A Mechanism for Stabilization of Membranes at Low Temperatures by an Antifreeze Protein

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ABSTRACT Polar fish, cold hardy plants, and overwintering insects produce antifreeze proteins (AFPs), which lower the freezing point of solutions noncolligatively and inhibit ice crystal growth. Fish AFPs have been shown to stabilize membranes and cells in vitro during hypothermic storage, probably by interacting with the plasma membrane, but the mechanism of this stabilization has not been clear. We show here that during chilling to nonfreezing temperatures the α-helical AFP type I from polar fish inhibits leakage across model membranes containing an unsaturated chloroplast galactolipid. The mechanism involves binding of the AFP to the bilayer, which increases the phase transition temperature of the membranes and alters the molecular packing of the acyl chains. We suggest that this change in acyl chain packing results in the reduced membrane permeability. The data suggest a hydrophobic interaction between the peptide and the bilayer. Further, we suggest that the expression of AFP type I in transgenic plants may be significant for thermal adaptation of chilling-sensitive plants.

INTRODUCTION

Antifreeze glycoproteins (AFGPs) and antifreeze proteins (AFPs) found in the blood and tissues of organisms that live in freezing environments inhibit ice crystal growth in a noncolligative manner (Davies and Sykes, 1997; Doucet et al., 2000; Graham et al., 1997). A variety of terrestrial plants possess such proteins (Doucet et al., 2000; Urrutia et al., 1992; Worrall et al., 1998; Duman, 1994; Meyer et al., 1999; Sidebottom et al., 2000), but their effects on ice crystal growth are of doubtful adaptive significance for freezing tolerance, as the freezing point is depressed by only a fraction of a degree (Doucet et al., 2000; Worrall et al., 1998; Sidebottom et al., 2000). The fish AFPs make up two distinct classes: the heavily glycosylated AFGPs and the nonglycosylated AFPs. The latter fall into at least four subclasses, as characterized by amino acid composition and structure (Davies and Sykes, 1997). Recently, there has been great interest in AFP type I, an α-helical peptide of ~3 kD (Yang et al., 1988). The amino acid composition of this peptide consists of ~60% Ala, with four key Thr residues in alignment on one side of the peptide. Structural evidence from several groups suggests that these four Thr (Haymet et al., 1998, 1999; Zhang and Laursen, 1998), possibly along with the adjacent Ala residues (Baardsnes et al., 1999), are important for the peptide-ice interaction, which is hypothesized to be primarily hydrophobic (Chao et al., 1997; Haymet et al., 1998, 1999; Zhang and Laursen, 1998; Baardsnes et al., 1999).

Rubinsky et al. (1990, 1991) found that fish AFPs also protect membranes during low temperature stress. Bovine and porcine oocytes maintained a membrane potential after they had been chilled to 4°C and rewarmed in the presence of AFPs. This protection was attributed to an interaction between the antifreeze peptides and integral membrane proteins of the cell membranes. However, chilling membranes through the liquid-crystalline to gel phase transition (T_m) is well known to result in a transient increase in membrane permeability (Quinn, 1985; Clerc and Thompson, 1995), which is thought to lead to chilling damage. Subsequently, Hays et al. (1996) showed that AFGPs from polar fish inhibit leakage from liposomes composed solely of phospholipids as they are chilled through their T_m, indicating that the AFGPs confer membrane protection through interactions with lipids. Similar results were obtained with studies on human blood platelets, which are extremely sensitive to cold. Human platelets undergo physiological activation when they are chilled below 20°C (White and Krivit, 1967; Zucker and Borrelli, 1954), and this activation has been correlated with the temperature range over which the platelets pass through their membrane phase transition (Tablin et al., 1996; Crowe et al., 1999; Tsvetkova et al., 1999). Bloodbanks currently store platelets at 22°C, for a maximum of 5 days, because of the chilling sensitivity of platelets. In the presence of AFGPs, platelets can be stored at 4°C for at least 21 days, after which they respond normally to various platelet agonists in vitro (Tablin et al., 1996).

However, no evidence for a direct interaction between the antifreeze peptides and cellular or model membranes was found in any of the previous studies. The AFPs and AFGPs did not alter the phase behavior of either phospholipid

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liposomes or platelet membranes (Hays et al., 1996; Tablin et al., 1996), and the peptide secondary structure was not affected in the presence of the liposomes (Hays, 1998). Additionally, AFPs had only slight protective effects on single phospholipid systems, which were either *trans*-monounsaturated or fully saturated lipids (Hays et al., 1996; Hays, 1998).

We have now investigated the effects of AFPs on membranes containing the plant lipid digalactosyldiacylglycerol (DGDG) obtained from chloroplast thylakoid membranes. DGDG is a highly unsaturated lipid, whose acyl chains are composed predominately of 16:3 and 18:3 fatty acids (Quinn and Williams, 1983). In sharp contrast with previous results with phospholipid liposomes (Hays et al., 1996), AFP type I completely abolished chilling damage in these DGDG-containing model membranes and significantly altered their membrane phase behavior. The galactolipid-containing model membrane system has given us insight into the mechanism of interaction between the AFPs and lipids during low temperature stress, as well as the first evidence for a direct interaction between the peptides and the lipid bilayer. In contrast, we find that AFGPs do not inhibit leakage from the DGDG-containing membranes during chilling, nor do they alter the phase behavior of these liposomes. These results are in stark contrast to studies using pure phospholipid liposomes where the AFGPs inhibited leakage completely as the liposomes were chilled through their T_m (Hays et al., 1996). The current study clearly demonstrates that the lipid composition of a membrane dictates whether or not an AFP will protect and/or interact with the bilayer as it is chilled through its phase transition temperature.

EXPERIMENTAL PROCEDURES

Materials

Dimyristoylphosphatidylcholine (DMPC) and *N*-biotinyl-phosphatidylethanolamine were purchased from Avanti Polar Lipids (Alabaster, AL) and DGDG was purchased from Lipid Products (Redhill, Surrey, UK). AFP type I was a gift from A/F Protein (Boston, MA) and was visualized as a single band after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and silver staining.

Leakage experiments

DGDG:DMPC liposomes (1:1, w/w) were made using a Liposofast hand-held extruder (Avestin, Ottawa, Canada) with 100-nm pore filters (Poretics, Livermore, CA; MacDonald et al., 1991) at a concentration of 20 mg/ml lipid in 100 mM carboxyfluorescien (CF) (Molecular Probes, Eugene, OR), 10 mM TES, and 0.1 mM EDTA (pH 7.4). The liposomes were then passed over a Sephadex G-50 (Pharmacia, Uppsala, Sweden) column in TEN buffer (10 mM Tes, 0.1 mM EDTA, and 50 mM $NaCl_1$ pH 7.4) (TEN buffer, pH 7.4) to separate the liposomes from the free, external CF. Leakage experiments were performed as previously described (Hays et al., 1996) immediately after the liposomes were prepared, as the liposomes become unstable after storage for more than 3 hs. Briefly, 5 μl of liposomes (10 mg/ml lipid after gel filtration) were combined with TEN buffer and protein in a cuvette to reach the final concentrations indicated in the figures in a total volume of 1.75 ml. The samples were stirred continuously while cooled to 4°C at a rate of 0.5°C/min by a circulating ethylene glycol bath connected to a cuvette holder in a Perkin-Elmer LS 50B fluorescence spectrometer (Norwalk, CT). Sample temperature was measured by a digital thermometer connected to a thermocouple that was placed directly into a sample. Fluorescence readings were taken as the samples were cooled and after the samples were rewarmed quickly, and fluorescence corresponding to 100% leakage was measured after the addition of 50 μl 1% Triton X-100 to the cuvette.

Fourier transform infrared spectroscopy (FTIR) analysis

Infrared (IR) spectra were recorded with a Perkin-Elmer Spectrum 2000 FTIR spectrometer equipped with a liquid nitrogen-cooled mercury/cadmium/telluride detector and analyzed with Perkin-Elmer Spectrum software. Liposomes were extruded as described above in TEN buffer, and proteins were added outside the liposomes in a 1:1 weight ratio (0.233:1, protein:lipid molar ratio). The temperature of the sample holder was controlled with a Peltier device (Paige Instruments, Davis, CA), which holds the sample between two CaF_2 windows. The sample temperature was measured with a thermocouple placed directly on the windows. A resolution of 4 cm^{-1}, 4 coadded interferograms, 2 cm s^{-1} mirror speed, and a 3500–900 cm^{-1} wavenumber range were used. The sample chamber was continually flushed with dry air to keep the relative humidity near 0%.

Fluorescence anisotropy

For fluorescence anisotropy measurements, extruded liposomes (2 mg/ml lipid) were incubated with diphenylhexatriene (DPH) or trimethylammonium diphenylhexatriene (TMA-DPH) (Molecular Probes) in a 200:1 lipid:probe molar ratio in the dark under $N_{2(g)}$ for 30 min (Hincha et al., 1999). TEN buffer (1.75 ml) with or without AFP type I (final concentration 1.0 mg/ml; 4.1:1, protein:lipid molar ratio) was cooled to 5°C in cuvettes, after which 50 μl liposomes were added and the samples were warmed through T_m. The anisotropy of the membrane lipids was determined as the depolarization of the fluorescence emitted from the probes (Lentz, 1993) at excitation and emission wavelengths of 350 and 460 nm, respectively.

Pelleting liposomes

DGDG:DMPC liposomes containing 1 mol % *N*-biotinyl-phosphatidylethanolamine were made as described above, in the presence of 2 mM $CaCl_2$. Liposomes were incubated for 1 h in microfuge tubes with AFP type I, in a 1:1 weight ratio of protein:lipid (0.233:1 molar ratio), and 20 μg streptavidin-coated beads (Dynabeads, Dynal A.S., Oslo, Norway) at room temperature or on ice, and pelleted by centrifugation at 50 × g for 30 s (Zhan, 1999). Supernatant and pellet samples were lyophilized and resuspended in electrophoresis sample buffer. Proteins were fractionated by SDS-PAGE (18% acrylamide) according to Laemmli (1970) and visualized by silver staining (BioRad, Hercules, CA). Gel images were taken with a digital camera and the bands were quantitated by laser densitometry (Image Quant Software, Molecular Dynamics, Sunnyvale, CA).

NBD-Labeling of AFP type I and fluorescence resonance energy transfer (FRET)

AFP type I was *N*-terminally labeled with 4-fluoro-7-nitrobenzofurazan (NBD) (Molecular Probes) according to Rapaport and Shai (1991). AFP type I was lyophilized as a trifluoroacetic acid salt, and mixed with free NBD in a 5:1 weight ratio and an excess of dry dimethylformamide. This

mixture was incubated for 24 h in the dark under $N_{2(g)}$ at room temperature. Subsequently, the solvent was dried off under a stream of $N_{2(g)}$ and the resulting sample was placed under vacuum to remove any residual solvent. The sample was suspended in TEN buffer and the solution pH was checked and adjusted to 7.4, if necessary. The sample was subjected to gel filtration (Sephadex G-15, Pharmacia) and the fractions containing NBD-labeled protein were pooled. Circular dichroism showed that the NBD-labeled AFP folded in the same temperature-dependent manner as the unlabeled AFP (M.M.T., unpublished results).

FRET experiments were performed by labeling liposomes with DPH as described above, followed by incubation for 1 h with NBD-labeled protein at room temperature or on ice. In all cases, the samples were kept in the dark under $N_{2(g)}$ to prevent peroxidation of the unsaturated DGDG acyl chains during the course of the experiments. Liposomes (50 μl) were combined with TEN buffer at room temperature in a cuvette to a final volume of 2 ml, and fluorescence was determined at an excitation wavelength of 357 nm and emission wavelengths of 460 nm (DPH) and 518 nm (NBD). Measurements were taken immediately after rewarming and after an additional 3 h incubation at 22°C. FRET was measured as a decrease in DPH fluorescence intensity, the donor fluorophore, along with a corresponding increase in NBD fluorescence, the acceptor fluorophore.

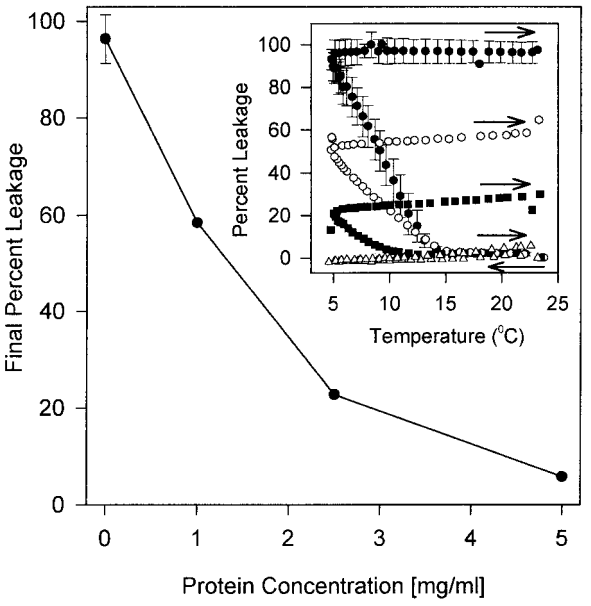

FIGURE 1 AFP type I inhibits leakage from DGDG:DMPC liposomes during chilling. Liposomes in the presence of increasing concentrations of AFP type I were cooled to 4°C and rewarmed; final percent leakage is shown. (*Inset*) Percent leakage from liposomes as they were cooled and rewarmed (*arrows*) at 0.5°C/min in the presence of increasing AFP type I concentrations; ● = control; ○ = 1.0 mg/ml (8.2:1, protein:lipid molar ratio); ■ = 2.5 mg/ml (20.4:1, protein:lipid molar ratio); △ = 5.0 mg/ml (40.8:1, protein:lipid molar ratio). $n = 3$ for all data points.

RESULTS

Leakage experiments

We first wanted to determine whether AFPs inhibited leakage from model membranes composed of equal weight fractions of DMPC and DGDG. When these liposomes were cooled through their phase transition temperature (T_m), leakage was inhibited by AFP type I in a concentration-dependent manner (Fig. 1). Most of the leakage occurred during cooling (Fig. 1, *inset*), as the liposomes passed through their T_m (between 10° and 20°C, Fig. 3). Increasing concentrations of AFP shifted the onset of leakage to lower temperatures, until leakage was essentially eliminated at 5.0 mg/ml AFP (Fig. 1). No significant leakage occurred during rewarming of the liposomes in the presence of AFP type I (Fig. 1, *inset*), suggesting that any interaction between the liposomes and the protein may be stable after the liposomes pass through their T_m. Strikingly different results were obtained when the liposomes were chilled in the presence of small molecular mass AFGPs (2.7–7.9 kD) that are similar in molecular mass to AFP type I (~3 kD). AFGP fractions 6,7,8, and 8 alone did not inhibit leakage compared with the controls, nor did the control non-AFPs ovomucoid and ovotransferrin (data not shown). Thus, the interaction between AFP type I and these membranes containing a plant galactolipid is distinctly different from the interactions observed with membranes composed of phospholipids alone, in which the effects of AFGPs and AFPs on membrane permeability are reversed (Hays et al., 1996).

FTIR Spectroscopy

We used FTIR to investigate whether the reduced membrane permeability of DMPC/DGDG vesicles during chilling by AFP type I was attributable to a direct interaction between the proteins and the lipids. AFP type I could limit the permeability of the membrane by altering the order of the lipid acyl chain region, which can be observed using FTIR. To ensure that the DGDG and DMPC were well mixed in the lipsomes, we used perdeuterated DMPC, in which all the methylenes are deuterated (CD_2), (d54DMPC) in liposomes along with fully protonated DGDG to determine the phase behavior of each lipid independently. The higher mass of the d54DMPC CD_2 shifts its IR vibration to a lower wavenumber than the DGDG methylene CH_2 (Mendelsohn and Moore, 1998), which allows simultaneous determination of the phase transition temperature of the two lipids. The data in Fig. 2 clearly show that the two lipids were well mixed over the temperature range used in this study, as the thermotropic responses of both lipids were identical.

Then we determined the phase transition temperature of fully protonated liposomes using FTIR spectroscopy (Mantsch and McElhaney, 1991) by monitoring the symmetric CH_2 stretch vibration over temperature with and without AFP. In Fig. 3, the wavenumber of the symmetric CH_2 stretch vibration of the liposomes in the presence and absence of AFP is plotted as a function of temperature. There was a clear increase in the T_m of the liposomes in the presence of AFP from 14.9° to 18.4°C during cooling (Fig. 3 *A*). The wavenumber position of the symmetric CH_2

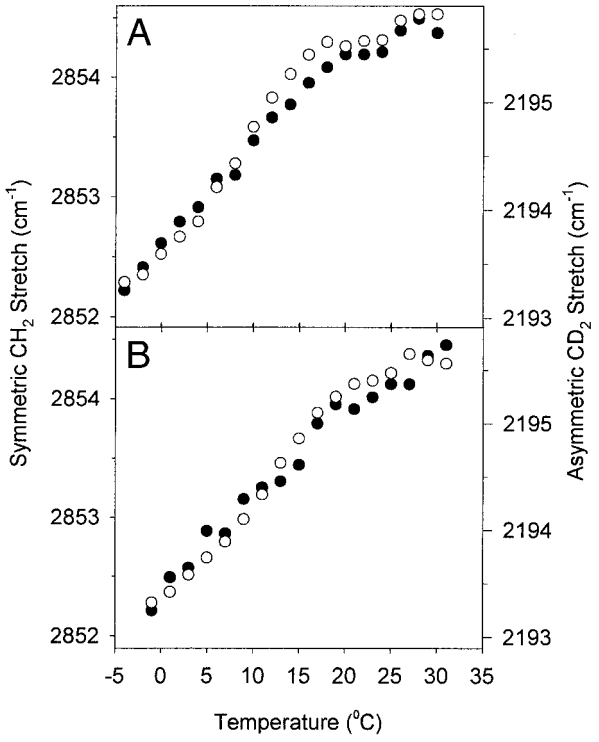

FIGURE 2 DGDG and DMPC are well mixed in the liposomes over the temperature range used. The symmetric CH_2 stretch of fully protonated DGDG (○) and the asymmetric CD_2 stretch of d54 DMPC (●), as measured by FTIR, are plotted as a function of temperature during cooling (*A*) and warming (*B*).

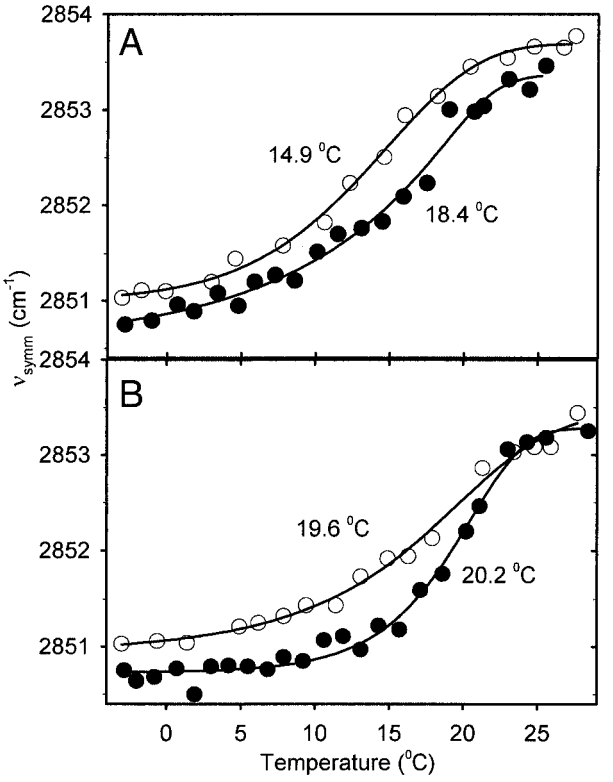

FIGURE 3 AFP type I increases the phase transition temperature of 1:1 DGDG:DMPC liposomes. The symmetric CH_2 stretch of the lipids was measured with FTIR in the absence (○) and presence (●) of AFP type I as a function of temperature. Phase transition temperatures (T_m) are indicated and were determined during cooling (*A*) and warming (*B*) as the midpoint of the curves.

stretch vibration was shifted to lower wavenumbers in the presence of AFP type I in both liquid-crystalline and gel states of the lipids. This suggests that the peptide induced a more ordered bilayer in both liquid crystalline and gel phases. During warming in the presence of the AFP, the liposomes remained in gel phase at a higher temperature than the liposomes alone (Fig. 3 *B*). The wavenumbers of the experimental and control samples were clearly different in the liquid-crystalline phase before cooling and in the gel phase. However, after the samples had been cooled and rewarmed through T_m, that difference in wavenumber disappeared (Fig. 3 *B*). This indicates that there may be differential effects of the AFP on the gel and liquid-crystalline phases. The increase in T_m induced by AFP type I, indicating tighter packing of the lipid molecules, is consistent with the suggestion that an increase in the molecular packing density of the lipids in the vesicles results in reduced bilayer permeability.

With mixed lipid systems it is common to observe that the cooling scan T_m is lower than the warming scan T_m because of an increased heat requirement to melt the system after it is in the gel phase (Chapman and Urbina, 1974). In control experiments, we found that there was no contribution of the AFP type I alone in the methylene stretching region of the spectrum (between 3000 and 2800 cm^{-1}, data not shown). We also investigated whether lower ratios of protein:lipid would cause any shift in the T_m of the liposome, and found there was no effect on T_m with either a 1:10 or 1:5 protein:lipid ratio (w/w, data not shown). Additionally, AFGP fractions 6,7,8, and other control non-AFPs did not cause a shift in the T_m of these liposomes (data not shown).

Fluorescence anisotropy

The FTIR and leakage experiments were performed at different concentrations and ratios of lipid to protein because of constraints of experimental design. FTIR requires a highly concentrated sample in a small volume to see clear IR absorbances. In contrast, fluorescence spectroscopy requires low turbidity and a relatively large sample to measure a clear signal without underlying noise from light scattering. Therefore, we needed a method that could bring together the leakage experiment results with the FTIR data to determine whether the shifts we observed in the FTIR correlated with the inhibition of leakage monitored by fluorescence. For this purpose, we used fluorescence anisotropy, which can be

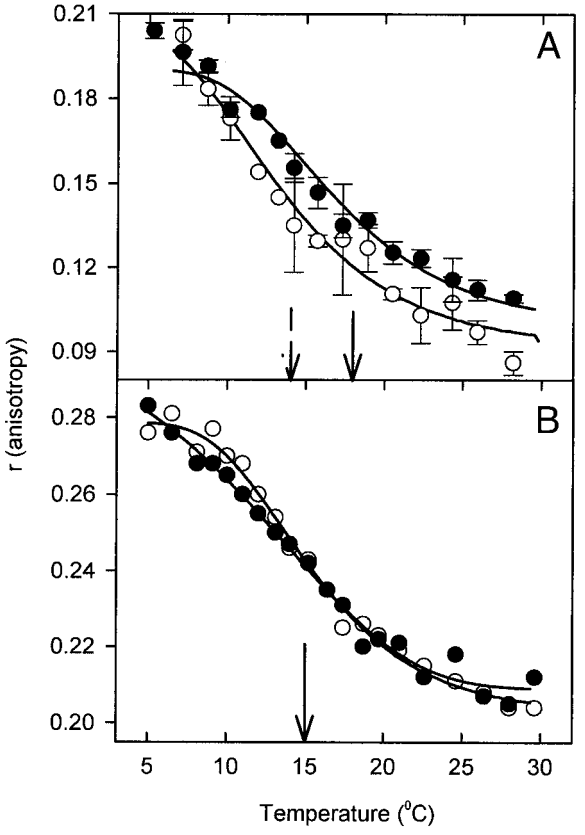

FIGURE 4 Phase transition temperatures and acyl chain order measured by fluorescence anisotropy. (*A*) DPH and (*B*) TMA-DPH indicate the local order of the core and interfacial regions of the lipid bilayer, respectively, in the presence (●) and absence (○) of 1.0 mg/ml AFP type I during warming. In *a*, the T_m in the presence of AFP is indicated by the solid arrow, without AFP by the dashed arrow.

used to monitor the relative order of the bilayer in a solution of low concentration.

Changes in local order of the bilayer core in the presence of AFP were observed with fluorescence anisotropy, using probes that are located at different depths in the bilayer. When DPH, which preferentially partitions into the hydrophobic core of the bilayer (Kaiser and London, 1998), was incorporated into the liposomes, a marked increase in anisotropy was observed in the presence of AFP during and above the phase transition as the liposomes were warmed through T_m (Fig. 4 *A*), indicating a more ordered bilayer core. These data confirmed the increase in T_m seen with FTIR (Fig. 3), which indicated AFP type I alters the packing order of the lipid acyl chain region, and show that these changes occur at the same protein:lipid ratios used in the liposome leakage experiments. By contrast, when the liposomes were labeled with the interfacial probe TMA-DPH there was no difference in the anisotropy between the control liposomes and the liposomes in the presence of AFP type I during warming from 5° to 30°C (Fig. 4 *B*). This indicated that AFP type I did not affect the order at the interfacial region of the bilayer. With FTIR, there was no change in the spectral region corresponding to the galactolipid headgroups of the DGDG (data not shown), which is in agreement with the TMA-DPH fluorescence anisotropy data. We also looked at the lipid carbonyl stretch region with and without AFP in the FTIR, between 1755 and 1730 cm^{-1}. After baseline correction and Fourier self-deconvolution of this region, there was very little difference between the carbonyl stretch of the liposomes in the presence of the AFP and the liposomes alone (data not shown), which again confirms the TMA-DPH results. These data suggest that the proteins interact with the bilayer hydrophobically, perhaps through the Ala and Thr residues that predominate in the primary sequence of AFP type I, because the AFP affected the acyl chain region and not the aqueous interface. This hypothesis is similar to the recent proposal, based on structural evidence, suggesting that AFP type I inhibits ice crystal growth through hydrophobic interactions between the Thr and/or the Ala residues and the surface of the ice (Haymet et al., 1998, 1999; Zhang and Laursen, 1998; Baardsnes et al., 1999; Chao et al., 1997).

SDS-PAGE analysis

It has remained unclear whether the low-temperature AFP/AFGP stabilization of membranes is a direct result of peptide-membrane binding or an indirect effect of the peptides on bilayer structure and permeability. To distinguish between these possibilities, we pelleted biotin-labeled liposomes with high-density streptavidin-coated beads in the presence or absence of AFP type I to look for direct AFP-membrane interaction. This technique is convenient to look for weak or transient interactions between peptides and bilayers because it requires only a very low-speed centrifugation to pellet the liposomes (Zhan, 1999). Therefore, if the AFP is associated with the bilayer it will preferentially be found in the pellet (Zhan, 1999). In samples incubated at room temperature, 36% of the AFP was associated with the pellet (Fig. 5, *lanes 4* and *5*). In contrast, 76% of the peptide was associated with the pellet after incubation on ice for 1 h (Fig. 5, *lanes 6* and 7). These results indicate that the majority of the AFP was associated with the lipid bilayer after passing through the T_m and that the interaction was stable, as the liposomes were pelleted after the sample was rewarmed to room temperature. The apparent stability of interaction after the liposomes have been chilled and rewarmed in the pelleting experiment is in agreement with the lack of leakage from the liposomes in the presence of AFP type I during rewarming (see Fig. 1, *inset*). SDS-PAGE analysis also showed a 4-kD peptide from the streptavidin-coated beads (Fig. 5, *lanes 3* and *8*) near the ~3-kD AFP type I. However, using densitometry we could clearly distinguish between the two bands. As a control we incubated liposomes with either ovomucoid or lucifer yellow, and found no measurable ovomucoid and <10% of the lucifer

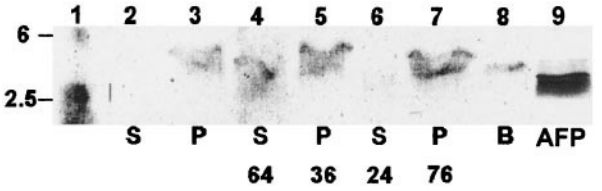

FIGURE 5 AFP type I associates with pelleted liposomes. Samples from biotin-PE labeled liposomes incubated with and without AFP type I, and pelleted with streptavidin-coated beads were subjected to SDS-PAGE followed by silver staining. *Lane 1*: molecular weight markers; *lanes 2 and 3*: liposomes without AFP type I; *lanes 4 and 5*: liposomes with AFP type I incubated at room temperature; *lanes 6 and 7*: liposomes with AFP type I incubated on ice; *lane 8*: control with streptavidin beads only (*B*); *lane 9*: AFP type I alone (much greater amount of protein than in experimental samples). S, supernatants; *p*, pellets. Numbers under the lanes refer to the percent of AFP type I in the supernatant and pellet of the respective samples. The molecular weight marker protein masses are given in kilodaltons on the left side of the figure.

yellow in the pellet (data not shown). Taken together, these data indicate that the AFP type I directly associates with the lipid bilayer when the liposomes are chilled through their phase transition temperature and that this interaction is specific and stable.

FRET

We used FRET between NBD-labeled AFP type I and DPH-labeled liposomes to confirm the direct interaction that was implied by the liposome pelleting experiments. FRET occurs when donor and acceptor fluorophores approach sufficiently close, on average, to transfer fluorescence excitation energy. Thus, there should be FRET between the DPH, which preferentially partitions into the bilayer core (Kaiser and London, 1998), and the NBD-AFP when the *N*-terminus of the peptide inserted into the membrane. A significant amount of FRET was observed when the DPH-labeled liposomes were incubated with NBD-AFP at room temperature (Fig. 6), but when the liposomes were incubated on ice for 1 h in the presence of 2.0 mg/ml NBD-AFP (0.22:1, protein:lipid molar ratio), FRET increased 2.5-fold (Fig. 6), indicating that the *N*-terminal NBD label on the peptide came into close proximity with the DPH in the bilayer core after the membrane had passed through its T_m. All samples were incubated at room temperature for an additional 3 h, and FRET was measured again. It is clear from Fig. 6 that when the samples were chilled for 1 h and then rewarmed to room temperature and incubated for 3 h, FRET increased compared with the samples that were not chilled and was not diminished with time at room temperature. Thus, the interaction seems to be stable once it has been established. As noted in the methods, the NBD-AFP secondary structure folding profile was identical to that

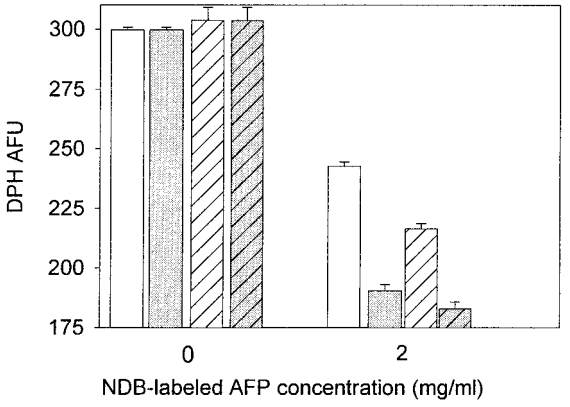

FIGURE 6 NBD-labeled AFP type I inserts into the hydrophobic region of the bilayer. FRET between the NBD-labeled AFP and DPH in the hydrophobic core was measured by the decrease in DPH fluorescence. Liposomes were incubated at room temperature (*white background*) or on ice (*gray background*) for 1 h, after which the DPH fluorescence was measured at room temperature (*open bars*). All liposomes were incubated at room temperature for an additional 3 h, and DPH fluorescence was measured again (*bars with diagonal lines*).

of the unlabeled AFP type I, as monitored by circular dichroism (data not shown).

DISCUSSION

We have shown that a fish AFP, AFP type I, inhibits leakage across membranes containing a chloroplast galactolipid when they are chilled through their T_m (Fig. 1). We propose that the mechanism involves direct interaction between the peptide and the bilayer, which alters the order of the acyl chains in the hydrophobic bilayer core as shown by FTIR and fluorescence anisotropy experiments (Figs. 3, 4). Three different experimental methods suggest that the interaction between the protein and the liposomes is stable after the liposomes have passed through their T_m. The liposome pelleting experiments and the FRET measurements (Figs. 5, 6), were both performed after the chilled samples had been rewarmed to room temperature, indicating that the interaction between the peptide and the bilayer was stable. As well, there was an almost complete inhibition of leakage observed during rewarming of the liposomes after they have been chilled through their T_m with AFP type I (Fig. 1, *inset*). We propose that the AFP causes these effects by partially inserting into the membrane during chilling (Figs. 5, 6).

The FTIR and fluorescence anisoptropy data show that the peptide had a great effect on the order of the bilayer core but not on the aqueous interface (Figs. 3, 4), a suggestion that is consistent with recent data indicating a hydrophobic interaction between AFP type I and ice (Haymet et al., 1998, 1999; Zhang and Laursen, 1998; Baardsnes et al., 1999; Chao et al., 1997) and data on small amphipathic peptides that insert into membranes (Tamm, 1991). Mutations in the

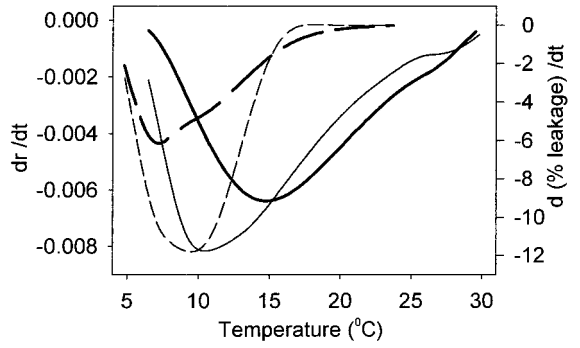

FIGURE 7 Inhibition of leakage is not correlated with the change in phase transition temperature. The first derivative of the cooling leakage curves of the control (*thin dashed line*) and 1.0 mg/ml AFP type I (*bold dashed line*) from Fig. 1 are plotted along with the first derivatives of the anisotropy curves of the control (*thin solid line*) and 1.0 mg/ml AFP (*bold solid line*) from Fig. 5 to compare the temperature at which the leakage occurs with the temperature of the phase transition. The first derivatives were determined using Peak Fit version 4 software (Jandel Scientific).

Thr and adjacent Ala of AFP type I suggest that these residues play key roles in the interaction between AFP type I and ice (Haymet et al., 1998, 1999; Zhang and Laursen, 1998; Baardsnes et al., 1999). It is not clear whether these amino acids are involved in membrane stabilization, but, clearly, the interactions in both cases have a predominantly hydrophobic component.

Closer examination of the leakage (Fig. 1) and the fluorescence anisotropy phase transition (Fig. 4) data suggests that these events are not related in the samples in the presence of AFP. In fact, leakage occurs at progressively lower temperatures as the protein concentration is increased (Fig. 1), whereas the phase transition occurs at progressively higher temperatures under the same conditions (Figs. 3 and 4). Inspection of the first derivatives of both curves (Fig. 7) makes it clear that the leakage and phase transition are in good agreement in the controls, as expected. However, in the presence of the AFP, the leakage and phase transition curves show only minimal overlap (Fig. 7), suggesting that they are indeed unrelated events. This conundrum, which may be related to the change in chain packing induced by the AFPs, clearly requires further investigation.

The difference in total concentrations and protein:lipid ratios used in the FTIR versus the leakage and fluorescence anisotropy experiments seem to leave an important question unanswered: how many proteins interact per lipid after the liposomes have been chilled through their phase transition? If we assume that the AFP is only associated with the outer leaflet of the liposome and ~75% of the protein is associated with the liposomes after pelleting (Fig. 5), then the ratio of protein:outer leaflet lipid in the pellet is 1:2. We feel that this calculation allows an approximation of the number of proteins associated per lipid. However, we do not know whether all the protein in the pellet is directly associated with the bilayers, or whether some of the protein is associated with the liposomes through protein-protein interactions. We do not propose that the peptide inserts into the bilayer in a transmembrane orientation. Studies of small α-helical peptides that insert across the bilayer actually cause leakage from liposomes (Dempsey, 1990; Epand et al., 1995; Tamm, 1991). In contrast, AFP type I inhibits leakage when it associates with the bilayer. Therefore, we propose that the AFP inserts only partially into the bilayer, and the FRET results suggest that it may be the *N*-terminus of the peptide that inserts (Fig. 6).

A recent paper suggests that the low temperature membrane protection conferred by AFPs may not be specific to AFPs (Wu and Fletcher, 2001). This is puzzling because similar low temperature protection has not been reported in other studies of AFP-membrane interaction (Hays et al., 1996; Rubinsky et al., 1990, 1991; Tablin et al., 1996), and also has not been observed in the present investigation. A possible explanation for this discrepancy could be found in the observation that control liposomes sometimes leak only a fraction of the amount of the average percentage of leakage during chilling. The cause of this has not been identified, but may be attributable to buffer or lipid contaminants (Hays et al., 2001; M.M.T., unpublished data). Therefore, a false positive can easily be obtained if a control liposome sample is not run simultaneously with the experimentals. All controls performed in the present study indicate that the interactions observed between AFP type I and the DGDG:DMPC liposomes are specific.

Previously, Hincha et al. (1993) reported that AFGPs and AFPs actually damage thylakoids during freezing, and these results have been confirmed with subsequent freezing studies with intact thylakoids and liposomes containing thylakoid lipids (Tomczak et al., 2001). Perhaps not surprisingly, in view of these results, attempts to increase freezing tolerance of plants through transformation with the AFP type I gene have met with little or no success (Kenward et al., 1993; Cutler et al., 1989; Hightower et al., 1991). Based on the results of our study, we suggest that transformation of plants with the gene for AFP type I could reduce chilling sensitivity by conferring low-temperature protection to cellular membranes.

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