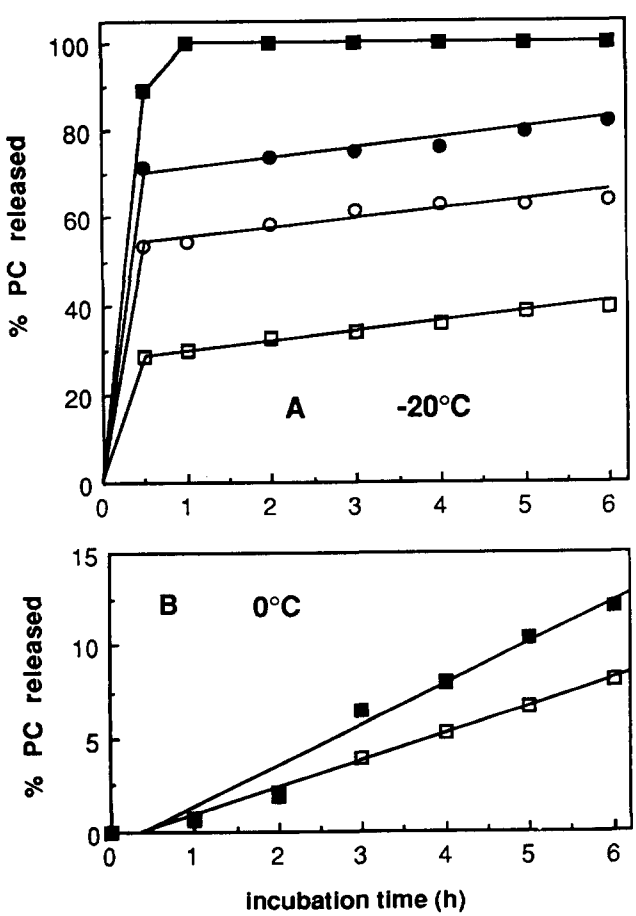

Fig. 2. Toxic effects of the antifreeze protein from antarctic eel pout (AFP-AB) on spinach thylakoid membranes. See legend to Fig. 1 for experimental details ($r = 0.99 - 0.94$).

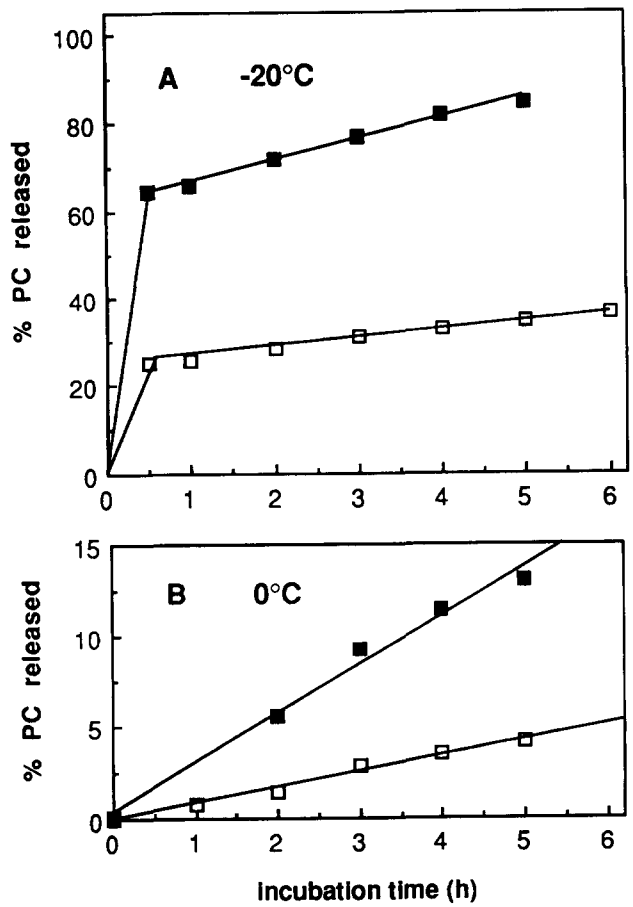

Fig. 3. Effect of AFGP on time dependent plastocyanin (PC) release from thylakoids suspended in an artificial stroma medium. See legend to Fig. 1 for further information ($r = 0.99$ for all curves). Only samples containing no AFGP (□) or 1 mg/ml (■) were compared.

The experimental results in Figs. 1A, 2A and 3A show that both AFPs and the physiological mixture of AFGPs increased plastocyanin release after a freeze-thaw cycle in a concentration dependent way. In seperate experiments we found that AFGP concentrations as low as 50 μg/ml resulted in a measurable increase in freeze-thaw damage to thylakoids (data not shown). In the presence of all proteins investigated, the rapid component of plastocyanin release was strongly increased. Only the AFGPs, however, influenced the slow, linearly time dependent release of plastocyanin at −20°C (Fig. 3A). The rate of plastocyanin release approximately doubled in the presence of 1 mg/ml AFGP as compared to the controls. On a weight per volume basis, the AFP-AB were the most cryotoxic proteins, followed by AFP-SF and AFGP.

In the absence of freezing, AFP-SF at 1 mg/ml showed no effect on plastocyanin release (Fig. 1B). Damage was slightly increased by AFP-AB (Fig. 2B) and strongly by AFGP (Fig. 3B) at the same concentrations, indicating an interaction with the membranes that is independent of ice crystal formation.

AFGP are composed of a peptide backbone with attached disaccharides (see Introduction for details). In an attempt to evaluate the roles that the different components play in cryotoxicity, we have compared the effects of the two sugars making up the disaccharides on freeze-thaw damage to thylakoids. Figure 4A shows that galactose is clearly protective while GalNAc had no effect on the release of plastocyanin after a freeze-thaw cycle at concentrations up to 10 mM. The higher overall amount of plastocyanin released from the thylakoids in these experiments is probably due to seasonal variations in the frost-hardiness of the plant material used to isolate the membranes [25].

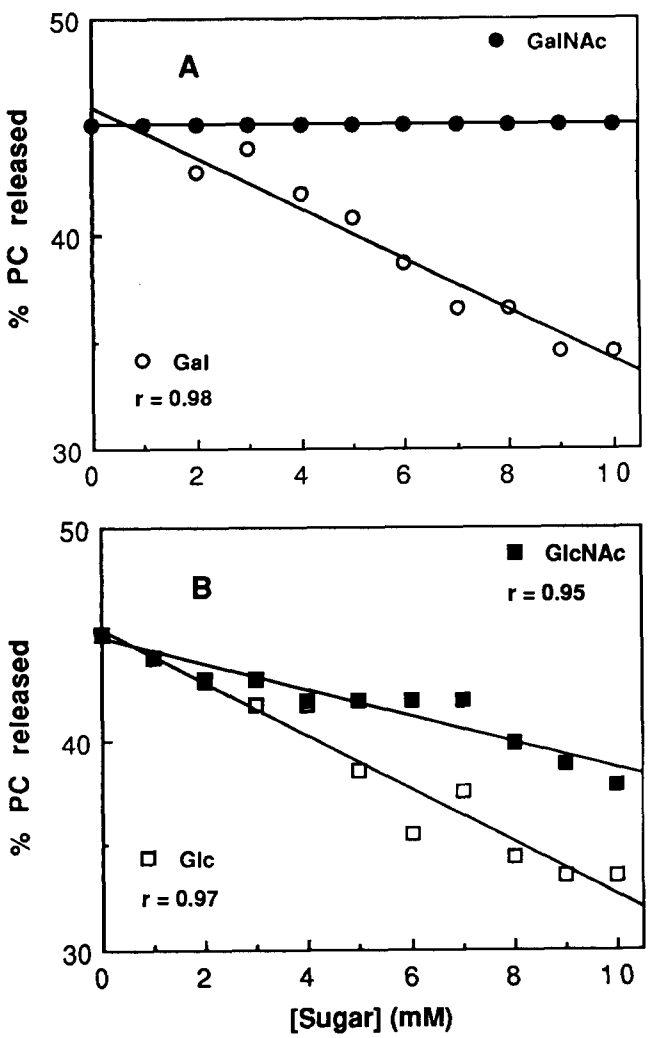

Fig. 4. Cryoprotection of isolated thylakoids by glucose, galactose and the respective *N*-acetylamines in an artificial stroma medium (compare Fig. 1). Samples were incubated at −20°C for 3 h. Freeze-thaw damage was measured as the release of plastocyanin (PC) from the membrane vesicles. All lines were fitted to the data by linear regression analysis. Where appropriate, the correlation coefficients are indicated.

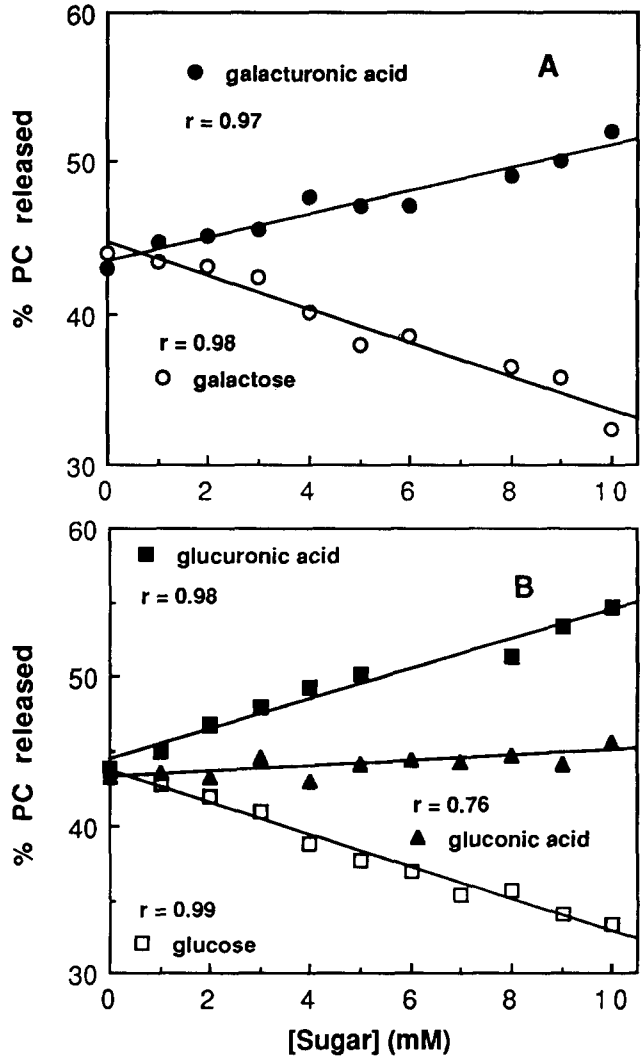

Fig. 5. Cryotoxic effects of the sugar acids derived from galactose (A) and glucose (B). Thylakoids were suspended in an artificial stroma medium. Sugar acids were used as their sodium salts. For other details compare Figs. 1 and 4.

Comparative experiments with glucose and *N*-acetylglucosamine (Fig. 4B) showed that the presence of the acetylamine group in glucose also lead to reduced cryoprotection, although quantitatively the effect is much more pronounced with galactose. Since GalNAc was the first sugar we had found that showed no concentration dependent cryoprotection (compare Refs. 26 and 27) we were interested to see whether this was a unique case. By screening different sugars we found that the addition of an amino group to galactose or glucose had no measurable effect on their cryoprotective efficiency (data not shown), indicating that the acetyl group was a decisive factor.

Surprisingly, sugar acids were found to be much more toxic (Fig. 5). The addition of a carboxyl group at

the C6 position in galactose (Fig. 5A: galacturonic acid) or glucose (Fig. 5B: glucuronic acid) transformed these cryoprotective sugars into effectively damaging solutes. When the carboxyl group was located at position C1 in glucose (Fig. 5B: gluconic acid) this lead to reduced toxicity as compared to glucuronic acid. This indicates that very specific structural requirements exist for a sugar to be either cryoprotective or cryotoxic.

In order to see whether the cryotoxic effects of the sugars are due to an increase in the fast or slow components of plastocyanin release, we performed time dependence experiments as shown in Figs 1–3, with thylakoids in the presence of 10 mM of the respective sugars (Fig. 6). We found that none of the sugars had an effect on the rapid phase of freeze-thaw damage. They did, however, increase the rate of plastocyanin release seen at longer incubation times. This indicates that the differential effects of the sugars can be traced to their ability to increase the solute permeability of the membranes and thereby time dependent osmotic rupture. An apparently neutral solute such as GalNAc increased time dependent plastocyanin release when compared to glucose or galactose and thereby overcame the protective effect that is generally associated with an increase in osmolality [17]. Quantitative differences between the values shown in Figs. 4 and 5, and in Fig. 6 are again attributable to seasonal variations in the plant material used. The qualitative differences between the sugars, however, were always apparent in these experiments (Fig. 6).

An important difference between the mode of action of the AFGP and AFP-AB on the one hand, and AFP-SF and the cryotoxic sugars on the other is that the former lead to increased plastocyanin release already at 0°C (Figs. 2B and 3B) while AFP-SF (Fig. 1B) and the *N*-acetylamine derivatives (at 10 mM) and the sugar acids (tested at concentrations up to 0.5 M) had no measurable effect under these conditions (data not shown).

Discussion

From experiments with mammalian cells and organs it was concluded that AFGP and AFP have the ability to interact with biomembranes (see Introduction for references). Our results, obtained with spinach thy-

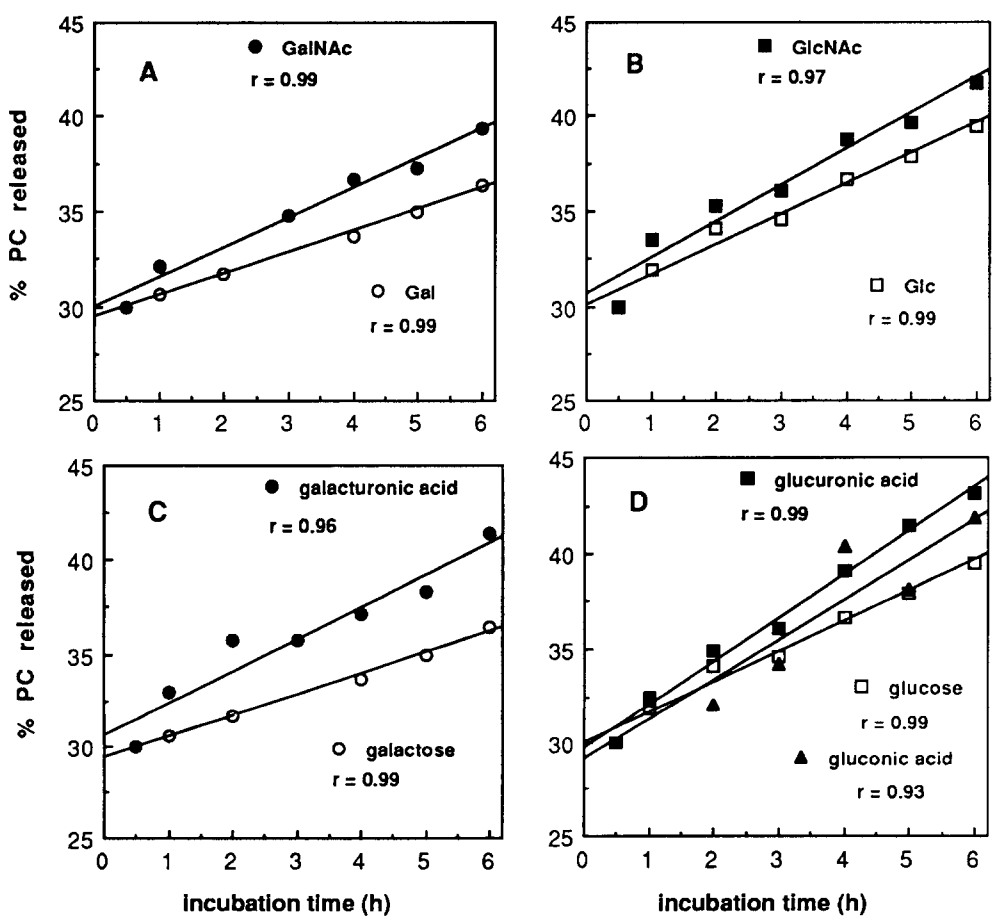

Fig. 6. Effect of different sugars on time dependent plastocyanin (PC) release at −20°C from thylakoids suspended in an artificial stroma medium (see legend to Fig. 1 for experimental details). The values shown are means of three independent experiments. Standard deviations were between 0.5 and 2.

lakoids, support this conclusion (Figs. 1–3). But while in the case of animal cells the interaction enhanced the stability of the plasma membrane, thylakoids were severely damaged. This striking difference may have its basis in the lipid composition of the two membranes. While the membranes of animal cells contain mostly phospholipids, thylakoids contain approx. 80% galactolipids and only 10% phospholipids [28].

This argument implies that the antifreezes interact in some way with the lipid phase of the membranes, despite their hydrophilicity. This is possibly related to their amphiphilc structure. Their solution structure based on CD and NMR studies suggests that they exist in the form of extended 3-fold, left-handed helices such that the threonines and their linked disaccharides are aligned along one side of the helix [29,30]. An X-ray crystallographic investigation of the alanine-rich winter flounder AFP has shown that the molecules form α-helical amphiphilic structures [4]. Investigations with synthetic signal peptides [31] and hemolytic peptides [32] have shown that these are the characteristic structures that lead to the penetration of such peptides into phospholipid bilayer membranes.

AFP-AB shows no α-helical structures in aqueous solutions [33]. It has, however, been shown for the signal sequence of a mitochondrial protein that in some cases peptides may only show α-helix conformation in the presence of detergent micelles or lipid bilayers [34]. Both mitochondrial signal peptides and a chloroplast transit peptide showed a strong preference for interaction with specific lipid classes [34,35].

These findings give us a basis for the hypothesis that the differential effects on mammalian cells and thylakoids are the result of the widely differing lipid composition of the respective membranes. Further experiments will be necessary to test this hypothesis and clarify the different modes of interaction of antifreezes with phospholipid- and galactolipid-rich membranes. Also, the differential cryotoxicity of the proteins might be related to a differential ability to penetrate the lipid bilayer.

Another property of antifreezes that has been related to their cryoprotective activity is recrystallization inhibition during warming of apparently vitrified aqueous solutions. It has been demonstrated recently [36] that red blood cells are protected from freeze-thaw damage under such conditions at low AFP concentrations (5–150 μg/ml) while higher concentrations were damaging. We found no protective effect of AFGP for thylakoids at concentrations from 5–50 μg/ml and increased damage at higher concentrations (data not shown). Also, under our conditions of relatively slow freezing, recrystallization after vitrification can be excluded as a cause of damage.

All antifreezes increased the rapid phase of plastocyanin release during a freeze-thaw cycle (Figs. 1A, 2A, 3A). Cryoprotective plant proteins are the only substances we have found so far that have an effect on the rapid phase of freeze-thaw damage [19] albeit by decreasing plastocyanin release.

The rate of plastocyanin release during the later phase is only increased by AFGP (Fig. 3A). It can also be mediated by a variety of other solutes such as cryoprotective leaf proteins [19] and several different sugars [26,27]. The sugar derivatives tested in this paper (Figs. 4 and 5) also exert their effects on the slow phase of damage (Fig. 6), presumably by altering the solute permeability of the membranes [17].

From our data it seems possible that the increased rate of plastocyanin release measured both at -20°C and at 0°C in the presence of AFGP (Fig. 3) may be enhanced by the GalNAc moiety of the disaccharide. This sugar lead to an increase in the rate of plastocyanin release in frozen samples when compared to glucose or galactose (Fig. 6). The toxicity of GalNAc must, however, be increased by the protein backbone since it is much more efficient as part of the AFGP than as a free solute. The GalNAc concentration in a solution containing 1 mg/ml AFGP is approx. 1.7 mM. Assuming a linear correlation between GalNAc concentration and the increase in the rate of plastocyanin release this would mean that the AFGP-bound GalNAc is about 50-fold more effective than the free sugar if the polypeptide is not involved in this effect at all. This seems a reasonable assumption since both non-glycosylated AFPs show no effect on slow plastocyanin release at -20°C (Figs. 1A, 2A).

This seems not unreasonable if we consider the strong effects already minor changes in the molecular structure of sugars can have on their cryotoxicity (Figs. 4 to 6). A possible partitioning of the AFGP into the membranes, as discussed above, would bring the sugars into much closer contact with possible target sites. This might also increase the cryotoxic effect of GalNAc.

Why substances such as GalNAc, GlcNAc and the sugar acids lead to increased rates of plastocyanin release during the slow phase of damage during freezing can not be explained on the basis of the available data. In the case of the sugar acids, it is certainly not a general effect of organic acids, since most simple organic acids have been shown to be cryoprotectants [37], as are many amino acids [38,39]. The fact that the artificial stroma medium already contains 150 mM glutamate also excludes the occurence of non-specific effects of additional carboxylates at concentrations up to 10 mM. The strong difference between gluconic and glucuronic acid (Fig. 5) which only differ in the carbon atom carrying the carboxyl group, emphasizes the importance of specific molecular structures that we have yet to understand.

The availability of an array of sugars and proteins with widely differing effects on freeze-thaw damage to

thylakoid membranes will enable us in further experiments to clarify the mechanisms by which these substances cause damage or confer protection and to clarify the relationships between molecular structure and functional properties.

On the basis of the data presented in this paper we would suggest that the expression of AFP genes in transgenic plants could lead to severe membrane damage during freezing, especially if the proteins are targeted to the chloroplast compartment of the cells. The plasma membrane might be less susceptible for damage, because it contains no galactolipids [40] and is therefore closer in its lipid composition to a mammalian membrane. However, since the plant plasma membrane contains more than 50 mol% sterols and sterol derivatives, most of which are unique to plant membranes, and also approx. 15 mol% glucocerebrosides [40], it is not possible to extrapolate from either mammalian or thylakoid membranes to the cryobehavior of the plant plasma membrane in the presence of AFPs.

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