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Antifreeze proteins differentially affect model membranes during freezing

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Abstract

Over the past decade antifreeze proteins from polar fish have been shown either to stabilize or disrupt membrane structure during low temperature and freezing stress. However, there has been no systematic study on how membrane composition affects the interaction of antifreeze proteins with membranes under stress conditions. Therefore, it is not possible at present to predict which antifreeze proteins will protect, and which will damage a particular membrane during chilling or freezing. Here, we analyze the effects of freezing on spinach thylakoid membranes and on model membranes of varying lipid composition in the presence of antifreeze protein type I (AFP I) and specific fractions of antifreeze glycoproteins (AFGP). We find that the addition of galactolipids to phospholipid model membranes changes the effect each protein has on the membrane during freezing. However, the greatest differences observed in this study are between the different types of antifreeze proteins. We find that AFP type I and the largest molecular weight fractions of AFGP induce concentration dependent leakage from, and are fusogenic to the liposomes. This is the first report that an antifreeze protein induces membrane fusion. In contrast, the smallest fraction of AFGP offers a limited degree of protection during freezing and does not induce membrane fusion at concentrations up to 10 mg/ml.

Keywords: Antifreeze protein; Liposome; Freezing stress; Membrane fusion; Galactolipid

1. Introduction

Antifreeze proteins (AFP) and antifreeze glycoproteins (AFGP) lower the freezing point of water non-colligatively and inhibit ice recrystallization during rewarming of frozen solutions. Such proteins have been found in polar fish, cold hardy plants, and over-wintering insects [1–4] and are structurally extremely diverse. Their physiological role in fish and insects is to lower the freezing point of body fluids in a non-colligative manner, a process termed thermal hysteresis, and thereby help the animals to avoid lethal freezing events. The fish proteins show a thermal hysteresis of between 0.6 and 1.5°C [4], while insect antifreeze proteins decrease the freezing point of solutions by up to 6°C [5]. In contrast, plant antifreeze proteins exhibit a smaller degree of thermal hysteresis, typically 0.2–0.4°C, but are extraordinary inhibitors of ice recrystallization [6–9]. Since these plants can survive freezing events, the physiological role of

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AFPs may be to protect plants during rewarming after freezing.

In addition to their role as inhibitors of ice crystal growth, AF(G)Ps affect membrane stability during low temperature and freezing stress. Enhanced survival of mammalian oocytes in the presence of AFGPs and AFPs was observed after hypothermic exposure [10,11] and during freezing in the presence of AFGPs [12,13]. Subsequently, Hays et al. [14] found that AFGPs could prevent leakage from liposomes composed solely of phospholipids as they are cooled through their phase transition temperature (T_m), suggesting that the peptides interact with lipids to stabilize membranes at low temperature.

Studies of freezing cells and tissues with antifreeze proteins also have shown that these peptides can have a toxic effect on viability, which, in some applications, can be beneficial. For example, high concentrations of AFPs have recently been proposed as adjuvants during cryosurgery to increase cell destruction during freezing of malignant tumors [15]. Freezing studies have shown that a 'physiological' mixture of the eight AFGP size fractions (2.3–34 kDa), AFP I and AFP III are cryotoxic to plant thylakoids, as determined by the leakage of plastocyanin, a soluble thylakoid lumen protein [16]. In addition to the damage after freezing, AFGPs and AFP III even caused leakage of plastocyanin when the samples were incubated at 0°C. The smallest molecular weight AFGP fraction, fr. 8, only had a minor influence on the stability of thylakoids during freezing, while the larger fractions, fr. 3 and 4, were clearly cryotoxic [17].

However, the membrane topology of thylakoids, which contain approx. 80% galactolipids [18], is very different from mammalian cell membranes, where phospholipids predominate. Therefore, it is not possible to extrapolate the findings from thylakoid studies directly to other systems. Now we have used model membranes containing either the thylakoid lipids monogalactosyldiacylglycerol (MGDG), or digalactosyldiacylglycerol (DGDG), or only phospholipid to clarify whether lipid composition determines the effects of AFGPs or AFP I on membrane stability during freezing. We also investigated whether all AFGP fractions were indeed cryotoxic to membranes of these different lipid compositions and determined that, while AFP I and AFGP fr. 1–5 and fr. 3,4 are fusogenic during freezing, AFGP fr. 8 protects DGDG-containing membranes during freezing.

2. Materials and methods

2.1. Lipids and proteins

Egg phosphatidylcholine (EPC), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Galactolipids (MGDG and DGDG) were purchased from Lipid Products (Redhill, Surrey, UK). AFGP fractions were isolated as described from serum of the antarctic fish *Trematomus borchgrevinki* [19]. AFP type I was a gift of A/F Protein (Boston, MA, USA) and, with SDS-PAGE analysis followed by silver stain, was visualized as a single band (data not shown). Proteins were further purified by acetone precipitation or ether extraction, as described by Hays et al. [14], in order to remove any hydrophobic contaminants. Mass spectrometry analysis of the AFGP fractions showed the presence of a 300 MW contaminant at a concentration of less than 0.1%, which was removed by both the acetone and ether.

2.2. Liposome preparation

Liposomes were composed (on a weight basis) of 50% DGDG/50% EPC, 15% MGDG/85% EPC, or 100% EPC. MGDG is a non-bilayer lipid and, therefore, liposomes are severely destabilized with higher concentrations in the membranes [20]. Liposomes for leakage studies were made as previously described [20]. Briefly, 12 mg of lipid were hydrated in 600 μl of 100 mM carboxyfluorescein (CF; Molecular Probes, Eugene, OR, USA; purified according to [21]) in 10 mM TES and 0.1 mM EDTA (pH 7.4) and extruded using a Liposofast hand-held extruder ([22]; Avestin, Ottawa, Canada) with 100 nm pore filters (Poretics, Livermore, CA, USA). To remove external CF, the liposomes were passed over a Sephadex G-50 column in 10 mM TES, 0.1 mM EDTA and 50 mM NaCl (TEN buffer, pH 7.4). Liposomes

for fusion assays were made with the same lipid composition as for leakage, with the addition of 1 mol% each of the fluorescent probe pair NBD-PE and Rh-PE.

2.3. Liposome freezing experiments

Equal volumes of liposomes (10 mg/ml lipid) and protein solutions in TEN were combined (40 μl/sample) to reach the final protein concentrations indicated in the figures. Samples were frozen rapidly in an ethylene glycol bath precooled to −18°C, and incubated at that temperature for 3 h. After incubation, samples were warmed quickly to room temperature in a water bath. Controls were incubated in the dark on ice for 3 h.

CF fluorescence is self-quenching when the dye is trapped inside the liposomes at high concentrations. When CF leaks out of liposomes into solution, it is diluted and fluorescence is increased. Leakage was determined by mixing 5 μl liposome sample with 3 ml TEN buffer in a cuvette, with continuous stirring, and measuring fluorescence at room temperature with an Hitachi F2000 fluorometer (San Jose, CA, USA) at excitation and emission wavelengths of 460 nm and 550 nm, respectively. Total liposome CF content (100% leakage) was determined by adding 50 μl 1% Triton X-100 to the cuvette. Fluorescence of unfrozen samples without protein was set as 0% leakage.

Liposome fusion after freezing and thawing was determined using fluorescence resonance energy transfer [23] as described in detail [20]. Two liposome samples were prepared: one sample was labeled with both NBD-PE and Rh-PE, while the other sample was unlabeled. The two samples were combined after extrusion in a 1:9, labeled:unlabeled ratio, resulting in a final lipid concentration of 10 mg/ml. The liposomes were mixed with protein solution in the same manner as for the leakage experiments. After the 3 h incubation, fusion was measured by FRET with an Hitachi F2000 fluorometer at excitation and emission wavelengths of 450 and 530 nm, respectively. For this measurement, 20 μl of the liposome sample was mixed with 2 ml TEN buffer, and fluorescence was measured before (F) and after (F_0) the addition of 50 μl 1% Triton X-100. The energy transfer efficiency (E) between the two fluorophores was calculated as $E = 1 - F/F_0$. E was calculated for both control samples incubated on ice (E_0) and for samples frozen at −18°C (E_s). Vesicle fusion was calculated as % fusion $= 100 - ((E_s/E_0) \times 100)$. Therefore, fusion was detected as the decrease in energy transfer between the two fluorescent probes, as the probes were diluted into the unlabeled liposomes.

2.4. Graph normalization

Data on graphs were normalized in order to compare relative changes in leakage under different conditions. The data sets were adjusted individually so that each set started at the same level of leakage at 0 mg/ml protein. This allows quantitative comparisons to be made between the different lipid compositions over the indicated protein range.

2.5. Thylakoid isolation

Thylakoids were isolated from spinach purchased at local markets in Davis, CA, USA, as described previously [24], with some minor modifications. Briefly, spinach leaves (180 g) were homogenized in 180 ml 300 mM NaCl, 30 mM tricine, 3 mM $MgCl_2$, 0.5 mM EDTA (pH 7.8), filtered through four layers of cheesecloth and centrifuged to 7700 rpm (4°C), and the centrifuging was immediately stopped. Pellets were resuspended in 10 mM $MgCl_2$ and 20 mM K_2SO_4 and pelleted at 3000 rpm for 5 min (4°C); this wash was repeated once. Thylakoids were then suspended 1:1 (v/v) in 300 mM Na-glutamate and 100 mM sucrose (incubation buffer, pH 8.0). Chlorophyll concentration was determined according to Arnon [25].

2.6. Thylakoid freezing experiments

Protein and incubation buffer were combined with a volume of thylakoids containing 50 μg chlorophyll to reach the indicated protein concentrations in 40 μl samples. One set of samples was placed in the dark on ice and another set was rapidly frozen in an ethylene glycol bath precooled to −18°C. The samples were incubated for 3 h. After incubation, samples were pelleted at 12 000 rpm at 4°C for 5 min. Control samples were centrifuged directly following isolation. Total plastocyanin content was determined by soni-

cating thylakoid aliquots with a pulse sonicator (Virtis, Gardiner, NY, USA) in three brief pulses, while the sample remained on ice. The sample was pelleted and supernatant pipetted off, placed at −20°C for 1 h and pelleted again.

All sample supernatants were subjected to SDS-PAGE [26] analysis (15% acrylamide). Polypeptides were transferred to PVDF membranes (Millipore, Bedford, MA, USA) by electroblotting [27]. Leakage of proteins from thylakoids was determined immunologically with anti-plastocyanin antibody [28,29]. The membrane was blocked with 4% non-fat dried milk in Tris buffered saline (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) and subsequently incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Bio-Rad, Hercules, CA, USA). The protein was visualized with ECF substrate (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) on a STORM phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA), from which a densitometry plot was determined (Image Quant Software, Molecular Dynamics).

3. Results and discussion

The present study differs from that of Hincha et al. [16] and extends their study in several important ways. First, we use proteins from different species of fish than used in the previous report. Namely, AFGP from *T. borchgrevinki* and AFP I from the winter flounder *Pleuronectes americanus*, as opposed to AFGP from *Dissostichus mawsoni* and AFP I from *Platichthys stellatus* in the earlier study. Therefore, we performed freezing experiments with intact thylakoids to test the effects of these proteins on membrane stability. Additionally, we extended the concentration range of the proteins from 0–1 mg/ml [16] to 0–15 mg/ml. Most importantly we studied how clearly defined fractions of AFGP affect model membranes of varying composition during freezing.

Fig. 1 shows that the proteins used in the present study behaved similar to those investigated earlier. Using densitometry after Western blotting we were able to determine that AFGP fr. 1–5 induced more plastocyanin leakage during freezing, 87% at 15 mg/ml, than AFGP fr. 8, which caused 55% leakage at the same concentration. AFP I had the strongest effect on plastocyanin leakage, inducing nearly 100% leakage at 15 mg/ml. There were no clear effects of any of the proteins on thylakoid stability when the samples were incubated at 0°C. This is not surprising, as the effects reported previously [16] were small and probably not detectable on Western blots.

In order to determine whether the cryotoxicity of antifreeze proteins for thylakoids is related to the presence of chloroplast galactolipids, we prepared liposomes containing either mixtures of MGDG and EPC, DGDG and EPC, or EPC alone, and performed freezing experiments. The liposomes were

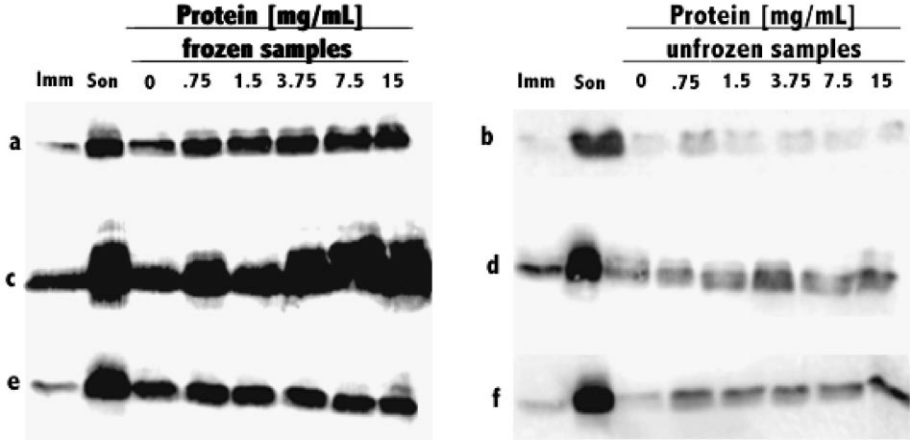

Fig. 1. Plastocyanin leakage from thylakoids after freezing with antifreeze proteins. Thylakoids incubated with: (a,b) AFGP fr. 1–5, (c,d) AFP I, (e,f) AFGP fr. 8 (unprecipitated). Panels a, c and e are frozen samples; b, d and f are unfrozen samples. Imm, supernatant taken from samples immediately after thylakoids were isolated; Son, supernatant from sonicated samples.

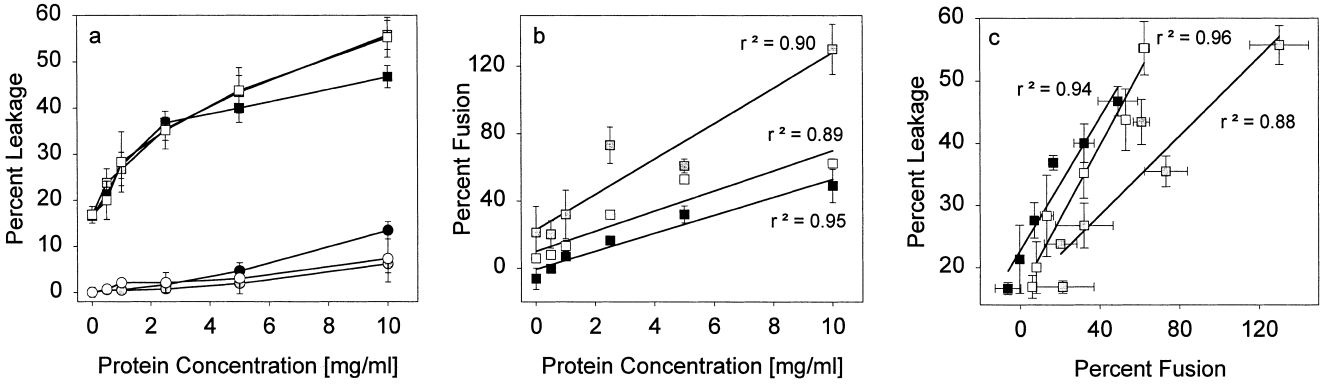

Fig. 2. AFP I is damaging to liposomes during freezing. (a) CF leakage from samples frozen at −18°C (squares) or stored at 0°C (circles) for 3 h. (b) Percent fusion of frozen samples plotted against protein concentration. (c) Percent leakage plotted as a function of percent fusion for frozen samples. Linear regressions in b and c were calculated with a 99% confidence interval. Solid symbols denote samples with EPC liposomes, gray symbols liposomes containing 15% MGDG/85% EPC, and open symbols liposomes containing 50% DGDG/50% EPC. The values are the means from at least three samples. Where standard error bars are not visible, they are smaller than the symbol.

frozen under the same conditions as the thylakoids, and the proteins were added only to the outside of the vesicles to make the experiments comparable to both the thylakoid experiments and previous liposome chilling experiments [14]. We found that AFP I was cryotoxic to all three types of liposomes, already causing a significant increase in CF leakage over the control at a protein concentration of 1 mg/ml (Fig. 2a). At 10 mg/ml, the EPC liposomes lost 30% more CF than the frozen control, and the MGDG- and DGDG-containing liposomes lost 40% more CF than the control (Fig. 2a). AFP I also led to a concentration-dependent, 10% increase in CF leakage in the EPC unfrozen samples. In contrast to these data, we found that AFP I completely inhibited leakage from DGDG-containing liposomes during chilling through their T_m (M.M. Tomczak et al., unpublished data). This illustrates that antifreeze proteins can have different effects on membranes under different conditions.

In order to determine a possible physical mechanism of the cryotoxicity of antifreeze proteins, we

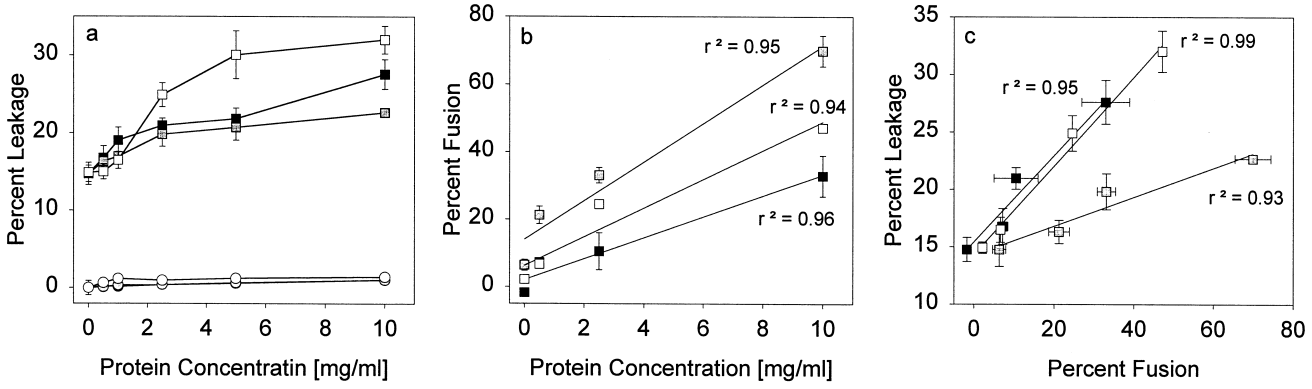

Fig. 3. AFGP fr. 3,4 causes damage to liposomes during freezing. (a) Leakage from samples frozen at −18°C (squares) and stored at 0°C (circles). (b) Percent fusion of frozen samples plotted against protein concentration. (c) Percent leakage plotted as a function of percent fusion for frozen samples. Linear regressions were calculated with a 99% confidence interval. Solid symbols denote samples with EPC liposomes, gray symbols liposomes containing 15% MGDG/85% EPC, and open symbols liposomes containing 50% DGDG/50% EPC. For all samples, $n = 3$.

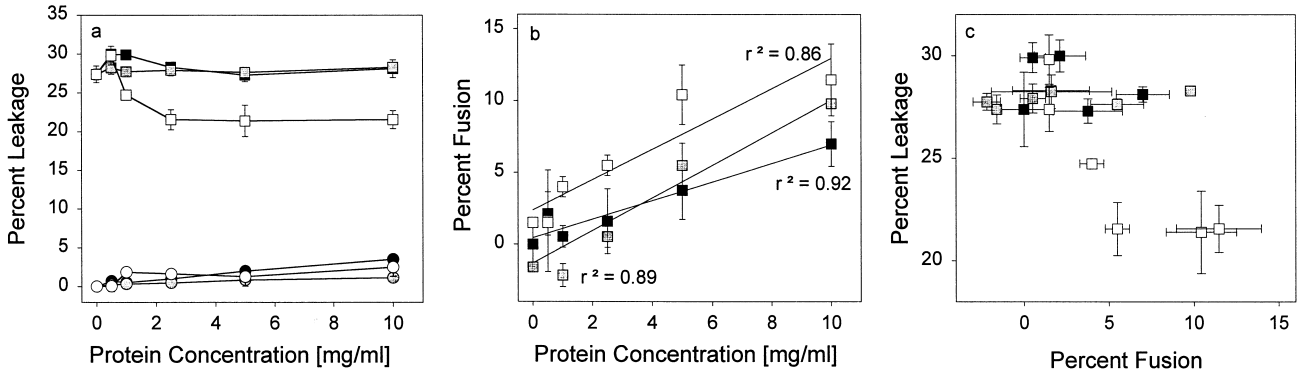

Fig. 4. AFGP fr. 8 inhibits leakage from DGDG-containing liposomes and does not induce membrane fusion. (a) Percent leakage from liposomes incubated with AFGP fr. 8, $n=9$; squares represent samples frozen at −18°C for 3 h and circles represent samples stored at 0°C. (b) Percent fusion of frozen liposomes plotted as a function of protein concentration. Linear regressions were calculated with a 99% confidence interval. (c) Percent leakage of frozen samples plotted as a function of percent fusion. EPC liposomes are represented by solid symbols, 15% MGDG/85% EPC liposomes by gray symbols, and 50% DGDG/50% EPC liposomes by open symbols.

also measured membrane fusion under the same conditions. In Fig. 2b membrane fusion is plotted as a function of protein concentration for the frozen samples. Clearly, there was a linear correlation between fusion and protein concentration for all three types of liposomes during freezing with AFP I. This correlation was very similar for pure EPC and DGDG-containing liposomes, while significantly more fusion occurred in the MGDG-containing liposomes. At high protein concentrations, the fusion of the MGDG:EPC liposomes was calculated to be greater than 100%. This occurred because the measured fluorescence values were not corrected for the quenching of fluorescent probes by Triton X-100. This quenching results in a proportional overestimation of fusion values for all samples, so the results from different samples still can be compared relative to each other. Therefore, we conclude that the MGDG-containing liposomes undergo significantly more fusion in the presence of AFP I during freezing than the other two types of liposomes. This may be due to the fact that MGDG is a non-bilayer lipid, and such lipids are known to promote membrane fusion.

In order to determine if leakage and fusion were correlated, we plotted leakage as a function of fusion (Fig. 2c) and found that the relation between the two is linear. These results suggest that the increase in leakage and fusion are related events caused by increasing protein concentration during freezing. There was no fusion in the samples incubated on ice, regardless of protein concentration (data not shown).

In the experiments with AFGP we used three different protein preparations: AFGP fr. 1–5 (32–10 kDa), AFGP fr. 3,4 (20, 17 kDa) and AFGP fr. 8 (2.7 kDa). Since AFGP fr. 1–5 and AFGP fr. 3,4 had almost identical effects on liposome stability, we present only results obtained with AFGP fr. 3,4. Fig. 3a shows that, although AFGP fr. 3,4 increased CF leakage from liposomes during freezing, this increase was much smaller than for AFP I. MGDG-containing liposomes showed the smallest effect of increasing protein concentration on leakage, while DGDG-containing liposomes showed the largest effect. There was no increase in CF leakage at 0°C from any of the liposomes in the presence of AFGP fr. 3,4. Surprisingly, the effect of AFGP on membrane fusion during freezing was qualitatively very similar to the effect of AFP I. There was a linear increase in membrane fusion with increased protein concentration after freezing, and percent fusion and percent leakage were linearly correlated after freezing with AFGP fr. 3,4, as well (Fig. 3b,c), although AFGP fr. 3,4 induced much less fusion in all membranes than AFP I (see Fig. 2b,c). Again, MGDG-containing liposomes showed the greatest degree of membrane fusion of all three liposome types. This suggests that the antifreeze proteins take advantage of the fact that MGDG is a non-bilayer lipid in

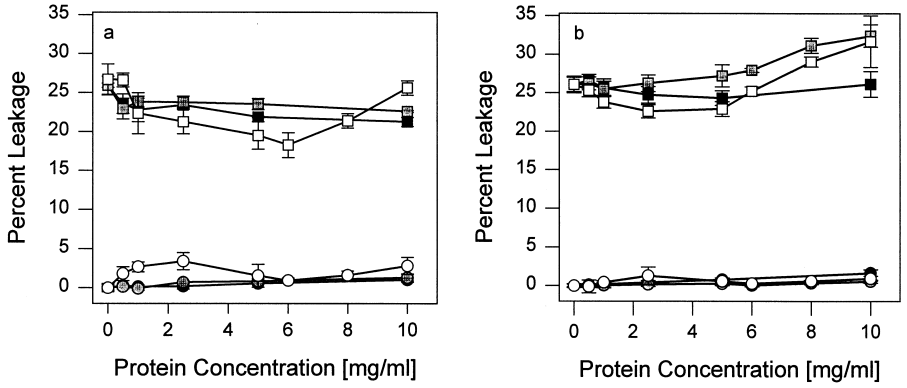

Fig. 5. AFGP fr. 8 variants at low concentrations inhibit leakage from DGDG liposomes. Percent leakage from liposomes incubated with (a) acetone precipitated AFGP fr. 8; (b) *E. gracilis* AFGP fr. 8. $n=3$. Squares represent samples frozen at −18°C, and circles represent samples stored at 0°C. Solid symbols denote samples with EPC liposomes, gray symbols liposomes containing 15% MGDG/85% EPC, and open symbols liposomes containing 50% DGDG/50% EPC.

order to induce relatively more membrane fusion after freezing than the other liposome types. Since DGDG-containing liposomes are less affected than MGDG-containing liposomes, the increase in fusion seems not to be related to the presence of glycolipids in general.

Three variations of AFGP fr. 8, the smallest molecular weight AFGP, were used in this study. AFGP fr. 8 was either used without further purification (Fig. 4) or after acetone precipitation (Fig. 5a). The third type comes from the arctic fish *Eleginus gracilis* (Fig. 5b), and contains a carboxy terminal Arg-Ala motif in addition to the 14 amino acids normally present in AFGP fr. 8, rendering the peptide slightly more basic [30]. The addition of *E. gracilis* AFGP fr. 8 did not alter the sample pH.

After freezing with unprecipitated AFGP fr. 8 there was a decrease in CF leakage with increasing protein concentration from DGDG:EPC liposomes (Fig. 4a). Also in contrast to the results with the larger molecular weight fractions (see Fig. 3), the MGDG:EPC and pure EPC liposomes showed no significant increase in leakage during freezing in the presence of AFGP fr. 8 (Fig. 4a). We then tested whether AFGP fr. 8 had an influence on fusion during freezing. As shown in Fig. 4b, there was a slight increase in membrane fusion of the liposomes with increased protein concentration, but this level of fusion is negligible when compared to the fusion induced at 10 mg/ml by AFGP fr. 3,4 (Fig. 3b) or AFP I (Fig. 2b). Additionally, there was no linear relationship between fusion and leakage for any liposome type when leakage was plotted as a function of fusion (Fig. 4c). This is in contrast to the linear relationships observed with both AFP I and AFGP fr. 3,4 (Figs. 2c and 3c, respectively).

A decrease in leakage from DGDG:EPC liposomes was also seen during freezing with acetone precipitated AFGP fr. 8 at concentrations up to 6 mg/ml (Fig. 5a). CF leakage increased at higher concentrations. There was a slight decrease in leakage at 0.5 mg/ml acetone precipitated AFGP fr. 8 from both the MGDG:EPC and pure EPC liposomes compared with the frozen control, and this was maintained over the entire protein concentration range studied (Fig. 5a). Acetone precipitation or ether extraction of AFP I or AFGP fr. 3,4 did not change the freeze-induced leakage from any of the liposomes compared with the unprecipitated protein described above (data not shown). These results are in contrast with the report from Hays et al. [14], who found that acetone precipitation could greatly enhance the membrane stabilizing effects of the protein during chilling.

E. gracilis AFGP fr. 8 caused a slight initial decrease in leakage from DGDG:EPC liposomes at low concentrations (Fig. 5b). This was followed by an increase in CF leakage between 6 and 10 mg/ml. The MGDG:EPC liposomes followed a leakage pattern similar to the DGDG:EPC liposomes; however, no initial decrease in leakage was seen after freezing (Fig. 5b). Leakage from pure EPC liposomes was unchanged with increased *E. gracilis* AFGP fr. 8 concentration. The data from the experiments with

the different AFGP fr. 8 suggest that it is not cryotoxic to thylakoids or to liposomes during freezing, especially at low protein concentrations. Indeed, AFGP fr. 8 offers a small degree of protection to DGDG:EPC liposomes.

In order to determine if the effects seen with the different AFGP fractions were due to interactions of the sugar groups with the membranes, liposomes were frozen with galactose or *N*-acetyl galactosamine. Neither sugar had an influence on CF leakage from the liposomes during freezing at concentrations up to 10 mg/ml (data not shown). Incubation of the liposomes with the glycoproteins ovotransferrin or ovomucoid (25% carbohydrate by weight), neither of which have antifreeze activity, also had no influence on leakage at the same protein concentrations that were used for the antifreeze proteins (data not shown). These results suggest the effects reported above are specific for the AFGPs, and not non-specific effects of sugars or glycoproteins.

In conclusion, our study has shown that not all AFGPs are cryotoxic to membranes containing thylakoid galactolipids. AFGP fr. 8 offered protection, however slight, to DGDG-containing liposomes and had little effect on MGDG and pure EPC liposomes during freezing. In contrast, this study and the previous study [16] show that AFP I is cryotoxic to thylakoids and liposomes. The damaging effects of AFGP fr. 3,4 depend critically on lipid composition, with striking differences between membranes containing MGDG or DGDG. In addition, we report for the first time that antifreeze proteins can induce membrane fusion and that the degree of fusion also depends on membrane lipid composition, with the highest degree of fusion observed in membranes containing the non-bilayer lipid MGDG.

The greatest differences observed in this study, however, were the differences between the different antifreeze proteins, regardless of membrane composition. The results suggest that AFP I may be damaging to membranes in general after freezing, while AFGP fr. 8 might have a slightly protective effect. However, it is difficult to determine if the findings of the liposome studies will extend to whole cells or tissues. These findings stress the complexity of the interactions that take place between antifreeze proteins and membranes. The current data suggest that AFP I will be cryotoxic to most membranes; however, a prediction of whether an antifreeze protein will protect or damage a membrane after freezing does not appear possible without experimentation.

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