J Bioenerg Biomembr (2008) 40:389–396
DOI 10.1007/s10863-008-9164-8

Effects antifreeze peptides on the thermotropic properties of a model membrane

Hagit Kun · Refael Minnes · Yitzhak Mastai

Received: 15 May 2008 / Accepted: 28 July 2008 / Published online: 27 September 2008

Abstract In this paper, we report on the effect of short segments of type I antifreeze protein (AFP I) on the thermotropic properties of a model membrane. Two different types of dimyristoylphosphatidylcholine model membranes were used, multilamellar vesicles and small unilamellar vesicles. The membrane properties were studied by differential scanning calorimetry (DSC) and fluorescence anisotropy. With the incorporation of AFP I and its short segments, the order of the model membrane increased both in the gel state and in the liquid crystalline state. The interaction of AFPs with the model membrane caused a shift in the phase transition to lower temperatures, which is accompanied by a broadening of the DSC thermogram. This preferential stabilization to a more ordered phase by the AFPs could be due to ordering the hydrophobic membrane core and separation into domains. Overall, this approach of employing short segments of AFP I simplifies the correlation between antifreeze protein characteristics and the effect of these parameters on the interaction mechanism of AFP with cell membranes. The success of this approach can lead to the identification of short peptides with high antifreeze activity.

Keywords Antifreeze peptides · Thermotropic properties · Peptide–lipid interactions · Model membrane

H. Kun · Y. Mastai (✉)
Department of Chemistry and the, Institute of Nanotechnology,
Bar-Ilan University,
Ramat-Gan 521900, Israel
e-mail: mastai@mail.biu.ac.il

R. Minnes
Department of Physics, Bar-Ilan University,
Ramat Gan 521900, Israel

Introduction

Freezing is almost always lethal to cellular organisms as it deprives biological processes of the fluid aqueous environment that they require, induces denaturing of biomolecules, and ruptures cell membranes. Despite this, polar and near-polar fish typically survive in seas where the temperature is subzero, frequently as low as −1.9°C. Publications, first by Devries et al. (1971), Devries and Wohlschl (1969), Komatsu et al. (1970), Somero and Devries (1967) and later by others, described the existence of a glycoprotein in the serum of Antarctic fish that lowered the freezing temperature without increasing the osmotic pressure. Since these early studies, there have been many reports on these and similar-acting proteins, some of which are not glycoproteins. A significant number of studies have been focused on their characteristics (Barrett 2001; Davies and Hew 1990; Feller and Gerday 1997; Fletcher et al. 2001), while other studies have been dedicated to revealing the principle and the mechanisms behind their functionality (Duman et al. 1991; Feeney et al. 1986; Yeh and Feeney 1978; Yeh and Feeney 1996).

The two main families of proteins that possess the capability of depressing the freezing temperature are the *antifreeze glycoproteins*, or AFGPs, and the *antifreeze proteins,* or AFPs. The structures of both the AFGPs and AFPs have been extensively studied for several decades. Antifreeze glycoproteins are carbohydrate rich, 2.6–34 kDa, proteins containing an (ala-ala-thr) repeat unit with a disaccharide (Bouvet and Ben 2003). AFPs are classified as AFP Type I to IV, based on their composition and secondary structures. Type I AFPs are small, 3.3 to 4.5 kDa, alanine rich, α-helical, and constructed of 11 amino acid repetitions. The most studied Type I AFP, called HPLC6 (or TTTT), consists of 37 amino acids present in

the winter flounder. Because of their simple structure, our interest will be directed towards Type I AFPs.

One very important aspect of the research on AFPs is their interaction with cell membranes. Most cells are damaged when they are cooled below physiological temperatures due to the extensive stress exerted on the membrane. The ability of antifreeze proteins to protect cell membranes from hypothermic damage was first demonstrated by Rubinsky et al. 1990, 1992. In addition, it was also hypothesized that AFP selectively suppressed calcium and potassium currents of porcine granulosa cells, suggesting that the mechanism of antifreeze protein action involved the blockage of ion channels (Negulescu et al. 1992). Hays et al. showed that AFGPs from polar fish inhibit leakage from liposomes composed solely of phosphatidylcholines, as they are chilled through their main phase transition temperature (Hays et al. 1996). Their studies indicated that the calcium leakage through the membrane is inhibited not because of the blockage of the ion channels, but by interactions with the lipids only. Evidence consistent with this hypothesis comes from studies on human blood platelets (Crowe et al. 1999; Tablin et al. 1996), which also demonstrated that AFPs were able to inhibit cold-induced morphological changes initiated when the platelets pass through their phase transition temperature. Recently, Tomczak and Crowe proposed that the mechanisms of membrane stabilization and the protection of AFGPs and Type I AFP differ from each other (Tomczak and Crowe 2002). While AFGPs are expected to form a monolayer covering the membrane to prevent leakage, Type I AFPs are inserted into the membrane. To reveal the effect of AFGPs and AFPs on the cell membrane, lipid vesicles (Hincha et al. 1993; Ricker et al. 2003) have been employed as model membranes, and a range of experimental techniques were applied, e.g., differential scanning calorimetry (DSC), Fourier transform infrared (FTIR) spectroscopy, liposome binding and leakage tests (based on fluorescence assays), fluorescence anisotropy, and fluorescence resonance energy transfer (FRET). Furthermore, model membranes with diverse compositions have been used in order to fully understand the nature of the interactions between AFPs and cell membranes. It was shown that these interactions are lipid specific, i.e., the lipid composition of the bilayer dictates whether or not a certain AFPs or AFGPs will protect/interact with the membrane (Hays et al. 2001; Tomczal et al. 2001, 2003; Wu and Fletcher 2000). Finally, the majority of research on the effect of AF(G)Ps on cells or model membranes has been performed using native proteins or their mutants.

Fluorescence anisotropy has been extensively used in the literature to study membrane-related changes. Overall fluorescent probes used in fluorescence anisotropy are completely or partially lipophilic, and their incorporation into membranes occurs within a specific domain, independent of any cell pre-treatments (Borenstain and Barenholz 1993; Trevors 2003; vanderHeide et al. 1996). Several different probes have been designed and employed to study a variety of membrane dynamics (Trevors 2003). The most commonly used probe is DPH (1,6-diphenyl-1,3,5-hexatriene), an extremely hydrophobic, symmetrical, rod-like trans-polyene that penetrates the hydrophobic core, orienting itself parallel to the fatty acid side chains (Adler and Tritton 1988; Kaiser and London 1998). Knowing the probe location is important in the interpretation of experimental observations (Borenstain and Barenholz 1993). The physical and spectral properties of DPH and other probes are ideally suited for studies in membrane structure and motion (Borenstain and Barenholz 1993). The typically high quantum yield of the probes allows a lower ratio of probe-to-lipid (Borenstain and Barenholz 1993; Adler and Tritton 1988; Denich et al. 2003; Shinitzky and Barenholz 1978). The theoretical relationship quantifies the degree of the depolarization of light emitted by the membrane-embedded fluorescent probe as a measure of the membrane's state (Trevors 2003; Adler and Tritton 1988; Sklar 1984). The degree of polarization is defined by several parameters, of which polarization ratio (P) and polarization anisotropy (r) are most commonly used, as described by Shinitzky and Barenholz (Shinitzky and Barenholz 1978). The probe is subject to the rotational restriction imparted by the lipid order. For example, acyl chain interactions in a highly structured gel-phase membrane will decrease the probe motion and result in a higher degree of retained polarization. As such, the polarization ratio and membrane fluidity are inversely proportional, whereby a liquid crystalline membrane with reduced acyl chain interactions will facilitate probe rotation, thus resulting in a lower polarization ratio (Trevors 2003). Fluorescence anisotropy was previously used to determine changes in the local order of a lipid bilayer core in the presence of AFP, using probes located at different depths of the bilayer. When DPH was incorporated into the liposomes, a marked increase in fluorescence anisotropy was observed in the presence of AFP during and above the phase transition temperature as the liposomes were warmed through T_m, indicating a more ordered bilayer core. These data confirmed the results seen with FTIR, which indicated that Type I AFP alters the packing order at the lipid acyl chain region. When liposomes were labeled with the interfacial probe, TMA-DPH, Type I AFP did not affect the fluorescence anisotropy of the liposomes during warming, indicating that the head group region is not influenced, as was concluded by FTIR. These results suggest that the AFPs interact preferentially with the bilayer hydrophobic core, and less with the aqueous interface.

The second and more commonly used method of analyzing additives effect on membranes is DSC. DSC is

a nonperturbing thermodynamic technique that has proven of great value in studies of additives effect on the thermotropic phase behavior of phospholipids bilayers. DSC allows observation of various thermal events including phase transitions. Phase transitions of model membranes are well characterized by DSC (Blandamer et al. 1995). Furthermore, the DSC thermogram enables to determine to what extent the phospholipids molecules of the model membrane ''melt'' cooperatively. The cohesion of the phospholipids molecules during the phase transition would therefore produce a sharp phase transition peak. The use of DSC allowed a rapid assessment of the effect of AFP on phase transition temperature and cooperativity of the model membrane. Also, the effect of added AFP on the width of the phase transition peak in DSC thermogram is an indication of the cooperativity of the model membrane.

Recently, Mastai et al. (Kun and Mastai 2007) introduced a new approach to the study of AFP activity and interactions with membranes, based on employing short segments of AFP instead of using entire proteins. This methodology proved capable of clarifying the antifreeze activity of Type I AFP. It was shown that short segments of Type I AFP can possess about 60% of the antifreeze activity of the native protein. In addition, it was also demonstrated that the AFP fragments exhibit non-zero thermal hysteresis, and caused modification of the ice crystal habits and a decrease in the crystallization rates of ice.

In the present paper, we extended our approach by studying the effect of Type I AFP and its short segments on the physical properties of model membranes. The focus of this paper is to examine the AFP interactions with cell membranes and to characterize the resulting changes in the membrane'' properties, and to some extent shed light on the molecular mechanism. Our research will focus on Type I AFPs, mainly because of their ordered repetitive sequence and simple α-helical structure. Our working strategy is to employ Type I AFPs, but also to emphasize the use of various short fragments instead of the whole proteins. Such antifreeze short peptides can be regarded as "molecular tools" or "nano-indicators" that would disclose how various fractions of AFPs interact with membranes. This approach simplifies the establishment of a correlation between AFP characteristics such as hydrophilicity or hydrophobicity and charge, and the effects of these characteristics on the interaction with membranes. With respect to the membrane stability conferred by the AFPs, it is not known what changes in the mechanical properties of the membrane are potentially induced by AFPs.

In addressing the question about the effect of AFPs on membranes, we use dimyristoylphosphatidylcholine (DMPC) vesicles as model membranes. It should be mentioned that although DMPC is oversimplified as a model for cell membranes, it is still a very effective model system for examining AFP membrane interactions due to its well-defined phase transitions. Moreover, two different types of DMPC model membranes were used, multilamellar vesicles and small unilamellar vesicles. This selection of multilamellar and small unilamellar vesicles was based on the method employed to investigate the interactions of Type I AFP and its short segments with DMPC model vesicle systems.

Materials and methods

Peptide synthesis Three short segments of HPLC6 (DTAS DAAAAAALTAANAKAAAELTAANAAAAAAAATAR-COOH) were prepared. HK1 (TAANAAAAAAAATAR-COOH, 14 residues), HK2 (DTASDAAAAAAL-NH_2, 12 residues) and HK3 (TAANAKAAAEL-NH_2, 11 residues) were synthesized using Fmoc chemistry on Rink Amide (0.51 mmol/g, 1 mmol) resin or on Wang (0.66 mmol/g, 1 mmol) resin. Using TFA deprotection and THF cleavage with triisopropylsilane and water as scavengers, the crude peptides were extracted from the resin by gravitational filtering; then dissolved in water and analyzed by a C_{18} HPLC column with a gradient of 0–100% ACN in aq. 0.1% TFA. HPLC was synthesized by C S Bio Co. HPLC6 was purchased in powder form from CS Bio Co. and analyzed by mass spectra and HPLC.

Model membrane DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) was purchased from Avanti Polar Lipids and used as a model system to study AFP-cell membrane interactions. We used two membrane models; one consisting of unilamellar vesicles and the other of multilamellar vesicles. DMPC was dissolved in a TES buffer, pH=7.5 and vortexed for 5 min to prepare the multilamellar vesicles. The unilamellar vesicles were prepared by dissolving DMPC in a TES buffer, pH=7.5 followed, by 1 min vortex and 20 min probe sonication.

Differential Scanning Calorimetry (DSC) Antifreeze activity was also determined using a DSC Mettler Toledo DSC 822 device equipped with Mettler TA-STAR^e software. The samples were weighed and sealed in an aluminum pan. An empty aluminum pan was used as a reference. Solutions were at a weight ratio of 2:1 (DMPC 20 mg/mL:AFP 10 mg/mL). Phase transition temperatures were determined as max temperatures. ΔT_m is defined as $T_{m,DMPC}$–$T_{m,AFP}$. The enthalpy was calculated by integrating the area under the peak, and $\Delta T_{1/2}$ is the difference between the peak's left and right border at the half height of the peak. The samples were heated from 15 °C to 36 °C at rate of 1 °C/min. The measurements were performed with DMPC multilamellar vesicles at a concentration of 20 mg/mL.

Polarized fluorescence A polarized fluorescence method was used to measure the membrane model's core fluidity using a Perkin–Elmer (Norwalk, CT, USA) LS-50B digital fluorimeter. Small unilamellar vesicles were used as model membranes for measurements with DPH (1,6-diphenyl-1,3,5-hexatriene) as dye. Solutions were prepared at two weight ratios, 2:1 (0.5 mg/mL DMPC and 0.25 mg/mL AFP) and 11:1 (1.1 mg/mL DMPC and 0.1 mg/mL AFP). DMPC vesicles were prepared as described above, after which the AFP was introduced into the vesicles, to reach final volume of 3 ml. Finally, 3μl of DPH (1 mM) was added to the DMPC/AFP solution. Temperature-dependent experiments were performed from 28 °C down to 19 °C or 7 °C with a decrease of 0.5 °C. All measurements were carried out under continuous mixing. The degree of fluorescence polarization, P, was obtained according to the following equation: $P = \frac{I_v - I_h}{I_v + I_h}$, where I_v and I_h are the emission intensities, polarized vertically and horizontally to the direction of the polarization of the exciting light, respectively (Shinitzk and Inbar 1974; Fuchs et al. 1975; Andrich and Vanderkooi 1976).

Results and discussion

In this work, we use differential scanning calorimetry (DSC) and fluorescence anisotropy to study the thermotropic aspects of peptide–lipid interactions. DSC is used to examine peptide–lipid interactions by changing the melting point, the enthalpy, and the shape of the DMPC phase transition. Figure 1 shows the DSC thermograms of the phase transition of DMPC vesicles in the presence and absence of the AFPs. The phase transition of DMPC bilayers involves two stages, a pre transition at T_p=14.1 °C and a main phase transition at T_m=23.9 °C (Marsh 1990), both of which can be easily detected by DSC. The main phase transition, T_m, is related to the transformation from the gel to the liquid crystalline phase ($P_{\beta'} \rightarrow L_\alpha$). Figure 1 shows the thermogram of DMPC, which exhibited a sharp endothermic peak at T=23.65 °C with an enthalpy of 26.01 kJ/mol, corresponding to the reported temperature and energy values for the phase transition in DMPC (Marsh 1990). In Fig. 1 the phase transition of DMPC in the presence of the AFPs is shown, and the thermodynamic parameters of the phase transition are summarized in Table 1. As shown, the effects of the AFPs are reflected as the shift in the main phase transition temperature, (T_m), changes at the width at half height ($\Delta T_{1/2}$), and therefore, also changes in the transition enthalpy. It should be noted that DSC measurements were performed with DMPC multilamellar vesicles since small unilamellar vesicles gave distorted thermograms. The DSC thermogram of HPLC6 shows that it affected the thermodynamics of the DMPC phase transition by decreasing the enthalpy of phase transition by ca. 3.66 kJ/mol and by shifting the T_m to lower value by 0.32 °C. The thermogram of the HK3 segment indicated a similar enthalpy decrease and a decrease of 0.45 °C in the T_m value. The thermogram of HK1 segment indicated an enthalpy decrease of 1.15 kJ/mol and a decrease of 0.33 °C in the T_m value. The DSC thermograms of the HK2 segment displayed a phase transition enthalpy increase of 0.81 kJ/mol, while it had almost no effect on the T_m, ΔT_m=−0.02 °C.

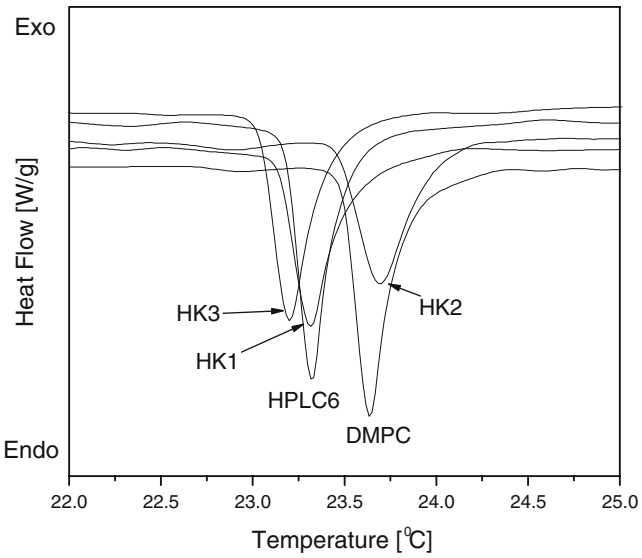

Fig. 1 Phase transition measurements of multilamellar DMPC vesicles in the absence and presence of AFPs using DSC at a concentration of 10 mg/ml (AFP) and 20 mg/ml (DMPC)

Table 1 DSC results of the effect of AFP segments on the model membrane phase transition

Sample	ΔT_m	ΔH (kJ/mol)	$\Delta T_{1/2}$ (°C)
Pure DMPC	0	26.01	0.18
HPLC6	0.32	22.35	0.19
HK1	0.33	24.86	0.22
HK2	−0.02	26.82	0.31
HK3	0.45	22.32	0.22

The AFPs also altered the DSC peak width at half height, $\Delta T_{1/2}$. All the segments and HPLC6 increased the $\Delta T_{1/2}$, HK2 being the most pronounced by 0.31 °C. The DSC thermogram showed that the $\Delta T_{1/2}$ increased by 0.19–0.31 °C.

Fluorescence polarization is a well-known method used to determine the polarization of the acyl chains in lipid membranes, and thereby, to determine the modification in the order of the lipid acyl chains (Tomczak et al. 2002a) in solutions at low concentration. Results of fluorescence polarization measurements of the temperature effect on the lipid fluidity in the presence of AFPs are shown in Figs. 2a

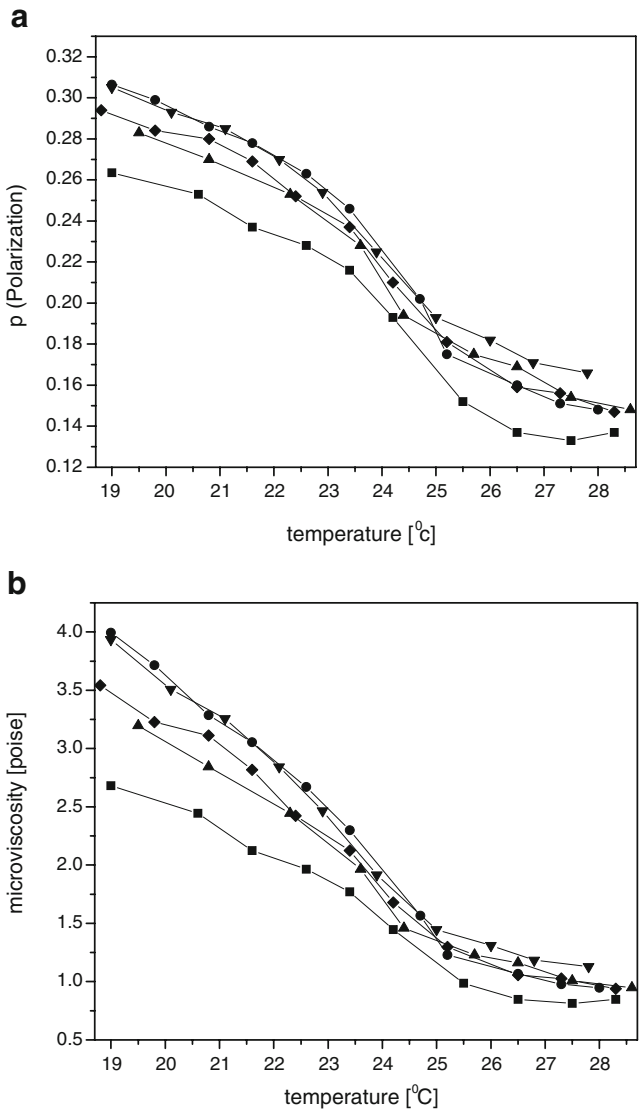

Fig. 2 **a** Phase transition measurements of unilamellar vesicles in the presence of Type I AFP (AFP:DMPC, 1:11) using polarized fluorescence. DMPC (*filled square*), HK1 (*filled triangle*), HK2 (*filled inverted triangle*), HK3 (*filled diamond*), HPLC6 (*filled circle*). **b** Microviscosity of the model membrane as calculated from polarized fluorescence measurements at the unilamellar vesicles in the presence of type I AFP (AFP:DMPC, 1:11). DMPC (*filled square*), HK1 (*filled triangle*), HK2 (*filled inverted triangle*), HK3 (*filled diamond*), HPLC6 (*filled circle*)

and 3. Figure 2a displays measurements performed at a AFP:DMPC weight ratio of 1:11. The results show that solutions of HK2 or HPLC6 have a similar effect below the phase transition temperature. However, above the phase transition, HK2 gave polarization values higher than those of HPLC6, indicating a stronger lipid ordering effect. HK3 and HK1 show a similar behavior, as much as 15% higher than that of DMPC below the phase transition temperature. Above phase transition they gave similar results to those of HPLC6. Figure 3 presents results of an AFP:DMPC weight ratio of 1:2. At this ratio, lower temperatures were measured. HK2 gave higher values than the other segments and rose above the values of DMPC. HK3 showed the same tendency as HPLC6.

The DSC results showed that the thermotropic phase behavior of DMPC is affected by the presence of AFP and its short segments. Generally, the presence of AFPs led to a shift in T_m, an increase in $\Delta T_{1/2}$, and a lowering of the enthalpy of the phase transition. All the segments and HPLC6 had an effect on the cooperativity of the lipid phase transition, when HK2 had the stronger effect. The observed phase transition temperature decrease indicated an increase in the stability of the liquid crystalline phase. The T_m decreased when HPLC6, HK1, HK3 were introduced into the model membrane, with HK3 having a slightly greater effect than the others. The moderate decrease of the enthalpy could indicate that the AFP "facilitated" the transition from the liquid crystalline phase to the gel phase by enhancing the order of the acyl chains in the membrane, as will be discussed below.

The DSC measurements enabled us to determine the change in the phase transition temperature. However, in order to determine the molecular change in the membrane, polarized fluorescence measurements were used. A few conclusions can be deduced from the polarized fluorescence results. First, the phase transition temperature of the model membrane decreased in the presence of the AFPs. Another important conclusion is that above the phase transition temperature at $T > T_m$, the order of the model membrane core, the acyl chain region, increased in the presence of AFP. Finally, below the phase transition temperature, $T < T_m$, the order of the model membrane core also increased.

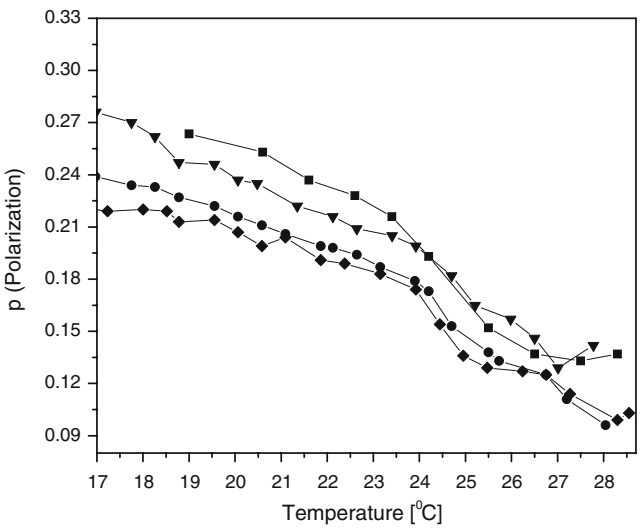

Fig. 3 Phase transition measurements of unilamellar vesicles in the presence of Type I AFP (AFP:DMPC, 1:2) using polarized fluorescence. DMPC (*filled square*), HK2 (*filled inverte triangle*), HK3 (*filled diamond*), HPLC6 (*filled circle*)

At temperatures higher than the phase transition temperature, HK2 induced order to the acyl chains, more than HK1, HK3 and HPLC6. Below the phase transition temperature, HK2 and HPLC6 showed the same effect on the acyl chains, HK1 and HK3 also induced order to the chains, but to a lower extent. When a higher weight ratio of AFP to DMPC was examined with polarized fluorescence, the AFPs still seemed to induce order to the membrane core. HK2 showed a higher polarization than HPLC6 and its polarization results were similar, and even higher, than those of DMPC. This can imply the great capability of HK2 to induce order to the acyl chains, despite the large concentration which normally disrupts the membranes'' integrity.

The apparent microviscosity, η, of the membrane medium was calculated according to the empirical equation, $\eta = \frac{2P}{0.46-P}$, where p is the degree of fluorescence polarization, as shown in Fig. 2b. The apparent microviscosity of lipid bilayers calculated from fluorescence polarization data indicated that the system became much more complex and the microviscosity was elevated both below and above the T_m, as can be seen in Fig. 2b. The effect below the phase transition temperature was larger than that above the T_m, as calculated by the equation of the microviscosity (η).

Overall, there are very few molecules capable of causing this effect on the microviscosity. Drugs and other additives interact with membranes and lead to a decrease in the membrane order and membrane microviscosity. The short AFPs exhibited unique effects on the membrane that are opposite to the effects of common additives introduced into membranes. There are two classes of materials capable of inducing the membrane's core order, cholesterol and saturated fatty acids. Each class has a different effect on the membrane phase transition. Cholesterol, when incorporated into the lipid composition of the membrane, maintains the T_m, but has an opposite effect on the membrane core below T_m and above T_m. Below T_m it reduces the order of the acyl chain in the membrane core, while above T_m it induces order to the acyl chains. This opposite effect on the different sides of phase transition temperature increases with the increase in the amount of cholesterol in the membrane lipids. While the T_m is not affected, it causes the transition to disappear at cholesterol/phosphatidylcholine molar ratios above 0.35 (Mabrey et al. 1978). When incorporated in the membrane lipid, saturated fatty acids change the T_m (Tomczak et al. 2002b), while maintaining the order of the membrane core both above and below the T_m. This causes the polarized fluorescence spectra to retain their shape while moving on the temperature scale. When comparing the effect of our AFPs on saturated fatty acids and cholesterol, it is clear that they posses altogether different attributes than the former membrane-effecting class. The AFPs examined here, like cholesterol, affect the order of the membrane core, and like the saturated fatty acids, they change the T_m. Unlike cholesterol, the AFPs induced order both above and below the T_m and increased the membrane fluidity below the T_m to much larger values than the fluidity increase below the T_m. It should also be noted that the effect of the AFPs was inversely dependent on the concentration, unlike cholesterol.

The DSC and polarized fluorescence results shown here are in disagreement with those of Crowe's group (Tomczak et al. 2002b). Crowe's group showed that HPLC6 had no effect on the T_m, or on the order of DMPC large unilamellar vesicles at 1:1 weighed ratio by FTIR. This discrepancy could be due to the different weight ratio used, the effect of AFP on the T_m could be inversely proportional to the concentration, as was seen in the DSC, and therefore the shift in the T_m was observed here at the lower weight ratio; different instruments used with different specifications regarding sensitivity or volume, which could lead to intermolecular interaction at higher volumes and concentration; and the different acidic C-cap HPLC6 used by us could lead to the different result due to effects at the molecular level. The inversely proportional concentration and T_m shift was not farther investigated as the fluorescence anisotropy instrument used had a margin error of more than 0.01 °C.

Examining both the DSC and polarized fluorescence results enabled us to propose the following mechanism to explain AFP activity on the model membrane. The order induced to the membrane core and the broadened phase transitions combined could indicate that the AFPs inserted into the membrane induced order in isolated domains, and therefore decreased the phase transition cooperativity. The domains can be in both layers of the DMPC membrane or only in one of the DMPC layers, depending on the depth of the AFP penetration. HK2 seems to affect one layer of the DMPC in the membrane, therefore causing an increase in the enthalpy of the transition, while HK1, HK3 and HPLC6 penetrated the outer layer and reached the inner layer, therefore decreasing the enthalpy. This theory of penetration will be examined by fluorescence probes. Moreover, the polarized fluorescence measurements show that the AFP/lipid ratio and the antifreeze effect on the order of the membrane are inversely proportional. The more AFP molecules penetrate the membrane the more domains are formed and the membrane becomes ''defective'. There seems to be a delicate balance between the order caused by AFP penetrating the membrane at low concentration and the disruption of the order in higher concentration of AFP.

When attempting to understand which of the 11 amino acid repetitions in the native protein, HPLC6, which are represented in each short segment, is the source of the antifreeze activity, it is seen that both HK1 and HK3 were responsible for the change in T_m. HK2, even though being

the side to first insert the membrane (Tomczak et al. 2002a), was ineffective in changing the thermodynamics of the phase transition, except for the cooperativity of the transition. However it was most effective in inducing order to the membrane core.

Summary

In this work, we employed differential scanning calorimetry (DSC) and fluorescence depolarization as preferable methods for exploring the effects of Type I AFP and short segments of Type I AFPs on the physical properties of a DMPC model membrane. Using DSC, we could measure the phase transition, and thus determine the effect of Type I AFPs and short segments on the model membrane. As specified above, DSC and fluorescence depolarization were utilized to determine the changes in the hydrophobic core of the model membrane. DSC and fluorescence anisotropy showed a gradual downward shift in the phase-transition temperature. This shows that the AFP segments stabilized the liquid crystalline phase more than the gel phase. This preferential stabilization of the liquid crystalline phase could be due to hydrophobic interactions in the model membrane core. Further work on the relevance of this stabilization on the permeability and vitality of model membranes is in progress. The importance of amide in the C-cap on the polarized fluorescence results will be examined further.

Acknowledgments Y. Mastai acknowledges financial support from Horvitz Foundation. We thank Prof. Meir Shinitzky from the Weizmann Institute of Science and Prof. Benjamin Ehrenberg form the department of Physics Bar Ilan University for useful discussions.

References

Adler M, Tritton TR (1988) Fluorescence depolarization measurements on oriented membranes. Biophys J 53:989–1005

Andrich MP, Vanderkooi JM (1976) Temperature-dependence of 1,6-diphenyl-1,3,5-hexatriene fluorescence in phospholipid artificial membranes. Biochemistry 15:1257–1261

Barrett J (2001) Thermal hysteresis proteins. Int J Biochem Cell B 33:105–117

Blandamer MJ, Briggs B, Cullis PM, Engberts JBFN (1995) Gel to liquid-crystal transitions in synthetic amphiphile vesicles. Chem Soc Rev 24:251

Borenstain V, Barenholz Y (1993) Characterization of liposomes and other lipid assemblies by multiprobe fluorescence polarization. Chem Phys Lipids 117–127:64

Bouvet V, Ben RN (2003) Antifreeze glycoproteins—structure, conformation, and biological applications. Cell Biochem Biophys 39:133–144

Crowe JH, Tablin F, Tsvetkova N, Oliver AE, Walker N, Crowe LM (1999) Are lipid phase transitions responsible for chilling damage in human platelets? Cryobiology 38:180–191

Davies PL, Hew CL (1990) Biochemistry of fish antifreeze proteins. Faseb J 4:2460–2468

Denich TJ, Beaudette LA, Lee H, Trevors JT (2003) Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. J Microbiol Meth 52:149–182

Devries AL, Wohlschl DE (1969) Freezing resistance in some antarctic fishes. Science 163:1073

Devries AL, Vandenhe J, Feeney RE (1971) Peimary structure of freezing point-depressing glycoproteins. J Biol Chem 246:305

Duman JG, Wu DW, Xu L, Tursman D, Olsen TM (1991) Adaptations of insects to subzero temperatures. Quart Rev Biol 66: 387–410

Feeney RE, Burcham TS, Yeh Y (1986) Antifreeze glycoproteins from polar fish blood. Annu Rev Biophys Bio 15:59–78

Feller G, Gerday C (1997) Psychrophilic enzymes: molecular basis of cold adaptation. Cell Mol Life Sci 53:830–841

Fletcher GL, Hew CL, Davies PL (2001) Antifreeze proteins of teleost fishes. Annu Rev Physiol 63:359–390

Fuchs P, Parola A, Robbins PW, Blout ER (1975) Fluorescence polarization and viscosities of membrane lipids of 3t3 cells. P Natl Acad Sci U S A 72:3351–3354

Hays LM, Feeney RE, Crowe LM, Crowe JH, Oliver AE (1996) Antifreeze glycoproteins inhibit leakage from liposomes during thermotropic phase transitions. P Natl Acad Sci U S A 93:6835–6840

Hays LM, Crowe JH, Wolkers W, Rudenko S (2001) Factors affecting leakage of trapped solutes from phospholipid vesicles during thermotropic phase transitions. Cryobiology 42:88–102

Hincha DK, Devries AL, Schmitt JM (1993) Cryotoxicity of antifreeze proteins and glycoproteins to spinach thylakoid membranes - comparison with cryotoxic sugar acids. Biochim Biophys Acta 1146:258–264

Kaiser RD, London E (1998) Location of diphenylhexatriene (DPH) and its derivatives within membranes: Comparison of different fluorescence quenching analyses of membrane depth. Biochemistry 37:8180–8190

Komatsu SK, Devries AL, Feeney RE (1970) Studies of structure of freezing point-depressing glycoproteins from an antarctic fish. J Biol Chem 245:2909

Kun H, Mastai Y (2007) Activity of short segments of type I antifreeze protein. Biopolymers 88:807–814

Mabrey S, Mateo PL, Sturtevant JM (1978) High-sensitivity scanning calorimetric study of mixtures of cholesterol with dimyristoylphosphatidylcholines and dipalmitoylphosphatidylcholines. Biochemistry 17:2464–2468

Marsh D (1990) Handbook of lipids bilayers. CRC, Boca Raton, Florida

Negulescu PA, Rubinsky B, Fletcher GL, Machen TE (1992) Fish antifreeze proteins block Ca entry into rabbit parietal cells. Am J Physiol Cell Physiol 263:C1310–C1313

Ricker JV, Tsvetkova NM, Wolkers WF, Leidy C, Tablin F, Longo M, Crowe JH (2003) Trehalose maintains phase separation in an air-dried binary lipid mixture. Biophys J 84:3045–3051

Rubinsky B, Arav A, Mattioli M, Fletcher GL (1990) The effect of antifreeze glycopeptides on membrane potential changes at hypothermic temperatures. Biochem Biophys Res Comm 173:1369–1374

Rubinsky B, Mattioli M, Arav A, Barboni B, Fletcher GL (1992) Inhibition of Ca2+ and K+ currents by antifreeze proteins. Am J Physiol 262:R542–R545

Somero GN, Devries AL (1967) Temperature tolerance of some antarctic fishes. Science 156:257

Tablin F, Oliver AE, Walker NJ, Crowe LM, Crowe JH (1996) Membrane phase transition of intact human platelets: Correlation with cold-induced activation. J Cell Physiol 168:305–313

Tomczak MM, Crowe JH (2002) The interaction of antifreeze proteins with model membranes and cells. In Fish Antifreeze Proteins World Scientific Publ., UK

Tomczak MM, Hincha DK, Estrada SD, Feeney RE, Crowe JH (2001) Antifreeze proteins differentially affect model membranes during freezing. Bba-Biomembranes 1511:255–263

Tomczak MM, Hincha DK, Estrada SD, Wolkers WF, Crowe LM, Feeney RE, Tablin F, Crowe JH (2002a) A mechanism for stabilization of membranes at low temperatures by an antifreeze protein. Biophys J 874–881:882

Tomczak MM, Vigh L, Meyer JD, Manning MC, Hincha DK, Crowe JH (2002b) Lipid unsaturation determines the interaction of AFP type I with model membranes during thermotropic phase transitions. Cryobiology 45:135–142

Tomczak MM, Hincha DK, Crowe JH, Harding MM, Haymet ADJ (2003) The effect of hydrophobic analogues of the type I winter flounder antifreeze protein on lipid bilayers. Febs Lett 551:13–19

Trevors JT (2003) Fluorescent probes for bacterial cytoplasmic membrane research. J Biochem Bioph Meth 57:87–103

Shinitzk M, Inbar M (1974) Difference in microviscosity induced by different cholesterol levels in surface membrane lipid layer of normal lymphocytes and malignant lymphoma-cells. J Mol Biol 85:603–615

Shinitzky M, Barenholz Y (1978) Fluidity parameters of lipid regions determined by fluorescence polarization. Biochim Biophys Acta 515:367–394

Sklar LA (1984) Fluorescence polarization studies of membrane fluidity, where do we go from here. Plenum, New York

vanderHeide UA, vanGinkel G, Levine YK (1996) DPH is localised in two distinct populations in lipid vesicles. Chem Phys Lett 253:118–122

Wu YL, Fletcher GL (2000) Efficacy of antifreeze protein types in protecting liposome membrane integrity depends on phospholipid class. Bba-Gen Subjects 1524:11–16

Yeh Y, Feeney RE (1978) Anomalous depression of freezing temperature in a biological system. Accounts Chem Res 11: 129–135

Yeh Y, Feeney RE (1996) Antifreeze proteins: structures and mechanisms of function. Chem Rev 96:601–617