Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 6835–6840, June 1996
Biophysics

Antifreeze glycoproteins inhibit leakage from liposomes during thermotropic phase transitions

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Communicated by Peter Marler, University of California, Davis, CA, March 12, 1996 (received for review January 30, 1996)

ABSTRACT **Antifreeze glycoproteins (AFGPs), found in the blood of polar fish at concentrations as high as 35 g/liter, are known to prevent ice crystal growth and depress the freezing temperature of the blood. Previously, Rubinsky *et al.* [Rubinsky, B., Mattioli, M., Arav, A., Barboni, B. & Fletcher, G. L. (1992) *Am. J. Physiol.* 262, R542–R545] provided evidence that AFGPs block ion fluxes across membranes during cooling, an effect that they ascribed to interactions with ion channels. We investigated the effects of AFGPs on the leakage of a trapped marker from liposomes during chilling. As these liposomes are cooled through the transition temperature, they leak ≈50% of their contents. Addition of less than 1 mg/ml of AFGP prevents up to 100% of this leakage, both during chilling and warming through the phase transition. This is a general effect that we show here applies to liposomes composed of phospholipids with transition temperatures ranging from 12°C to 41°C. Because these results were obtained with liposomes composed of phospholipids alone, we conclude that the stabilizing effects of AFGPs on intact cells during chilling reported by Rubinsky *et al.* may be due to a nonspecific effect on the lipid components of native membranes. There are other proteins that prevent leakage, but only under specialized conditions. For instance, antifreeze proteins, bovine serum albumin, and ovomucoid all either have no effect or actually induce leakage. Following precipitation with acetone, all three proteins inhibited leakage, although not to the extent seen with AFGPs. Alternatively, there are proteins such as ovotransferrin that have no effect on leakage, either before or after acetone precipitation.**

In polar regions of the oceans, fish live in waters with temperatures near the freezing point of sea water (1–3). Several species of these fish synthesize antifreeze glycoproteins (AFGPs) or antifreeze proteins (AFPs), which lower the serum freezing temperatures noncolligatively (4–6) without affecting the melting point (7). During cooling, the proteins may bind to ice nucleators (8) or ice-like water clusters (9) in the blood, thus delaying the initiation of ice crystal growth. Besides freezing point depression, AFGPs may also function as inhibitors of ice recrystallization during warming (10, 11).

The AFGPs are found primarily in Antarctic Nototheniid and some northern cod (12). These proteins are repeats of the glycotripeptide Ala-Ala-Thr, with the disaccharide, galactose-*N*-acetylgalactosamine, bound to Thr. The protein tertiary structure has been characterized as a left-handed α-helix with the sugars coplanar, located on one side of the backbone (13, 14), but some gamma turns have been reported (15). Eight fractions of AFGP have been classified according to their molecular mass (33.7–2.6 kDa) (5, 16). Fractions 7 and 8, which are the smallest, account for ≈80% of the total AFGP in the serum and have certain alanine residues replaced with proline.

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Proteins with antifreeze activity are also known to be present in insects (17), and indirect evidence has been provided for their presence in a variety of terrestrial plants (18, 19). In these cases, it is difficult to designate a function to the proteins because the depression of the freezing temperature is often extremely small, on the order of 0.25°C, which would not appear to provide significant antifreeze protection. Rubinsky *et al.* (20) discovered an alternative function for AFGPs. They provided evidence that these molecules may protect membrane integrity during cooling by blocking ion fluxes across membranes (20, 21). They suggest that this effect may be due to a direct interaction of the AFGPs with ion channels, a suggestion that has become controversial (22–24). In the present work, we confirm the suggestions of Rubinsky *et al.* (21) that the proteins protect membranes during chilling, but we propose a distinctly different mechanism.

As membranes are cooled to low temperatures, one source of injury is often thermotropic phase transitions (25). During the liquid crystalline to gel phase transition, membranes become leaky, resulting in loss of intracellular contents (for review, see ref. 26). It is not entirely clear what causes the leakage during the phase transition, but it may be due to defects in packing of the hydrocarbon chains during the coexistence of gel and liquid crystalline domains (25, 27). Using pure phospholipid vesicles as a model membrane system, we report here that AFGPs can prevent leakage during this transition.

MATERIALS AND METHODS

AFGPs were isolated from the serum of two Antarctic fish species: *Dissostichus mawsoni,* and *Trematomus borchgrevinki.* The separation and purification of the AFGP were carried out as previously described (5). Some fractions, such as *D. mawsoni* 2–6+, contain fractions 2 through 6 plus a fraction that has a molecular mass slightly higher than 4.6 kDa. AFP type I from winter flounder was a gift from A/F Protein (Boston). These peptides possess no carbohydrates but may contain up to 60% alanine, as well as some hydrophilic amino acids (28). Freeze-dried AFGPs and AFPs were readily dissolved in water to make concentrations ranging between 1 mg/ml and 10 mg/ml. Concentrations of AFGPs and AFPs in the fish blood during the winter months can reach 35 mg/ml (29), so the concentrations used here are physiologically realistic.

In later experiments, we further purified the proteins by acetone precipitation. The proteins (10 mg) were dissolved in 1 ml of water to which 3 ml of cold acetone was added. This resulted in precipitation of the AFGPs. The supernatant was removed by aspiration, after which the residual acetone was

Abbreviations: AFGPs, antifreeze glycoproteins; AFPs, antifreeze proteins; DEPC, dielaidoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; egg PC, egg phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; T_m, transition temperature; CF, carboxyfluorescein; DSC, differential scanning calorimetry.

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evaporated with N_2 gas. The proteins were then resuspended in water and freeze-dried.

Dielaidoylphosphatidylcholine (DEPC), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and egg phosphatidylcholine (egg PC) were purchased from Avanti Polar Lipids. Lipid was dried under nitrogen gas, placed under vacuum to remove all chloroform, and then dispersed in 200 mM carboxyfluorescein (CF) purchased from Molecular Probes and purified according to Weinstein *et al.* (30). Liposomes were prepared using a Liposofast hand-held extruder (Avestin, Ottowa) with 0.1 μm pore filters (Poretics, Livermore, CA). External CF was removed from the liposomes by passing them through a Sephadex G50–80 (Sigma) column (2 cm × 20 cm). The final concentration of the liposomes was ≈20 mg/ml. Liposomes (5 μl) were placed into a methacrylate cuvet containing 10 mM TES buffer, pH 7.5, a stirring bar, and a thermocouple. At a concentration of AFGP at 1 mg/ml, the protein/lipid ratio is ≈17:1 on a weight/weight basis. The total cuvet volume with or without protectant was 1.75 ml. The DEPC liposomes [transition temperature (T_m) = 12°C] were cooled from 20°C to 0°C at a rate of ≈0.5°C per min. Because four samples can be cooled simultaneously, a control was always run beside the experimental samples. The point of initiation of leakage with repeated controls varied ±1°C. Fluorescence and temperature were recorded by a microcomputer coupled to a Perkin–Elmer model LS-5 fluorometer and the thermocouple (Model 871 Digital Thermometer, Omega Engineering, Stamford, CT). After chilling to 0°C, the liposomes were lysed with Triton X-100, thus providing a measure of total trapped CF.

Freezing temperatures of solutions were measured with a freezing point osmometer (Advanced Instruments, Needham Heights, MA), which senses the heat of fusion on freezing. After solutions of the longer AFGPs are supercooled in a −4°C water bath, a vibrating wire initiates crystallization at −3°C (31). Solutions of the short AFGPs were vibrated at −1°C (32).

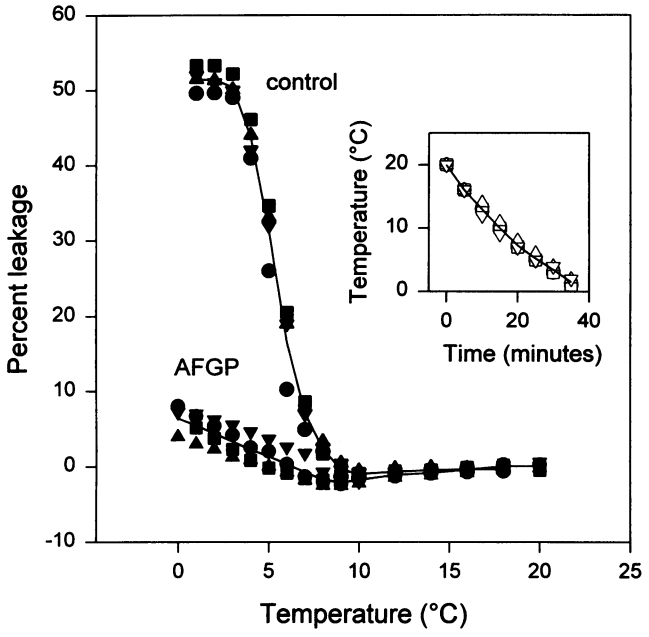

FIG. 1. Percent leakage of CF from DEPC liposomes upon cooling through the lipid phase transition temperature of 12°C. The lines show the average of four independent experiments; the raw data for those experiments are indicated by the points. AFGPs are fractions *D. mawsoni* 2–6+ at a concentration of 1 mg/ml. Control samples have only liposomes. There is 95% confidence that the AFGPs are significantly different from the control using the Student's *t* distribution. (*Inset*) The repeatability of the cooling rate from 20°C to 0°C. Results of four independent experiments are shown.

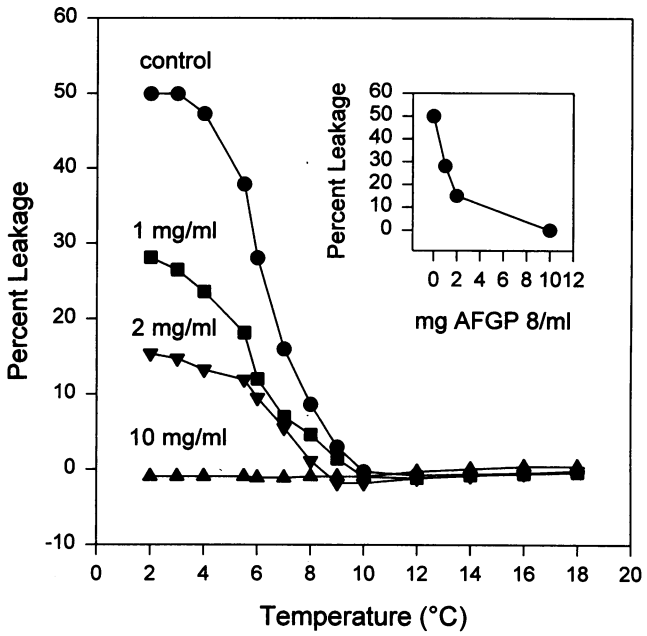

FIG. 2. Percent leakage of CF from DEPC liposomes upon cooling. Various concentrations of AFGP fraction 8 from *T. borchgrevinki* were added to the sample at 18°C. The control has no AFGP present. (*Inset*) A plot of AFGP concentration versus percent leakage of CF at 2°C.

With a thermocouple inserted into the solution, the freezing and melting temperatures of solutions were measured on a chart recorder.

Transition temperatures of lipid with and without AFGP were determined by two independent methods. The first was differential scanning calorimetry (DSC) of 1:4 on a weight/weight basis. Cooling and heating scans were performed with a high sensitivity DSC (Calorimetry Sciences, Provo, UT). Samples were scanned at a rate of 20°C per hr, which is similar to the leakage rate, and were neither normalized to equivalent units of lipid nor baseline subtracted.

Because the protein/lipid ratio was not the same in the DSC and leakage experiments, we looked for a sensitive method for measuring the transition that could be used at the same

Table 1. Leakage from liposomes with various protectants

Protectant	% leakage	% leakage after purification
Dm 2-6+, 1 mg/ml	7	
Tb 5–7, 1 mg/ml	10	0
Dm 2–4, 1 mg/ml	12	0
Tb 1-5, 1 mg/ml	20	0
Tb 6–8, 1 mg/ml	24	
Tb 8, 1 mg/ml	42	
Alanine, 1 mg/ml	44	
Trehalose, 1 mg/ml	45	
Galactose, 1 mg/ml	46	
Bovine serum albumin, 1 mg/ml	47	5
Control	50	
N-acetylgalactosamine, 1 mg/ml	59	
Threonine, 1 mg/ml	61	
Glycerol, 0.4 M	62	
AFP type I, 1 mg/ml	70	5
Bovine lactotransferrin, 1 mg/ml	73	94
Ovomucoid, 1 mg/ml	76	50
Ovotransferrin, 1 mg/ml	81	64
Phospholipase A_2, 1 mg/ml	98	100

Liposomes were cooled through T_m and values at 2°C are shown. All values have a standard error of ±2.5%. Dm, *D. mawsoni*; Tb, *T. borchgrevinki*.

Table 2. Permeability coefficients (cm/s) of CF during cooling of various liposomes

Lipid	P_{CF}	% control
DEPC		
Control	$9.6 \times 10^{-10} \pm 0.4 \times 10^{-10}$	100
AFGP	$3.4 \times 10^{-10} \pm 0.2 \times 10^{-10}$	35
DMPC/egg PC		
Control	$1.5 \times 10^{-10} \pm 0.2 \times 10^{-10}$	100
AFGP	0	0
DPPC		
Control	$7.4 \times 10^{-10} \pm 1.1 \times 10^{-10}$	100
AFGP	$3.4 \times 10^{-10} \pm 0.4 \times 10^{-10}$	46

All liposomes were cooled from liquid crystalline phase to gel phase with or without AFGP 5–7 at 1 mg/ml (not acetone precipitated). Calculated t statistics for control versus AFGP give $P < 0.01$ for all lipids. With 99% confidence, the control is significantly different from the AFGP.

protein/lipid ratio used in the leakage experiments. In this case, fluorescence anisotropy was inappropriate because measurements are slow, requiring changes in orientation of polarizing filters; thus, the heating and cooling rates are much different than those used in the leakage studies. Instead, we devised a sensitive, rapid method for determining the phase transition using liposomes made of DEPC and incorporating 0.5 mole per mole of DEPC for each of the resonance energy probes *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE) and *N*-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (rhodamine PE), which were obtained from Molecular Probes (33). With the probes incorporated into the same bilayer, quenching occurs, but as the liposomes go from gel to liquid crystalline phase, there is an increase in fluorescence that coincides with the DEPC transition measured by DSC. We suspect that the increase in fluorescence is due to some release of quenching as the lipid molecules occupy a larger volume in the liquid crystalline phase. We will provide a full report on this method elsewhere.

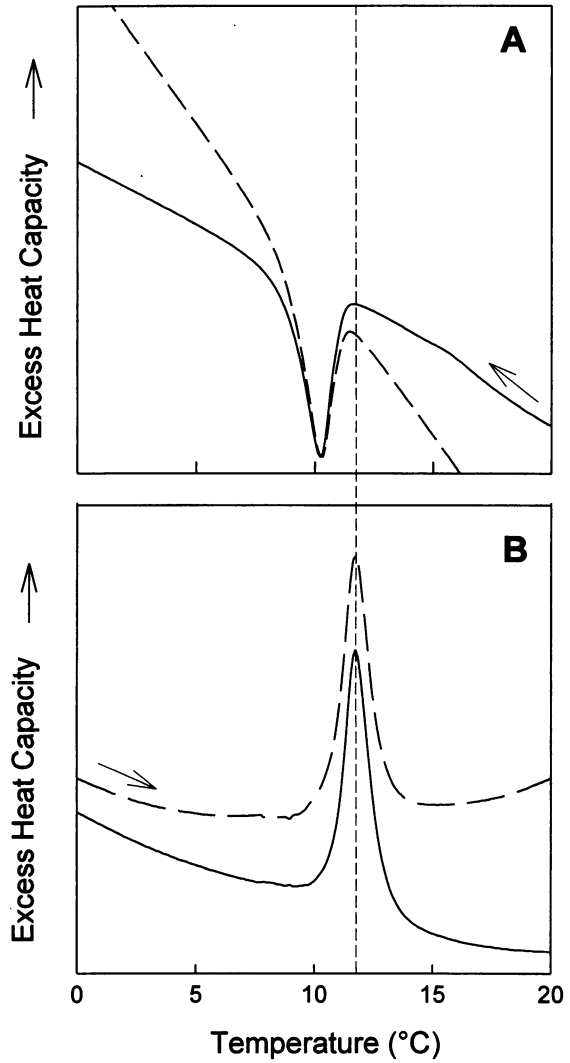

FIG. 3. Differential scanning calorimetry of liposomes (solid line) and liposomes plus 1 mg of AFGP 2–6+ per ml (dashed line) from *D. mawsoni*. (*A*) Cooling scan. (*B*) Warming scan. Samples were cooled at a rate of 20°C per hr and were neither normalized to equivalent units of lipid nor baseline subtracted.

RESULTS AND DISCUSSION

Effects of AFGPs on Leakage. As the DEPC liposomes are cooled through T_m from liquid crystalline to gel phase, they lose ≈50% of the total trapped CF (Fig. 1). Because this leakage depends on the time the lipids spend at the phase transition, it is critical to the experiments that the cooling rate and, consequently, the leakage be repeatable. The data for the controls shown in Fig. 1 demonstrate that this requirement is met. As shown in the Fig. 1 *Inset*, the sample temperature during cooling was repeatable, with the temperature at any given time varying by less than 1°C. In keeping with this repeatable cooling rate, the final values for leakage vary by about 2% on either side of the mean. During the phase transition, the variability is larger, quite likely because of small differences in cooling rates. With the addition of as little as 1 mg of AFGP fractions 2–6+ per ml from *D. mawsoni*, about 90% of the leakage during the phase transition is prevented.

Using fraction 8 from *T. borchgrevinki*, concentrations of AFGPs were increased from 1 mg/ml to 10 mg/ml. The results in Fig. 2 show that as liposomes are cooled through their transition, there is increased protection with increased concentration of AFGPs. In fact, at the highest concentration used

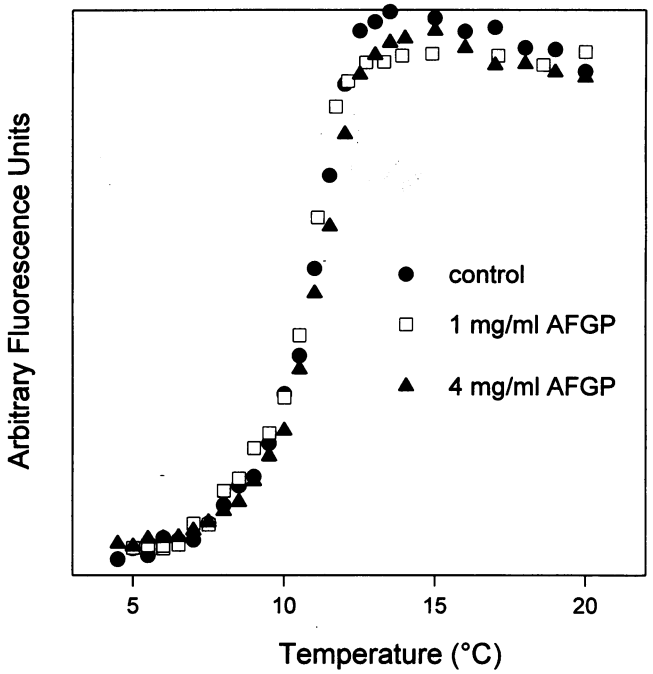

FIG. 4. Arbitrary fluorescence units due to cooling of DEPC liposomes made with NBD-PE and rhodamine-PE in the absence (●) or presence of AFGP at 1 mg/ml (□) and at 4 mg/ml (▲).

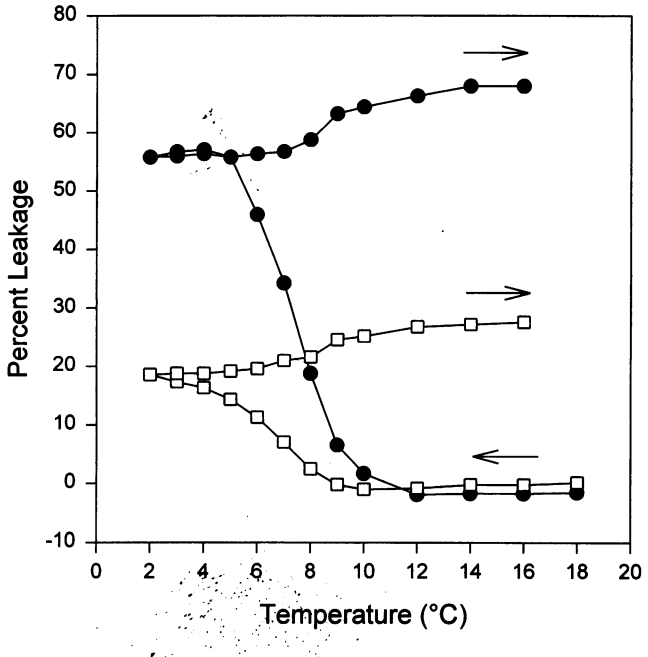

FIG. 5. Percent leakage of CF from DEPC liposomes that were cooled without (●) and with (□) of AFGP fractions 5–7 at 1 mg/ml (*T. borchgrevinki*) to 2°C and held for 20 min before rewarming to 16°C.

(10 mg/ml), leakage is completely inhibited. However, compared with larger AFGPs, much more of this smaller protein is required to inhibit leakage.

Other AFGP fractions tested gave qualitatively similar results in that they all inhibited leakage to varying degrees (Table 1). In every case, the differences between the samples with AFGPs (regardless of the source) and the controls are highly significant ($P < 0.01$).

Feeney and coworkers (32, 34) have reported an apparent cooperativity between effects of the large and small fractions of AFGPs on freezing temperature depression. We tested the possibility that such cooperativity may exist in the present case by recording leakage from liposomes chilled either with *D. mawsoni* 2–6+ at 0.25 mg/ml or with the same sample of fractions 2–6+, but with the addition of fraction 8 at 0.75 mg/ml. We saw no difference between the effects of these samples on leakage. Thus, the cooperativity seen with AFGPs on freezing temperature is not apparent in this case.

Other Lipids. We wished to ask whether the effects of AFGPs on DEPC liposomes might be due to a solution property seen only (and coincidentally) at the transition temperature for that lipid. Thus, the effects of AFGPs on leakage from liposomes as they pass through the phase transition were observed in two alternate lipid systems with widely varying transition temperatures: DPPC (T_m = 41°C) and a DMPC/egg PC mixture (1:1 ratio; T_m = 15°C). Like DEPC, leakage of these liposomes corresponds with the measured T_m. To compare the three different liposome compositions, we calculated the permeability coefficients of carboxyfluorescein across the membrane as it is cooled through T_m, using the following equation (35–37), where P_{CF} is the permeability coefficient for CF:

$$P_{CF} = \frac{\text{Flux}_{CF}\ (\text{mol CF} \cdot \text{cm}^{-2} \cdot \text{s}^{-1})}{\text{CF gradient}\ (\text{mol CF} \cdot \text{cm}^{-3})}$$

The data in Table 2 show that with each lipid, there is a highly significant inhibition of leakage when AFGPs are added. Thus, the effects of AFGPs on leakage from liposomes at the phase transition appear to be generalized, and are clearly not a temperature dependent solution property of the AFGPs themselves.

Effects of Components of AFGPs on Leakage. We tested the effectiveness of the individual components of AFGPs free in solution: galactose, *N*-acetylgalactosamine, threonine, and alanine (Table 1). None of these molecules inhibited leakage from liposomes near the phase transition, so it appears that the intact AFGP is required. We also tested the possibility that other low molecular weight compounds that are known to stabilize bilayers during freezing (31) or drying (22) might inhibit the leakage at the lipid phase transition. Neither trehalose nor glycerol have any effect (Table 1).

Finally, a trivial explanation for the observed effects on leakage could be that the AFGPs quench the CF, which could be incorrectly interpreted as an inhibition of leakage. We tested that possibility by adding AFGPs to CF and saw no change in fluorescence intensity.

Effects of Other Proteins on Leakage. As controls, we have tested the ability of several other proteins to inhibit this leakage, including AFP type I, bovine serum albumin, ovotransferrin, bovine lactotransferrin, phospholipase A_2, and ovomucoid. When these proteins were used without additional purification, none caused a significant decrease in leakage compared with the control, and some actually increased leakage (Table 1). The lack of effectiveness of AFP type I was of some immediate concern because Rubinsky *et al.* (20, 21) had previously reported that this protein stabilized intact cells during chilling. As a result, we investigated the properties of this and other proteins further.

Purification of Proteins. Gel electrophoresis (38) of the AFGPs of *T. borchgrevinki* fractions 5–7 showed one band for each of the three fractions. However, this does not rule out the possibility of contamination by other compounds that might be involved in the stabilization of the liposomes. Thus, we purified the AFGPs further by precipitation of the proteins in acetone to remove possible hydrophobic contaminants. The treatment improved the effects of the AFGPs, decreasing leakage from the liposomes from ≈10% before purification to 0% afterwards. Acetone precipitation of AFP type I dramatically altered its effectiveness; leakage from DEPC liposomes dropped from ≈70% to 5% (Table 1). A similar effect was seen with bovine serum albumin, but the other proteins tested showed relatively less improvement in their effectiveness (Table 1). Thus, it is clear that a contaminant is not responsible for the inhibition of leakage; indeed, the contaminants found in these protein samples increased leakage. Furthermore, it is apparent from the data in Table 1 that although the AFGPs remain the most effective of the proteins tested, the ability of

Table 3. Permeability coefficients (cm/s) of CF due to cooling and rewarming of liposomes

	Cooling		Warming	
	P_{CF}	% control	P_{CF}	% control
Control	$9.6 \times 10^{-10} \pm 0.4 \times 10^{-10}$	100	$9.3 \times 10^{-10} \pm 0.2 \times 10^{-10}$	100
AFGP	$3.4 \times 10^{-10} \pm 0.2 \times 10^{-10}$	35	$2.9 \times 10^{-10} \pm 0.3 \times 10^{-10}$	31

Liposomes were cooled with or without AFGP 5–7 at 1 mg/ml to 2°C and held for 20 min before rewarming to 16°C. Calculated *t* statistics for control versus AFGP give $P < 0.01$ for both cooling and warming. With 99% confidence, the control is significantly different from the AFGP.

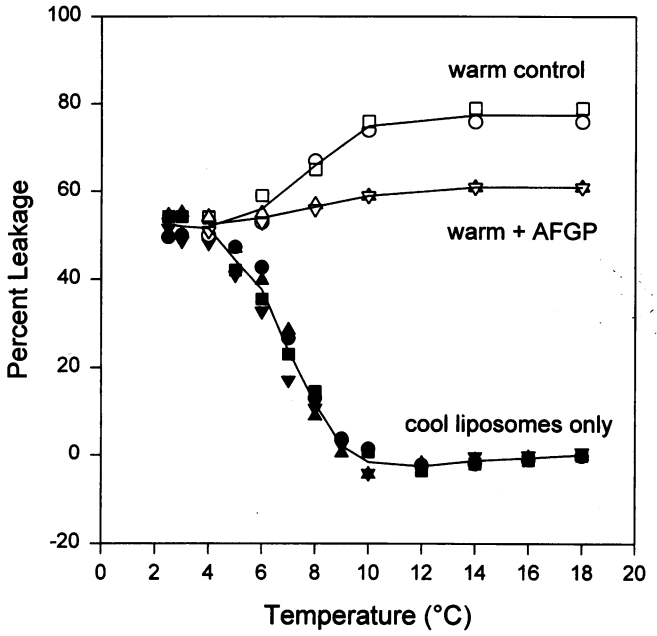

FIG. 6. Percent leakage of CF from DEPC liposomes that were cooled through T_m to 2°C and held for 20 min (solid symbols). The solid lines are the average of all data within a treatment; the raw data points are shown for each treatment. AFGPs fractions 5–7 were added to half of the samples to make a final concentration of 1 mg/ml and an equal volume of TES buffer was added to the other half, after which the samples were rewarmed to 18°C.

proteins to inhibit leakage across membranes during phase transitions may be a more general property than we previously suspected. We are testing a wide variety of proteins with this view in mind.

Stability of AFGPs After Heating. We previously reported that after heating, AFGPs lost their ability to inhibit leakage from liposomes, but not their antifreeze properties (39). In view of the findings presented above, we reinvestigated our results. A sample of *D. mawsoni* fractions 2–4 was heated to 80°C for 40 min in a water bath. This heat-treated sample no longer inhibits leakage from liposomes near the phase transition. However, when the same fractions of AFGP were acetone precipitated, we found that they can be heated similarly, and inhibition of leakage is not lost. We are presently investigating further and defining the contaminant responsible for these findings.

Mechanism of Inhibition of Leakage. Our investigation into the mechanism focused only on the AFGPs. The simplest hypothesis concerning the mechanism is that the AFGPs decrease the liquid crystalline to gel phase transition temperature, thus maintaining the lipids in liquid crystalline phase at low temperatures. We tested that hypothesis with DSC scans of DEPC liposomes prepared with and without AFGPs. The results (Fig. 3) show that the AFGPs have no effect on the transition during either warming or cooling. There is hysteresis between the warming scans (T_m = 12°C) and cooling scans (T_m = 10°C); the latter coincides with the onset of leakage (see Figs. 1 and 2).

Whereas the calorimetric results suggest that AFGPs do not depress the transition temperature of the phospholipids, there is a problem with these data: to detect the transition calorimetrically, it was necessary to use a much larger amount of lipid than was used in the leakage experiments. Under these conditions, it was not possible to elevate the AFGP concentration high enough to provide a ratio of AFGP to phospholipid similar to that used in the leakage experiments. Thus, we devised a fluorescence method with which we found that the lipid phase transitions could be measured at the same lipid and AFGP concentrations used in the leakage experiments (Fig. 4). The results show that during cooling, the transition starts at about 10°C, in good agreement with the onset of leakage (cf. Fig. 1). Addition of as much as 4 mg of AFGP per ml has no effect on the lipid phase transition. We conclude that the mechanism by which AFGPs inhibit leakage is not related to depression of T_m.

Mechanism of Inhibition of Leakage: Do AFGPs Inhibit Leakage During Warming? Another possibility is that the AFGPs interact directly with the liposomes during the phase transition and that this interaction is responsible for inhibiting the leakage. If this is the correct model, one might expect the access of AFGPs to the polar headgroups to be decreased in gel phase lipids, and that as a result the protection would be lost during warming. We tested this possibility through the following experiments.

1. Effects of adding AFGPs above T_m. As shown in Fig. 5, liposomes with and without AFGPs were cooled to 2°C and held at that temperature for 20 min so that all lipid molecules would be in the gel phase. In the presence of 1 mg of AFGP per ml, liposomes leaked about half as much as the controls described previously. Upon rewarming at a rate of 2°C per min, there is leakage from both samples at what superficially appears to be a similar rate (Fig. 5). However, because the concentration gradient of CF from the trapped volume to the external medium is much greater in the AFGP sample than in the control, apparently similar leakage rates measured as percentages may actually represent significantly different rates of diffusion. Thus, we calculated the permeability coefficients to take the magnitude of the concentration gradient into account. Table 3 shows the P_{CF} for the cooling of liposomes with and without 1 mg of AFGP per ml. It is clear from these calculations that AFGPs decrease the permeability of the liposomes during the gel to liquid crystalline phase transition (warming) to the same extent as during cooling.

2. Effects of adding AFGPs below T_m. The results shown in Table 3 do not rule out the possibility that the AFGPs have a direct interaction with the bilayer. In those studies, the proteins were added above T_m. To test the effectiveness of AFGP presented at gel phase, liposomes without AFGP were cooled to 2°C and held there for 20 min. AFGPs were then added to half of the samples, and all samples were rewarmed at a rate of ≈1.5°C per min (Fig. 6). Permeability coefficients were calculated for these experiments so that protection during warming and cooling could be compared. Table 4 shows that

Table 4. Permeability coefficients (cm/s) for CF when AFGPs are added below T_m

		Warming	
	Cooling, P_{CF}	P_{CF}	% control
Control	$8.7 \times 10^{-10} \pm 0.4 \times 10^{-10}$	$3.5 \times 10^{-9} \pm 0.2 \times 10^{-9}$	100
AFGP	—	$5.5 \times 10^{-10} \pm 0.3 \times 10^{-10}$	15

Liposomes were cooled without AFGP to 2°C. After being held at that temperature for 20 min, sufficient AFGP 5–7 was added to half of the samples to give a final concentration in the cuvette of 1 mg/ml. All samples were warmed to 18°C. Calculated t statistics for control versus AFGP give $P < 0.01$ for warming.

adding AFGPs below T_m decreases the permeability of the membranes to CF during warming, actually by a factor larger than that seen in the samples in which the AFGPs were added above T_m (cf. Table 3).

Based on the observation that the AFGPs inhibit leakage regardless of whether they are added above or below T_m, these experiments provide no evidence to support the hypothesis that a direct interaction with the bilayer is responsible for the remarkable properties of the AFGPs. However, it is still conceivable that such interactions may occur and the direct measurements of bilayer-AFGP interactions required to test this hypothesis are currently in progress.

This work was supported by grant USN N00014-94-1 from the Office of Naval Research and grant IBN 93-08581 from the National Science Foundation.

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