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Efficacy of antifreeze protein types in protecting liposome membrane integrity depends on phospholipid class

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Abstract

Antifreeze proteins have been reported to be capable of maintaining the membrane integrity of cold sensitive mammalian cells when exposed to hypothermic temperatures. However the mechanism(s) whereby these proteins exert this protective effect is unknown. The present study used liposomes as a model system to examine the nature of the interactions between four antifreeze (glyco)protein types (AFP I, II, III and AFGP) and albumin, with lipid membranes. Fluorescein isothiocyanate labelling indicated that all of the proteins bound to the three liposome types (dielaidoylphosphatidylcholine (DEPC), dielaidoylphosphatidylethanolamine (DEPE) and dielaidoylphosphatidylglycerol (DEPG)). AFGP was found to be highly effective at preventing leakage from all three liposome compositions as they were cooled through their phase transition temperatures. This was not the case for the other proteins. All four antifreeze types prevented zwitterionic DEPC liposomes from leaking as they were cooled through their phase transition temperature. However, albumin was equally as effective, indicating that this capacity was not unique to antifreeze proteins. All of the proteins, except AFGP, induced the negatively charged DEPG liposomes to leak prior to cooling, and were less effective than AFGP in preventing phase transition leakage from DEPE liposomes. It is proposed that many proteins, including antifreeze proteins, can protect zwitterionic liposomes, such as DEPC, by binding to the lipid bilayer thereby maintaining the ordered structure of the membrane during phase transition. However, when the membrane contains a negatively charged polar group, such as with DEPE and DEPG, proteins, although bound to them, may not be able to maintain sufficient membrane organization to prevent leakage during phase transition or, they may gain entry into the lipid bilayer, disrupt the structure and induce leakage. These results imply that the efficacy of antifreeze proteins in the cold protection of mammalian cells will not only depend on protein structure, but also on the lipid composition of the cell membrane.

Keywords: Antifreeze protein/glycoprotein; Ica; Cell membrane; Liposome; Phospholipid; Structure/function relationship

1. Introduction

During the past three decades a considerable amount of research has been conducted on the characterization of a group of proteins capable of inhibiting ice crystal growth. These proteins, named antifreeze proteins, were first discovered in fish because of their ability to lower serum freezing points and thus enable the fish to survive at subzero temperatures in the presence of ice [1,2]. Since that time these structurally diverse proteins have been found in plants, insects and bacteria [3–5]. To date, five distinct types of antifreeze (glyco)proteins (AF(G)P) have been isolated from a variety of north temperate and polar fish. These proteins are classified as AFP Type I to IV and AFGP based on their composition and tertiary structures [6]. Type I AFP are alanine-rich single α-helical peptides isolated from flounders and sculpins. Type II AFP described from sea raven, herring and smelt, are cystine-rich and highly homologous with the carbohydrate binding domains of C-type lectins. Type III AFP, from wolffish and ocean pout, have β-sheet structures, and Type IV, found in longhorn sculpin, contains a Glx-rich four-helix bundle structure. AFGP found in Antarctic notothenoids and Northern cods consist of glycopeptides made up of tripeptide units of Ala–Ala–Thr with galactosyl-*N*-acetyl galactosamine attached to each Thr [7].

Despite their structural diversity, all of the AF(G)P act by adsorbing to developing ice crystals, thereby inhibiting the addition of water molecules to the ice lattice. In addition to the action of antifreeze proteins on ice morphology and growth, these proteins also share the ability to interact with, and protect mammalian cells and tissues from hypo-

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thermic damage [2,4]. Numerous studies have been conducted to determine the mechanisms whereby antifreeze proteins bind to ice in order to block ice growth and inhibit ice recrystallization [8–14]. However, mechanism(s) whereby antifreeze proteins can protect mammalian cell membranes from hypothermic damage remains unclear. There is no evidence to indicate that membrane binding is a requirement for the AF(G)P protective effect, nor is it known whether antifreeze protein ice binding motifs are essential for such an effect.

The ability of antifreeze proteins to protect cell membranes from hypothermic damage was first demonstrated by Rubinsky et al. [15]. They found that AF(G)P significantly improved the fertilization of bovine and pig oocytes after being exposed to 4°C for 24 h. These proteins were also shown to protect the functional integrity of whole rat liver after exposure to hypothermic temperatures [16,17]. In addition, it was also demonstrated that AFP effectively suppressed calcium and potassium currents of porcine granulosa cells, and parietal cells of rabbit gastric glands suggesting that the mechanism of antifreeze protein action involved the blockage of ion channels [18,19]. On the other hand, AFGP has been shown to prevent leakage of liposomes as they were cooled through their phase transition temperatures [7,20]. These results suggested interactions of AFGP with the lipid bilayer of the cell membrane. One cause of cold induced cellular damage could arise when cells are cooled through the lipid membrane thermotropic phase transition temperature. During this liquid crystalline to gel phase transition, membranes become leaky, resulting in the loss of intracellular contents and an influx of extracellular materials [21]. Evidence consistent with this hypothesis comes from studies on human blood platelets where it was demonstrated that AFGPs from Antarctic notothenoids were able to inhibit the cold induced morphological changes that are initiated when the platelets pass through their phase transition temperature [22]. However, so far there is no direct evidence to show interactions between AF(G)P and the lipid bilayer.

In the present study, we investigated the effects of four different AF(G)P types (AFP 1, II and III and AFGP) and albumin on preventing the leakage of liposomes made of various lipid compositions. The results demonstrate that all of the protein types bind to the liposome membrane, and that the ability of these proteins to prevent leakage of the liposomes as they were cooled through their phase transition temperatures was dependent on protein structure and liposome lipid composition.

2. Materials and methods

2.1. Purification of antifreeze (glyco)proteins

Antifreeze glycoproteins were purified as described previously [7]. Briefly, thawed plasma was treated with 50% ethanol at 4°C and centrifuged to remove the precipitated proteins at 5000 rpm for 30 min. The resulting supernatant containing the antifreeze proteins was extensively dialyzed against 2.5 mM Tris–HCl pH 9.4 using Spectropore 3 dialysis tubing (molecular weight cut off: 3500). The dialyzed proteins were applied to a DEAE-Biogel column (5×50 cm) and eluted with a stepwise gradient of 2.5–250 mM Tris–HCl, pH 9.4. Fractions containing components 6–8 (2.7–3.8 kDa) from DEAE were pooled, freeze-dried and resuspended in deionized water (Millipore Milli-Q PF). Type I AFP from winter flounder, *Pleuronectes americanus* ($\sim$3.3 kDa) and Type III from ocean pout, *Macrozoarces americanus* ($\sim$6.6 kDa) were purified as described previously [23,24]. Briefly, thawed plasma was fractionated twice on a Sephadex G-75 gel permeation column and the G75 purified AFP was further purified using a DEAE-Biogel column as described for AFGP. Fractions eluted with 250 mM Tris–HCl, pH 9.4, containing AFP activity were used for the assays. Type II AFP (12–14 kDa) were purified from the plasma of sea raven *Hemitripterus americanus* by the method of Ng et al. [25]. Bovine serum albumin (BSA) was purchased from Sigma (Cat# A0281).

2.2. Liposome preparation and assays

Dielaidoylphosphatidylcholine (DEPC), dielaidoylphosphatidylethanolamine (DEPE) and dielaidoylphosphatidylglycerol (DEPG) were purchased from Avanti Polar Lipids. Liposome preparation and assays were performed as previously described by Wu et al. [7] with the following modifications. Small unilamellar liposomes (100 nm) of DEPC, DEPC/DEPE (4:1) and DEPC/DEPG (4:1) were prepared for liposome assays. Briefly, after drying, the lipid was dispersed in 100 mM carboxyfluorescein (CF) (Molecular Probes). External CF was removed from the liposomes by passing them through a Bio-spin 6 chromatography column (Bio-Rad Laboratories, Cat# 732-6002). The final concentration of the liposomes was approximately 30 mg/ml. Liposomes (2 μl) were added to a cuvette containing 2 ml of 10 mM TES, 0.1 mM EDTA, pH 7.4 and a stirring bar. Four samples were analyzed simultaneously using an automated programmable four cell turret Aminco.Bowman Series 2, luminescence Spectrometer. Liposomes were cooled from 20 to 0°C at a rate of 0.5°C/min. The temperature of the cuvettes was controlled by connecting the cuvette turret to a programmable water bath (Neslab, RTE-111) with the temperature sensor inserted into the inlet of the turret. At the completion of each temperature run the liposome vesicles were lyzed by the addition of a drop of Triton $\times$100 to provide a measure of total trapped CF.

2.3. Protein labelling and flow cytometry

Antifreeze proteins and albumin were labelled with flu-

orescein isothiocyanate (FITC) using a protocol developed by Katsikis and Roederer [26]. Briefly, AF(G)Ps were dissolved in a reaction buffer (500 mM carbonate, pH 9.5) at a final concentration of 10 mg/ml. FITC was added and mixed with the proteins to give a ratio of 10–20 μg/mg of proteins. The mixture was wrapped in foil and incubated at room temperature for 1 h. The unreacted FITC was removed by dialysis with a storage buffer (10 mM Tris, 150 mM NaCl, 3–4 drops of pHix (5 mg/ml pentachlorophenol in 95% ethanol) in a liter of solution, pH 8.2). The labelled proteins were lyophilized and tested to ensure that the addition of FITC did not change their thermal hysteresis activities or their ability to inhibit leakage from DEPC liposomes during thermotropic phase transition. For flow cytometry, lipids of various compositions were dispersed in 10 mM TES, 0.1 mM EDTA buffers. Liposomes (1 μm) were prepared using an extruder with a filter of a 1000 nm pore diameter. Labelled AF(G)Ps were added to the liposomes in a ratio of 1:1 or 2:1 by weight basis and the mixture was incubated for 1 to 3 h at room temperature or at 4°C. Unbound AF(G)Ps were removed by a Bio-spin 6 chromatography column.

Fluorescence was analyzed on a FACStar Plus flow cytometer (Becton Dickinson) calibrated with flow cytometry alignment beads (2.5 μm) (Molecular Probe) for 488 nm excitation (Department of Laboratory Medicine, Health Care Corporation of St. John's, Canada). The FACStar Plus flow cytometer is equipped with an argon laser emitting a 488 nm beam at 35–90 mW as excitation source and a 530/30 nm emission filter. For the measurement of binding at 4°C, the sheath buffer was maintained at 4°C during data acquisition. At least 10 000 events were counted and analyzed with Cell Quest software (Becton Dickinson) or Winmdi (University of Massachusetts, Amherst, MA, USA).

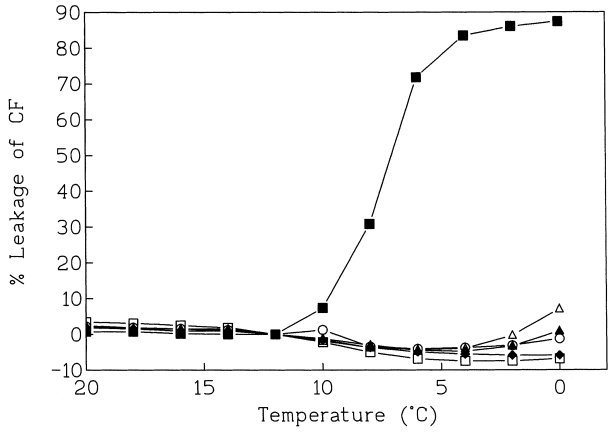

Fig. 1. Percent leakage of CF from DEPC liposomes upon cooling from 20 to 0°C. Each data point is the mean of four independent experiments. A protein concentration of 100 μg/ml was used for all samples. The control has no AF(G)P present (■). Proteins tested were AFP II (△), AFGP (◆), AFP I (○), AFP III (▲) and albumin (□).

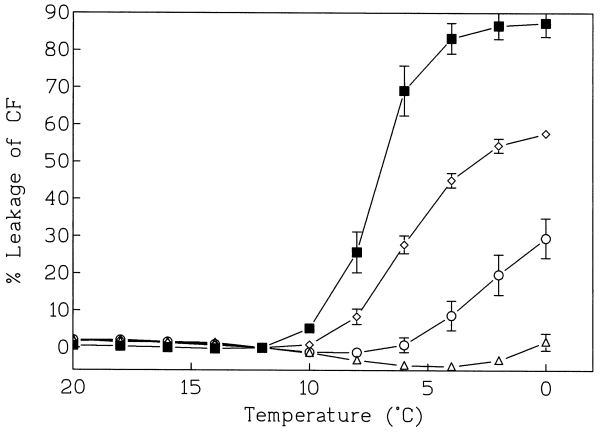

Fig. 2. Percent leakage of CF from DEPC liposomes upon cooling from 20 to 0°C. Each data point is the mean and standard error of four independent experiments. Type III AFP from ocean pout was used at a concentration of 100 μg/ml (△), 50 μg/ml (○) and 25 μg/ml (◇).

3. Results

3.1. Effects of AF(G)Ps on leakage of liposomes made of pure neutral lipids

All four antifreeze proteins studied suppressed leakage from DEPC liposomes as they were cooled from 20 to 0°C. At a concentration of 0.1 mg/ml the proteins completely inhibited liposome leakage. In contrast, leakage from control liposomes was greater than 80% (Fig. 1). This ability to completely block liposome leakage was not confined to the antifreeze proteins, as BSA was found to be equally effective at the same concentration (Fig. 1).

All of the proteins, including albumin, functioned in a dose dependent matter, with higher concentrations providing the greatest inhibition of liposome leakage. For example, Type III AFP completely blocked liposome leakage at a concentration of 0.1 mg/ml, while at 0.025 mg/ml leakage increased to approximately 66% of the control (Fig. 2).

3.2. Effects of AF(G)Ps on leakage of liposomes containing negative charge lipids

When liposomes made of a mixture of DEPC/DEPG(4:1) were studied, the abilities of the various proteins to prevent liposome leakage differed considerably. Type I AFP and albumin (0.1 mg/ml) induced liposome leakage immediately after they were added to the liposome suspension (Fig. 3). This leakage continued as the temperature decreased and was 15–20% greater than that of the control at 0°C. Type III and Type II AFP also induced leakage of the liposomes, albeit to a lesser extent than for Type 1 and albumin. In contrast to the other proteins tested, AFGP did not induce leakage and completely inhibited liposome leakage during phase transition (Fig. 3).

When the antifreeze proteins and albumin were added to liposomes prepared from a mixture of DEPC/DEPE

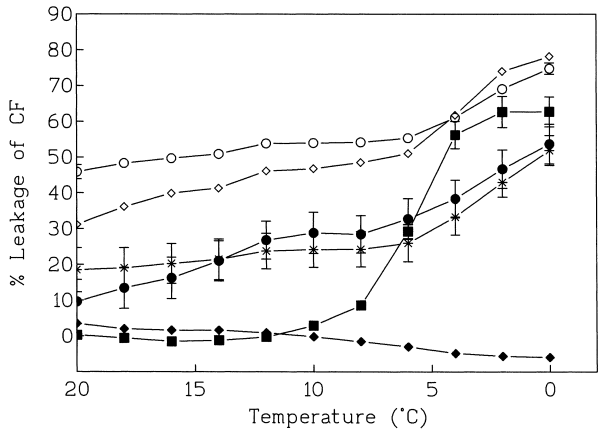

Fig. 3. Percent leakage of CF from DEPC/DEPG (4:1) liposomes when cooling from 20 to 0°C. Each data point is the mean and standard error of four independent experiments. A protein concentration of 100 μg/ml was used for all samples. The control has no AF(G)P present (■). Proteins tested were AFP II (*), AFGP (♦), AFP I (○), AFP III (●) and albumin (◇).

(4:1), there was no induction of liposome leakage. In addition all of the proteins suppressed leakage from the liposomes as they went through their phase transition temperature. However, the ability to protect the liposomes from leakage varied from protein to protein. Again, at a concentration of 0.1 mg/ml, AFGP completely prevented the liposomes from leaking (Fig. 4). At the same concentration, Type III and Type I AFP inhibited the leakage by 60%, while albumin and Type II AFP suppressed the leakage by approximately 40 and 15–20%, respectively (Fig. 4).

3.3. Binding of AF(G)Ps to lipid bilayer

All of the FITC labelled antifreeze proteins, and albumin, were equally as capable of blocking DEPC liposome

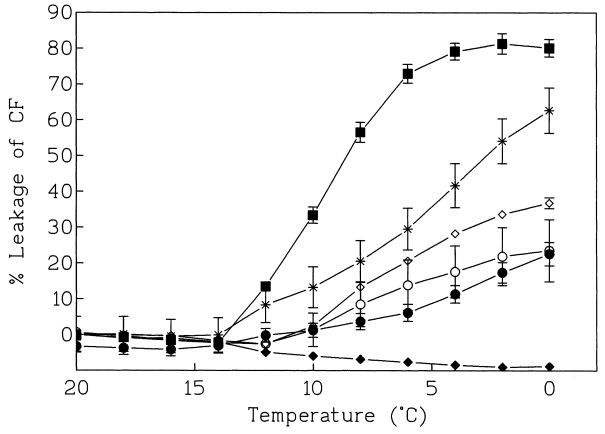

Fig. 4. Percent leakage of CF from DEPC/DEPE (4:1) liposomes upon cooling from 20 to 0°C. Cooling was performed from 45 to 0°C and leakage was not observed between 45 and 20°C. Each data point is the mean and standard error of four independent experiments. A protein concentration of 100 μg/ml was used for all samples. The control has no AF(G)P present (■). Proteins tested were AFP II (*), AFGP (♦), AFP I (○), AFP III (●) and albumin (◇).

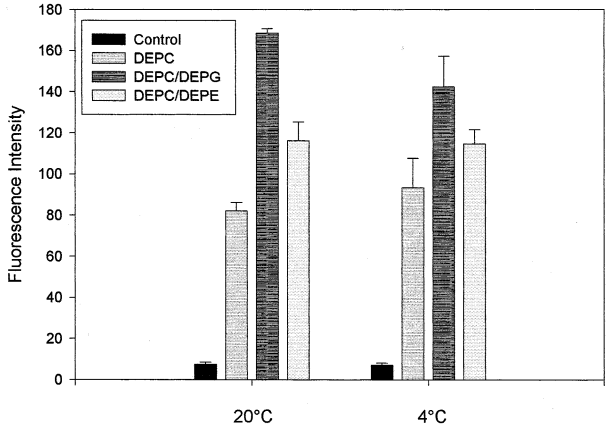

Fig. 5. FITC fluorescence intensity on liposomes made of different lipid compositions. 10 000 events were collected by a flow cytometer and analyzed with Cell Quest software for each experiment. Each column shows the mean of duplicate experiments with the values of each indicated as error bars. A final concentration of 50 μg/ml FITC labelled AFGP was used for all binding assays. The control (no labelled AFGP) was calibrated to about 10 for all types of liposomes. Liposomes made of different lipid compositions are indicated on the graph.

leakage as were their unlabelled counterparts (data not shown).

All three liposome types (1 μm) (DEPC, DEPC/DEPG (4:1) and DEPC/DEPE (4:1)) were used for the protein binding assays. All of the FITC labelled antifreeze protein types (AFP Type I and III, AFGP) and albumin bound to the liposomes. Since the data for all of the proteins illustrated the same point, only the results from the AFGP experiments will be presented here.

Mean fluorescence intensities (MFI) associated with liposomes in the presence of FITC labelled AFGP (50 μg/ml) were 10–22-fold greater than that associated with control liposomes (Fig. 5) clearly indicating that the AFGP bound to all three liposome types at room temper-

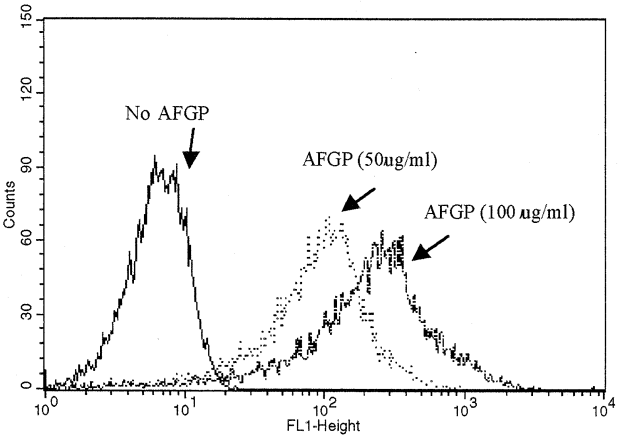

Fig. 6. Overlay of green fluorescence histograms. Liposomes (DEPC/DEPE, 4:1) were incubated with FITC labelled AFGP at a concentration of 50 and 100 μg/ml. Liposomes in the absence of labelled AFGP were used as a control. 10 000 events were collected by a flow cytometer and plotted with Cell Quest software. FL1-Height represents fluorescence intensity.

ature and at 4°C. Moreover the MFI associated with the liposomes increased 1.5–3-fold when the concentration of FITC labelled AFGP was increased from 50 to 100 μg/ml (Fig. 6).

4. Discussion

Antifreeze proteins have been reported to have the capacity to protect cold sensitive mammalian cells and organs from hypothermic damage [2,15,16,19,27]. Although this protective effect is believed to involve a direct interaction between antifreeze proteins and the cell membrane, the mechanism of this interaction is unknown.

In an attempt to gain understanding of the nature of the effects of antifreeze proteins on cell membranes, Hays et al. [20] using liposomes as a model, demonstrated that AFGP was effective in preventing liposomes, comprized of pure DEPC, DPPC, DMPC or egg phosphatidylcholine (PC), from leaking as they were cooled through their phase transition temperatures. This suggests the possibility that AFGP could, in general, prevent zwitterionic liposomes from leaking when cooled through their phase transition temperatures. The present study extended the observations of Hays et al. [20] indicating that all four of the antifreeze proteins could prevent DEPC liposomes from leaking. However, albumin and other proteins such as lactoferrin, transferrin, trypsin inhibitor and insulin (Wu et al., unpublished data) were also found to be highly effective at protecting these liposomes, therefore this functional property is not unique to the antifreeze proteins.

The lipid bilayer of cell membranes is considerably more complex than that of DEPC liposomes. Therefore, in order to determine whether antifreeze proteins could interact with cell membranes in a manner that was unique to this class of proteins, it was decided to work with somewhat more complex liposomes. In addition to PC, two of the more common cell membrane lipids are phosphatidylethanolamine and phosphatidylglycerol. In order to simplify data interpretation, DEPE and DEPG were chosen for study, because changes from DEPC to DEPE or DEPG only involve the polar head groups, while the non-polar fatty acid chain remains the same for all three molecules.

The flow cytometry results of the present study clearly indicate that all of the antifreeze proteins (Types I, III and AFGP) and albumin bind to the liposomes, and that the amount of protein bound depends directly on the protein concentration (Figs. 5 and 6). These observations indicate that the dose dependent effect of these proteins in blocking liposome leakage ([7] and Fig. 2) is the result of increasing amounts of protein binding to the liposomes. The question remains as to how protein binding prevents liposome membranes from leaking as they pass through their phase transition temperature and why AFGP is the most efficacious.

Peptide molecules that interact with lipid membranes are often found to have amphiphilic α-helical structure or β-structure [28,29]. A recent study on lipid binding using synthetic amphiphilic α-helical model peptides suggested that the binding of peptides to the lipid bilayer was largely dependent on the hydrophobicity of the peptides [29] These authors demonstrated that peptides with a hydrophobic face that was larger than the hydrophilic face, immersed their hydrophobic regions in the lipid bilayer. Peptides with a larger hydrophilic face only interacted with the anionic lipid head groups and cationic peptide residues on the liposome. AFGP is an amphiphilic molecule that is highly glycosylated with approximately 60% of the molecular mass attributable to carbohydrate. This renders the AFGP highly hydrophilic. Nuclear magnetic resonance studies suggest that AFGP adapts an unusual three-fold, left-handed helical conformation, with the carbohydrate moiety oriented on one side of the molecule and the hydrophobic amino acid backbone on the other [10,30]. Taking these points into consideration, we propose that the binding of AFGP to zwitterionic and negatively charged liposomes may be confined to the surface of the lipid bilayer. This hypothesis suggests that the carbohydrate moiety of the AFGP interacts with the polar head groups of the lipid bilayer through charged and/or hydrogen bond interactions. Another possibility is that the hydrophobic backbone of the AFGP partially immerses into the lipid bilayer rather than directly penetrating it, as penetration of the lipid bilayer may disrupt its structure and result in leakage.

Interactions between the other antifreeze protein types, albumin and the liposomes appears to be more complex than is the case for AFGP. All of these proteins induced DEPC/DEPG liposome leakage to varying degrees when added at room temperature (Fig. 3). This implies that these proteins penetrate the lipid bilayer and disrupt the ordered structure of the membrane. The reason for this disruption is likely attributable to the strongly negatively charged polar head groups of DEPG. It is clear from the data, that the strength of interactions between the proteins and the DEPG head groups, varies considerably from protein to protein.

DEPE also possesses a negatively charged polar head group. However in this case, none of the proteins induced leakage of the liposomes prior to cooling, and all of them provided some degree of protection as the liposomes were cooled through their phase transition temperatures (Fig. 4). The difference in results between the DEPE and DEPG liposomes could be attributable to the greater negative charge or the presence of -OH groups associated with DEPG.

By way of a general summary, it would appear that all of the proteins studied, and likely a wide array of other proteins, protect DEPC liposomes by binding to the membrane. In this way they maintain the ordered structure of the membrane during phase transition and, as a consequence, prevent leakage. The DEPE and DEPG liposomes

illustrate that by changing the charge of the polar head groups, proteins, although bound to the lipid bilayer, may not be able to maintain sufficient membrane organization to prevent leakage during phase transition or, they may gain entry into the lipid bilayer, disrupt the structure and induce leakage.

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