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ORIGINAL PAPER

A solid-state NMR study of the interaction of fish antifreeze proteins with phospholipid membranes

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Abstract Fish antifreeze proteins and glycoproteins (AF(G)Ps) prevent ice crystal growth and are able to protect mammalian cells and tissues from hypothermic damage in the sub-zero Polar oceans. This protective mechanism is not fully understood, and further data is required to explain how AF(G)Ps are able to stabilize lipid membranes as they pass through their phase transition temperatures. Solid-state NMR spectroscopy was used as a direct method to study the interaction of the 37-residue α-helical type I AFP, TTTT, and the low molecular weight fraction glycoprotein, AFGP8, with dimyristoylphosphatidylcholine membranes above and below the gel-fluid phase transition temperature. In contrast to previous studies in fluid phase bilayers these experiments have provided direct information regarding both the mobility of the phosphate headgroups and perturbation of the acyl chains at a range of temperatures under identical conditions on the same sample. At 5°C changes in the ^{2}H and ^{31}P spectra and a dramatic increase in the ^{31}P T_1 relaxation times were consistent with a significant disruption of the membrane by TTTT. Heating to 30°C appeared to expel the peptide from the lipid and re-cooling showed that the interaction of TTTT was not reversible. By contrast, ^{31}P spectra of the membranes with AFGP8 were consistent with interaction with the phosphate headgroups at both 5 and 30°C. Although both peptides interact with the phospholipid bilayer surface, which may stabilize the membrane at lower temperatures, the longer ^{31}P T_1 values and the ^{2}H NMR data obtained for TTTT compared with AFGP8 suggest that TTTT causes a greater reduction of phosphate headgroup mobility and has a greater effect on the lipid acyl chains at 5°C.

Keywords Antifreeze proteins · Glycoproteins · Solid-state NMR spectroscopy · Lipid membranes · Cryoprotectants

Abbreviations

ADP	Ammonium dihydrogen phosphate
AF(G)P	Antifreeze (glyco)protein
AFGP8	Low molecular weight fraction
CSA	Chemical shift anisotropy
DMPC	1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine
DMPC-d_{54}	1,2-Dimyristoyl(d_{54})-*sn*-glycero-3-phosphocholine
FTIR	Fourier transform infra-red
MLV	Multilamellar vesicles
MW	Molecular weight
ss-NMR	Solid-state nuclear magnetic resonance
TFE	Trifluoroethanol

Introduction

Fish antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) are structurally diverse proteins that have evolved in Arctic and Antarctic fish as a survival mechanism

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in the ice-laden sub-zero Polar oceans (DeVries 1988; Fletcher et al. 2001; Harding et al. 1999, 2003; Yeh and Feeney 1996). These remarkable molecules are characterised by their ability to modify the rate and shape of ice crystal growth and exhibit thermal hysteresis (Madura et al. 2000). Five classes of AFPs and a single class of AFGP have been identified and characterised. The most widely studied AFP is the type I 37-residue α-helical peptide, TTTT, from the winter flounder *Pseudopleuronectes americanus* (Duman and DeVries 1974) (Fig. 1a) which contains three 11-residue repeat units commencing with Thr. The AFGPs have proved more difficult to study due to the presence of multiple glycoforms that have been separated based on MW (Feeney 1974). As a consequence, studies have been generally performed on either the low MW fraction commonly referred to as AFGP8 (Fig. 1b) or the high MW fraction (AFGP5).

AF(G)Ps share the ability to protect mammalian cells and tissues from hypothermic damage and are also able to stabilize or disrupt membranes to leakage during low temperature and freezing stress (Tomczak and Crowe 2002). Hence these molecules have enormous potential as cryoprotective agents in medicine and biotechnology (Fletcher et al. 1999). Preliminary studies have shown promising results in applications including the enhanced storage of blood, sperm, embryos and other biological samples at reduced temperatures (Inglis et al. 2006). However, the mechanism whereby they exert these effects is unknown and the lack of understanding of both the antifreeze and cryoprotectant properties has not permitted the tailoring of AF(G)Ps for specific applications.

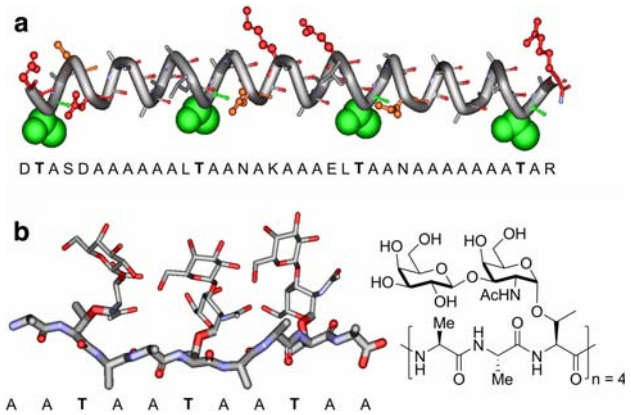

Fig. 1 **a** Primary sequence of TTTT (Also referred to as HPLC6 (Fourney et al. 1984)) from the winter flounder *Pseudopleuronectus americanus* with ribbon schematic from X-ray crystal structure (1wfa), hydrophilic (ball-and-stick) and Thr (space-filling) sidechains are shown (Yang et al. 1988). **b** Structure of the major component ($n = 4$) of the low molecular weight fraction of glycoprotein isolated from the rock cod *Gadus ogac* (commonly referred to as AFPG8); the ribbon schematic was generated from an NMR structure of a synthetic AFGP (Tachibana et al. 2004)

Crowe and co-workers have performed extensive studies on AF(G)Ps with phospholipids and plant lipids, using FTIR spectroscopy (Tomczak et al. 2002a, b, 2003). This technique was used to monitor the change in wavenumber (cm^{-1}) associated with the acyl chain methylene stretch frequency with temperature. Differential scanning calorimetry and fluorescence studies have also been applied to labelled AFP analogues (Hays et al. 1996, 1997; Tomczak et al. 2001, 2002a, b, 2003). These studies have shown that both low and high MW AFGPs and the type I AFP, TTTT, can interact with model membranes composed of only lipids, and that the lipid composition is important. Thus, while AFGPs inhibited leakage from phospholipid liposomes as they were chilled through their thermotropic phase transition, TTTT had marginal effects (Tomczak et al. 2002a, b). By contrast, TTTT was highly effective in inhibiting leakage from membranes containing plant galactolipids, but AFGPs offered no protection (Tomczak et al. 2002a).

In this paper, we report the results of the first solid-state NMR (ss-NMR) spectroscopy experiments with TTTT, AFGP8 and model membranes of dimyristoylphosphatidylcholine (DMPC). ss-NMR spectroscopy has provided significant insight in the study of membrane-peptide interactions (Graether et al. 2006; Lu et al. 2005; Cornell et al. 1988; Smith et al. 1992) and has the advantage of being a direct technique in which perturbations of the membrane head groups or acyl chains can be observed at multiple temperatures using one sample, without the addition of chemical probes or modification of the AF(G)Ps. ^{2}H NMR spectroscopy was used to probe any perturbation of the perdeuterated lipid chains as would be expected if AF(G)Ps interacted with the lipid core, while ^{31}P NMR spectroscopy and T_1 relaxation measurements provided information regarding the motions of the phosphate headgroups. The results confirm that the interaction of the α-helical AFP, TTTT, with DMPC is significantly different to AFGP8, and indicate that ss-NMR spectroscopy is a useful probe to identify AF(G)Ps that interact with different types of membranes.

Experimental

Materials

The naturally occurring proteins, TTTT and AFGP8, isolated from the winter flounder *Pseudopleuronectus americanus* and rock cod *Gadus ogac* respectively, were obtained from A/F Protein Inc. Pty Ltd (Waltham, MA, USA) and were used without additional purification. TTTT, isolated from blood plasma was >90% purity, while AFGP8 (also known as AFGP Fraction '8') was supplied as mixture of <4,000 Da glycopeptides between 80 and 90% purity, the

rest being small naturally occurring plasma peptides. Due to the highly hydrophobic nature of TTTT, amino acid analysis was performed by Auspep Pty Ltd (Melbourne, VIC, Australia), to accurately determine sample concentrations. Lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl(d_{54})-*sn*-glycero-3-phosphocholine (DMPC-d_{54}) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Water was degassed (Milli-Q). Chloroform and methanol were HPLC grade (Ajax Finechem). 2,2,2-Trifluoroethanol (TFE) was used as supplied from Aldrich.

Sample preparation

A stock solution of the DMPC-d_{54}/DMPC 1:1 (w/w) lipid mixture, prepared by dissolving 15 mg of each lipid in chloroform-methanol (3:1, 3 ml) was divided into three equal solutions (by mass). 10 mol% AFGP8 (3.8 mg) dissolved in the minimum amount of TFE (~0.5 ml) was added to the first solution, 10 mol% TTTT (6.1 mg) dissolved in the minimum amount of TFE (~0.5 ml) was added to the second solution and the third solution served as the control. The solvent was removed in vacuo (water bath at 30°C), the samples were dried under high-vacuum at RT for 15 h, and the dry samples were stoppered, sealed with parafilm and stored in the freezer until the spectra were acquired. Samples were allowed to warm to room temperature (20°C) and were hydrated with Milli-Q water (1 × 80 µl) using a vortex mixer. The volume of Milli-Q water was adjusted to account for the addition of 0.1 M acid/base to neutralise the charge of the added protein such that the total volume remained 80 µl. For the sample containing AFGP8, 0.9 µl of HCl (0.1 M) was added for every milligram of AFGP8, and for TTTT, 2.7 µl of NaOH (0.1 M) was added for each milligram of TTTT (by mass added). The lipid–water suspensions were transferred to 4 mm glass NMR tubes and samples were homogenised in the capped NMR tubes by vortexing for few minutes.

Solid-state NMR spectroscopy

Static ss-NMR experiments were performed with a Varian (Palo Alto, CA, USA) Inova 300 NMR spectrometer, fitted with a 4-mm Chemagnetics magic angle spinning probe operating at 121.38 MHz for ^{31}P and 46.02 MHz for ^{2}H. The chemical shift was referenced externally relative to the isotropic chemical shift of ammonium dihydrogen phosphate (ADP) ($\delta = 0$ ppm) by magic angle spinning for ^{31}P and using D_2O ($\delta = 0$ ppm) for ^{2}H. The 90° pulse width was determined before each experiment using ADP for ^{31}P and D_2O for ^{2}H experiments. For all experiments prior to processing, the FID was 'left-shifted' so that the FID commenced at the top of the echo. Data was Fourier transformed with zero-filling to 8k data points, with a line broadening of 100 Hz. ^{31}P CSA and Iso parameters were calculated using the lineshape analysis module in Topspin 1.3 (Bruker, Karlsruhe, Germany).

The temperature of the probe was calibrated using ^{1}H NMR of a sealed methanol sample (Van Geet 1970) and was set using the Chemagnetics temperature controller inline with a Bruker BCU05 cooling unit. Experiments were first performed at 5°C and then at 30°C, with the temperature recorded every 5 s over the course of an experiment. The standard deviation of the temperature ranged between 0.06 and 0.09°C, with the maximum drift of temperature ±0.5°C. Samples were left to equilibrate at ~4°C for 1 h before equilibration in the NMR at 5°C for 30 min. Samples were left at room temperature (~20°C) for 30 min before equilibration in the NMR at 30°C for 30 min.

^{31}P experiments used a spin echo (90°–τ–180°–τ–acquire) sequence with proton decoupling and typical acquisition parameters were: spectral width of 60 kHz, 90° pulse width 3.1 µs, echo time 50 µs, acquisition time 25 ms, recycle delay 1.5 s, 13 k transients. ^{2}H used the solid echo (90°–τ–90°–τ–acquire) sequence and typical acquisition parameters were: spectral width 150 kHz, 90° pulse width 2.2 µs, echo time 50 µs, acquisition time 8 ms, recycle delay 1 s, 32 k transients.

^{31}P T_1 experiments used the inversion recovery method (delay–180°–τ–90°–acquire with proton decoupling during acquisition). Acquisition parameters were the same as for the above ^{31}P experiments except for a recycle delay of 3 s and 6,144 transients. The array for τ was {0.001, 0.05, 0.1, 0.2, 0.5, 1, 2, 4, 6 s}. Due to the shorter experimental lifespan of the samples with TTTT at 30°C, the T_1 experiment was performed with 5,120 transients and the 0.001 and 6 s τ values were removed from the array. T_1 values were calculated by a non-linear curve fit of the peak intensity versus τ to the equation $M(\tau) = M(0)\exp(-\tau/T_1)$ using Origin 7.5, with r^2 values of the fits between 0.9946 and 0.9999 (average 0.9988).

Results

Experimental conditions

The effect of AFGP8 or TTTT on the DMPC-d_{54}/DMPC liposomes was studied by static ^{2}H and ^{31}P NMR ss-NMR spectroscopy. DMPC was chosen as a model membrane system as it has been well-characterised by ss-NMR and other spectroscopic methods (Seelig and Seelig 1980) and has been used in previous studies of binary lipid systems with AFPs (Tomczak et al. 2002a, b). However, the interaction of AF(G)Ps with DMPC alone has not been reported. Similar sample concentrations of TTTT and AFGP8 were used to those concentrations used in previous studies

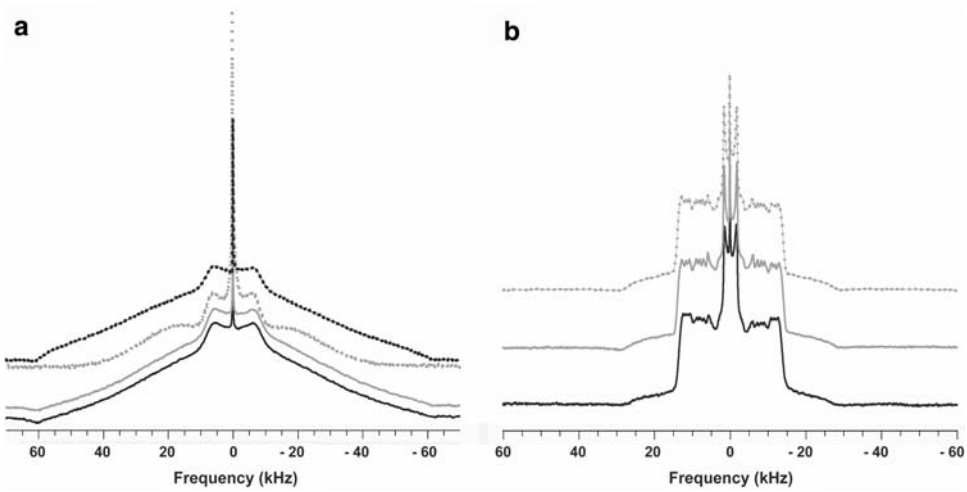

Fig. 2 ^{2}H Spectra of DMPC-d_{54}/DMPC (1:1) bilayers: **a** at 5°C control (*black solid*), with AFGP8 (*grey solid*), TTTT (*grey dash*) and TTTT after heating to 30°C and re-cooling to 5°C (*black dash*); **b** at 30°C control (*black solid*), with AFGP8 (*grey solid*) and TTTT (*grey dash*)

monitored by FTIR spectroscopy (Tomczak et al. 2002a, b) in order to allow comparison of the results using the different techniques.

Experiments were performed at 5°C as this temperature is relevant to the antifreeze activity of the AF(G)Ps. At this temperature, the DMPC liposomes exist in the gel phase but the water is not frozen, similar to Polar ocean conditions. Experiments were also performed at 30°C, which is well above the thermotropic phase transition temperature of the DMPC-d_{54}/DMPC (1:1) liposomes, which are present as multilamellar vesicles (MLV) (Lewis et al. 1987; Prenner et al. 1999).

The samples were analysed immediately after hydration and the experiments were performed sequentially with the 5°C spectra acquired before the 30°C spectra using the same sample. Experimental timeframes of ~96 h (AFGP8) and ~48 h (TTTT) were used. Longer times resulted in the loss of sample integrity, usually as the formation of a viscous gel (indicative of a cubic phase) or complete settling of the lipid suspension from the water (possibly indicating MLV aggregation). Including freeze/thaw cycles in the sample preparation did not change the resultant spectra.

Deuterium spectra

The ^{2}H spectra of pure DMPC as the control, and DMPC in the presence of either AFGP8 or TTTT are shown at 5°C (Fig. 2a) and 30°C (Fig. 2b). In all cases, the ^{2}H spectra showed an isotropic peak due to residual ^{2}H in the water. At 5°C, the control sample showed a typical gel phase Pake powder pattern (Fig. 2a) (Davis 1979). In the presence of AFGP8 there was no significant change to the spectrum suggesting that any interaction of AFGP8 with DMPC was not affecting the motion or structure of the lipid acyl chains significantly. By contrast, in the presence of TTTT, the powder pattern narrowed and a change in the lineshape between the 0 and 90° edges was observed. This result suggested a mixed or intermediate lipid phase and provided evidence of some interaction of TTTT with the membrane.

Samples were allowed to warm to 30°C and the spectra were acquired at this temperature (Fig. 2b). The spectrum of the control at 30°C was typical of a lamellar phase (Davis 1979), with a quadrupolar splitting of ~25 kHz for the 90° edge for the outermost CD_2 and ~3.4 kHz for the CD_3. No significant differences were observed in this spectrum or the quadrupolar splittings in the presence of either AFGP8 or TTTT. Compared with the control, the 5°C spectrum with TTTT (Fig. 2a) suggested some interaction with the membrane, which was no longer evident at 30°C. Due to the difficulty in quantifying deuterium splittings in the gel phase, changes in the CD_2 and CD_3 splittings were not quantified. Hence, the sample was cooled from 30 to 5°C in order to test the reversibility of interaction. The spectrum obtained on re-cooling was practically identical to the control, i.e. a typical gel phase lipid bilayer no longer showing any evidence of a mixed phase system.

Phosphorus spectra

The ^{31}P spectra of DMPC and DMPC in the presence of either AFGP8 or TTTT are shown at 5°C (Fig. 3a) and 30°C (Fig. 3b). The ^{31}P spectrum of the control at 5°C (Fig. 3a) was a typical broadened axially symmetric powder pattern with chemical shift anisotropy (CSA), $\Delta\sigma \approx -60$ ppm. The spectrum obtained in the presence of AFGP8 exhibited some isotropic component ($\delta = -1.4$ ppm) and a more pronounced 90° edge at $\delta = -20$ ppm than the control (Fig. 3a). In the presence of TTTT, there was a dramatic change in the spectrum with the 0° edge ($\delta = \sim 40$ ppm) difficult to resolve and the appearance of a large isotropic component ($\delta = -1.7$ ppm) (40% of the sample as estimated by curve-fitting of the peak areas).

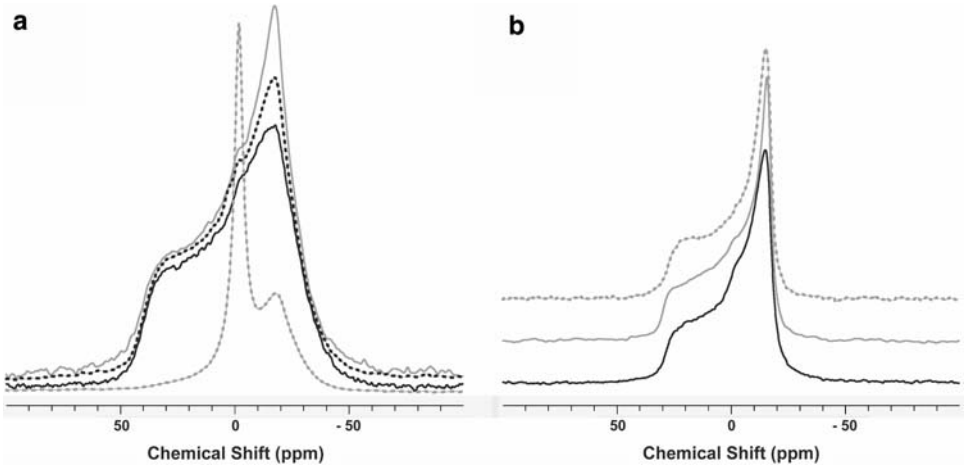

Fig. 3 ^{31}P Spectra of DMPC-d_{54}/DMPC (1:1) bilayers: **a** at 5°C control (*black solid*), with AFGP8 (*grey solid*), TTTT (*grey dash*) and TTTT after heating to 30°C and re-cooling to 5°C (*black dash*); **b** at 30°C control (*black solid*), with AFGP8 (*grey solid*) and TTTT (*grey dash*)

Table 1 ^{31}P CSA (ppm) and isotropic chemical shift (Iso) (ppm) for DMPC liposomes in the presence of AFGP8 or TTTT at 5 and 30°C

(ppm)	5°C		30°C	
	$\Delta\sigma$ (CSA)	δ (Iso)	$\Delta\sigma$ (CSA)	δ (Iso)
Control	−63.0	−1.0	−43.2	−1.6
With AFGP8	−58.2	−1.4	−45.6	−0.8
With TTTT	∼ −50	−1.7[a]	−45.4	−1.2

[a] Isotropic peak dominates spectrum

The ^{31}P spectrum of the control at 30°C (Fig. 3b) was a typical axially symmetric powder pattern ($\Delta\sigma \approx -45$ ppm), with an isotropic shoulder peak ($\delta = -1.6$ ppm). There was some increase in intensity of the 90° edge at $\delta = -15$ ppm, which was attributed to alignment of the lipids perpendicular to the magnetic field, as in more fluid systems the MLV elongate in the NMR spectrometer. The ^{31}P spectra in the presence of AFGP8 or TTTT were almost identical to the control and the chemical shift data is summarized in Table 1.

As observed with the ^{2}H NMR spectrum, the original ^{31}P powder pattern was not obtained when the TTTT sample was cooled back to 5°C from 30°C (Fig. 3a), and was practically identical to the control spectrum. Compared to the initial 5°C spectrum which was dominated by a strong isotropic component, the spectrum of the re-cooled sample showed a significantly reduced isotropic peak; the 0° edge was easily discerned while the 90° edge had the same intensity as this peak in the control.

T_1 relaxation measurements

In order to provide more insight into the interactions responsible for the changes observed by ^{2}H and ^{31}P spectroscopy, changes in the relaxation dynamics of the ^{31}P nuclei were investigated at 5 and 30°C. As T_1 relaxation pathways are driven by molecular motions on the nanosecond (ns) time scale (Separovic et al. 2000), these measurements were made in order to establish whether the motion of the headgroups was significantly affected by the AF(G)Ps. Table 2 summarises the ^{31}P T_1 values for the 0 and 90° edges and isotropic peak.

At 5°C, the T_1 of the DMPC control was 604 and 386 ms for the 0 and 90° edges of the powder pattern, respectively, which reflects an anisotropy in T_1 as previously reported for DMPC bilayers (Dufourc et al. 1992). The anisotropy in T_1 decreased at 30°C, with values of 484 and 392 ms, respectively, which reflect an increase in intensity of motions on the ns timescale and lead to a reduction in T_1 (Bonev et al. 2003; Pukala et al. 2007). An isotropic peak was not observed at 5°C but at 30°C the T_1 of the isotropic peak was 453 ms, intermediate between that of the extremities of the powder pattern.

Table 2 ^{31}P T_1 relaxation times of the 0° edge, isotropic peak and 90° edge at 5 and 30°C

DMPC liposomes	Temperature (°C)	0° edge (ms)	90° edge (ms)	Isotropic peak (ms)
Control	5	604 ± 52	386 ± 20	–
	30	484 ± 23	392 ± 5	453 ± 16
With AFGP8	5	1,170 ± 36	914 ± 9	966 ± 13
	30	663 ± 19	620 ± 5	607 ± 8
With TTTT	5	–	2,746 ± 377	1,784 ± 131
	30	1,333 ± 40	1,288 ± 6	–
	5[a]	1,142 ± 46	1,016 ± 31	–

[a] Result after sample warmed to 30°C and re-cooled to 5°C

Table 2 shows that in the presence of AFGP8, the T_1 of DMPC significantly increased at both 5 and 30°C. At 5°C, the T_1 was 1,170 and 914 ms for the 0 and 90° edges, respectively, while these values decreased to 663 and 620 ms at 30°C. The glycopeptide appeared to reduce the anisotropy in T_1 across the powder pattern, which is also reflected in the T_1 of the isotropic peak of 607 ms at 30°C. The presence of the peptide has increased T_1, which reflects a decrease in the intensity of motions on the ns timescale, possibly due to a decrease in long axis rotation of the lipid (Bonev et al. 2003; Pukala et al. 2007).

In the case of TTTT, an even greater increase in the T_1 values was observed at both temperatures. The T_1 values were 2,746 ms at 5°C and 1,288 ms at 30°C for the 90° edge of the powder pattern. The T_1 of the 0° edge was 1,333 ms at 30°C, which suggests that this peptide also reduces the anisotropy in the lipid dynamics. The 0° edge was difficult to distinguish at 5°C, so T_1 was not determined and likewise for the isotropic peak at 30°C. The T_1 of the isotropic peak at 5°C of 1,784 ms was approximately 40% shorter than the T_1 of the 90° edge, which suggests a different lipid phase for the isotropic component at the lower temperature. When the TTTT sample was cooled from 30 to 5°C, the T_1 observed were significantly shorter than first observed at 5°C, and the values were instead similar to those obtained for AFGP8 at the same temperature.

Discussion

α-Helical antifreeze protein

At 5°C, both the ^{2}H and ^{31}P spectra provide clear evidence for the interaction of TTTT with DMPC. The large isotropic ^{31}P NMR peak observed in the presence of TTTT at 5°C is indicative of a significant disruption of the lipid membrane (Lau et al. 2006). This conclusion is supported by the change in the ^{2}H spectrum at 5°C, which showed evidence of a phase intermediate between gel and fluid (Davis 1979). Thus, overall, the results are consistent with an interaction of the α-helical TTTT within the DMPC membrane in the gel phase, which disrupts the packing of the acyl chains. By contrast, at 30°C there was no evidence for significant interaction of TTTT with the lipid in the fluid lamellar phase and heating the sample from 5 to 30°C appeared to result in expulsion of the peptide from the lipid. Re-cooling the sample did not result in the interaction of the peptide with the lipid in the same manner. This result highlights the importance of the sample temperature in determining the mode of interaction between the AFP and the lipid.

Comparison of our results with those reported by Tomczak et al. (2002b) using FTIR spectroscopy needs to take into account the different sample preparation methods and the use of a *N*-terminal analogue of TTTT for some of the FTIR experiments. FTIR results reported that TTTT does not alter the gel-fluid phase transition, T_m, of extruded DMPC liposomes, but that the T_m of unsaturated DGDG-containing liposomes is increased (Tomczak et al. 2002a). In addition, liposome pellets showed no evidence for TTTT interacting with saturated DGDG/DMPC when incubated above or below their T_m (Tomczak et al. 2002b). However, liposome pelleting showed evidence of an interaction with unsaturated DGDG/DMPC in the gel-phase, leading to the conclusion that bilayer order is not affected in the presence of saturated liposomes. In our study the liposomes were multilamellar and not extruded bilayers. Even without freeze-thawing, most likely due to co-solubilizing the peptide and lipid, TTTT appears to have penetrated the MLV and disrupted the gel-phase lipid although this interaction is absent in the fluid-lamellar phase, in contrast to the conclusions of the FTIR studies with DMPC-containing liposomes. The formation of an isotropic phase at 5°C is consistent with formation of a toroidal pore rather than small lipid vesicles or micelles (Lau et al. 2006). The linewidth is too broad for small isotropically tumbling lipid structures, which also would be unlikely to reform into a bilayer when the sample was warmed to 30°C and cooled again to 5°C. The dramatic increases in the ^{31}P T_1 relaxation times are also consistent with a decrease in mobility on the ns timescale due to a reduction of long axis rotation of the lipid in the presence of the peptide (Pukala et al. 2007). At the high peptide concentrations used, formation of a toroidal pore would lead to significant averaging of the ^{31}P CSA and observation of an isotropic peak as the lipids diffuse over the pore (Lau et al. 2006). However, the averaging would be insufficient to reduce the ^{2}H quadrupolar splittings, which requires an order of magnitude faster motions ($\sim 10^{-5}$ s timescale) (Davis 1979; Lau et al. 2006; Separovic and Gawrisch 1996).

Antifreeze glycoprotein

The results with AFGP8 are significantly different to those obtained with TTTT, not surprisingly, given the structural differences between AFGPs and type I AFPs (Fig. 1). The reduction in the ^{31}P CSA (Table 1) in the presence of AFGP8 at 5°C indicated that there was an interaction between the protein and lipid resulting in some disorder of the headgroups. However, there was no evidence from the ^{2}H spectrum for disruption of the acyl chains. Thus, at 5°C the results are consistent with an interaction between AFGP8 and the phosphate head groups of the DMPC liposomes in the gel phase. At 30°C, there was less evidence for AFGP8 interacting with the liposome in the fluid lamellar phase but a small increase in ^{31}P CSA is consistent with a restriction of the phosphate group and the increase in T_1

indicated a reduction in long axis rotation of the lipid (Separovic et al. 2000).

Implications for low temperature applications

While AF(G)Ps interact with and protect mammalian cells and tissues from hypothermic damage and are able to stabilize membranes during low temperature and freezing stress, the mechanism(s) whereby they exert these effects is unknown. Mechanisms that involve the blockage or alteration of the flow of ions into cells (Rubinsky et al. 1990, 1991a, b) as well as the protection of cell membranes at they pass through their phase transition temperatures have been proposed (Tomczak and Crowe 2002). However, the relationship (if any) between antifreeze activity, and protection of cell membranes is unknown.

Tomczak and Crowe have suggested that in the case of AFGPs, the mechanism(s) of interaction that prevent transient leakage across the phospholipid membrane as it is cooled through its thermal phase transition temperature (T_m) may involve monolayer coverage of the surface of the membrane (Tomczak and Crowe 2002). In contrast, interactions between plant lipid-containing liposomes and the α-helical type I AFP, TTTT, which alters the order of the acyl chains in the hydrophobic core, have been proposed as the stabilisation mechanism during chilling *via* partial insertion of the *N*-terminal residues of the helical peptide into the membrane (Tomczak and Crowe 2002). However, this conclusion was based on fluorescence data obtained using a TTTT analogue labelled with 4-fluoro-7-nitrobenzofurazan at the *N*-terminus, and addition of a fluorescent probe to the liposome. Under these conditions, insertion of the *N*-terminal residues of the peptide into the membrane, mediated by the hydrophobic aromatic chromophore label, cannot be ruled out. Our data suggest that at similarly high concentrations (10:1), with the saturated neutral phospholipid bilayer, DMPC, the unlabelled, native TTTT causes a major disruption of the bilayer in the lipid gel phase. Conversely, when the bilayer is heated above T_m, TTTT appears to be expelled from the bilayer and not re-insert when cooled. The ^{31}P T_1 relaxation times together with the ^{2}H NMR data indicate that both TTTT and AFGP8 interact peripherally with the DMPC bilayer but also demonstrate that TTTT may insert into the bilayer, disrupting the motion of the acyl chains, depending on the thermal history or fluidity of the membrane.

The results of this study using ^{2}H and ^{31}P ss-NMR spectra of DMPC support the conclusions of previous studies that the interaction of AF(G)Ps is highly dependent on the lipid structure and exact composition of the membrane. Importantly, the investigation of a widely used component in binary lipid systems, DMPC, which has not previously been studied in isolation with AF(G)Ps, provides baseline data for future experiments with AF(G)Ps, including experiments with labelled peptides that may allow changes in the conformation(s) of the peptides to be monitored.

^{31}P ss-NMR spectroscopy has provided direct information regarding the mobility of the phosphate headgroups at a range of temperatures under identical conditions to data obtained from ^{2}H ss-NMR on the same sample. This data was not directly obtainable from previous FTIR experiments or other techniques. Changes in the ^{31}P spectra of the lipids may be attributed to the peptide being localised at the surface of the membrane and/or result from insertion of the peptide into the bilayer. The longer T_1 values obtained for TTTT compared with AFGP8 provides additional evidence that TTTT causes a greater reduction of phosphate headgroup mobility, and consistent with the ^{2}H spectra, that only TTTT affects the order or packing of the lipid acyl chains at 5°C, and hence causes significant disruption of the membrane. ^{2}H ss-NMR provides a direct screen for identification of perturbation of the lipid acyl chains by AF(G)Ps. What is unclear at the present time is the significance of headgroup and acyl chain perturbation to cryoprotective properties or to antifreeze activity.

The data from our study is consistent with hypotheses that there are different mechanisms of interaction for AFGP and TTTT with membranes (Tomczak and Crowe 2002). Further systematic studies using ss-NMR spectroscopy, as well as other techniques, with a range of lipid systems in which the headgroups, lipid charge, level of unsaturation and the membrane composition are systematically varied are required to develop a detailed structural model(s) of AF(G)Ps with membranes.

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