The effect of hydrophobic analogues of the type I winter flounder antifreeze protein on lipid bilayers

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Abstract The effect of four synthetic analogues of the 37-residue winter flounder type I antifreeze protein (AFP), which contain four Val, Ala or Ile residues in place of Thr residues at positions 2, 13, 24 and 37 and two additional salt bridges, on the binary lipid system prepared from a 1:1 mixture of the highly unsaturated DGDG and saturated DMPC has been determined using FTIR spectroscopy. In contrast to the natural protein, which increases the thermotropic phase transition, the Thr, Val and Ala analogues decreased the thermotropic phase transitions of the liposomes by 2.2°C, 3.4°C and 2.4°C, while the Ile analogue had no effect on the transition. Experiments performed using perdeuterated DMPC showed that the Ala and Thr peptides interacted preferentially with the DGDG in the lipid mixture, while the Val peptide showed no preference for either lipid. The results are consistent with interactions involving the hydrophobic face of type I AFPs and model bilayers, i.e. the same face of the protein that is responsible for antifreeze properties. The different effects correlate with the helicity of the peptides and suggest that the solution conformation of the peptides has a significant role in determining the effects of the peptides on thermotropic membrane phase transitions.

Key words: Antifreeze; α-Helical peptide; DGDG:DMPC liposomes; Lipid phase transition; Protein–membrane interaction

1. Introduction

In both pure water and aqueous solutions, 'antifreeze' proteins (AFPs) and glycoproteins (AFGPs) prevent the growth of ice crystals at temperatures below the equilibrium melting point [1–3]. While several classes of AFPs have been identified, the type I AFPs found in the blood of Alaskan plaice (*Pleuronectes quadritaberulatus*) and the sculpin (*Myoxocephalus scorpius*), in particular the type I AFP from the winter flounder (*Pleuronectes americanus*, TTTT, Table 1), have been most widely studied (for a review see [2]).

The 37 residue protein TTTT is highly α-helical at 0°C in aqueous solution, and in this conformation the four Thr residues at positions 2, 13, 24 and 35 are aligned on one face of the helix [4]. The low molecular weight of this protein has allowed production of >50 synthetic analogues (summarized in [2]). Structure–activity studies on these analogues have provided important insights into the roles of specific residues in the mechanism of inhibition of ice crystal growth [5–10]. Mutation of the Thr residues to Ser, Val, Ala and *allo*-Thr have shown that hydrophobic interactions and not hydrogen bonding involving the hydroxyl groups of the Thr residues are the crucial interactions that result in inhibition of ice growth.

AFPs and AFGPs also stabilize membranes during low temperature storage. These effects were initially attributed to an interaction between the proteins and integral membrane proteins in cells [11,12]. However, AFGPs also prevent leakage of a fluorescent dye from model membranes containing only lipids as they are passed through their thermotropic phase transition. Hence AFGPs may stabilize lipid bilayer structure during low temperature stress [13], although interaction with integral membrane proteins cannot be ruled out. In contrast to AFGPs, there have been limited studies on the interaction of AFPs with membranes. We have previously shown [14] that TTTT stabilizes model membranes as they are chilled through their phase transition and that the AFP alters the order of the acyl chain region without influencing the lipid headgroup region. In addition, the liposomes disrupted the temperature-dependent folding pattern of TTTT [16]. Although the detailed mechanism is unknown, it was proposed that, as for the ice growth inhibition properties, hydrophobic interactions play an important role in the interactions between TTTT and the bilayer [14].

In order to clarify the mechanism by which TTTT interacts with membranes, this study reports the effect of the synthetic analogues XXXX2KE (Table 1) on the phase transitions of model bilayers. The synthetic peptides contain the Thr residues mutated to Val, Ala and Ile respectively as well as two additional salt bridges. These sequences were studied in order to determine whether replacement of the four Thr residues in TTTT with hydrophobic residues resulted in a change in the interaction between the peptides and the bilayer. As the ice growth inhibition properties of these sequences have been reported previously [7,8,10], the studies with model bilayers were designed to assess whether the same residues are responsible for both the interaction between the type I AFPs and lipid bilayers, and the interaction between type I AFPs and

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ice. These results have important implications for the rational design of synthetic compounds that either stabilize or destabilize bilayers in a predictable manner.

2. Materials and methods

2.1. Lipids and peptides

Dimyristoylphosphatidylcholine (DMPC) and perdeuterated DMPC (d54 DMPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and digalactosyldiacylglycerol (DGDG) was purchased from Lipid Products (Redhill, Surrey, UK). Peptides XXXX2KE were synthesized and purified as described previously [7,8,10]. Peptide concentrations refer to weighed mass, which are not corrected for peptide content as determined by amino acid analysis.

2.2. Preparation of liposomes

DGDG:DMPC (1:1, w:w) unilamellar liposomes were extruded in 10 mM TES, 0.1 mM EDTA, and 50 mM NaCl (TEN buffer, pH 7.4) using a Liposofast hand-held extruder (Avestin, Ottawa, Canada) [15] with 100 nm pore filters (Poretics, Livermore, CA, USA) at a concentration of 20 mg ml^{-1} lipid. To test the effect of the synthetic peptides, XXXX2KE (20 mg ml^{-1}) was added to the DGDG:DMPC (1:1, w:w) liposomes, in an equal volume after extrusion, as described above, to give the final sample. This is referred to in the text as peptide:lipid (1:1, w:w).

2.3. Fourier transform infrared (FTIR) spectroscopy

Spectra were recorded with a Perkin Elmer Spectrum 2000 FTIR spectrometer equipped with a liquid nitrogen-cooled mercury/cadmium/telluride (MCT) detector and analyzed with Perkin Elmer Spectrum software (Norwalk, CT, USA). The temperature was controlled with a Peltier device (Paige Instruments, Davis, CA, USA), which holds the sample between two CaF_2 windows. The cooling rate was 2°C min^{-1} and the sample temperature was measured with a thermocouple placed directly on the windows. The sample chamber was continually flushed with dry air to keep the relative humidity near 0%. All experiments were performed in duplicate and gave reproducible results with measured wavenumbers ± 0.05 cm^{-1}. First derivatives were determined with Peak Fit software, version 4 (Jandel Scientific).

3. Results

3.1. FTIR spectroscopy of lipids

FTIR spectroscopy was used to monitor the effect of the synthetic peptides XXXX2KE on a binary lipid system prepared from a 1:1 mixture of the highly unsaturated DGDG and saturated DMPC as they are chilled through their thermotropic phase transitions. These two lipids (1:1) were chosen as they are well mixed in the resultant liposomes and as the physical stability of this system has been well characterized in previous studies [16–18]. The lipid phase transition can be determined by plotting the change in wavenumber (cm^{-1}) of the acyl chain symmetric CH_2 stretch (2855–2850 cm^{-1}) with temperature. In the FTIR spectrum of the DGDG:DMPC (1:1) liposome, the symmetric CH_2 stretching vibration moves to a lower wavenumber as the liposomes are chilled from 23°C to 0°C. The median wavenumber decreases and the peak nar-

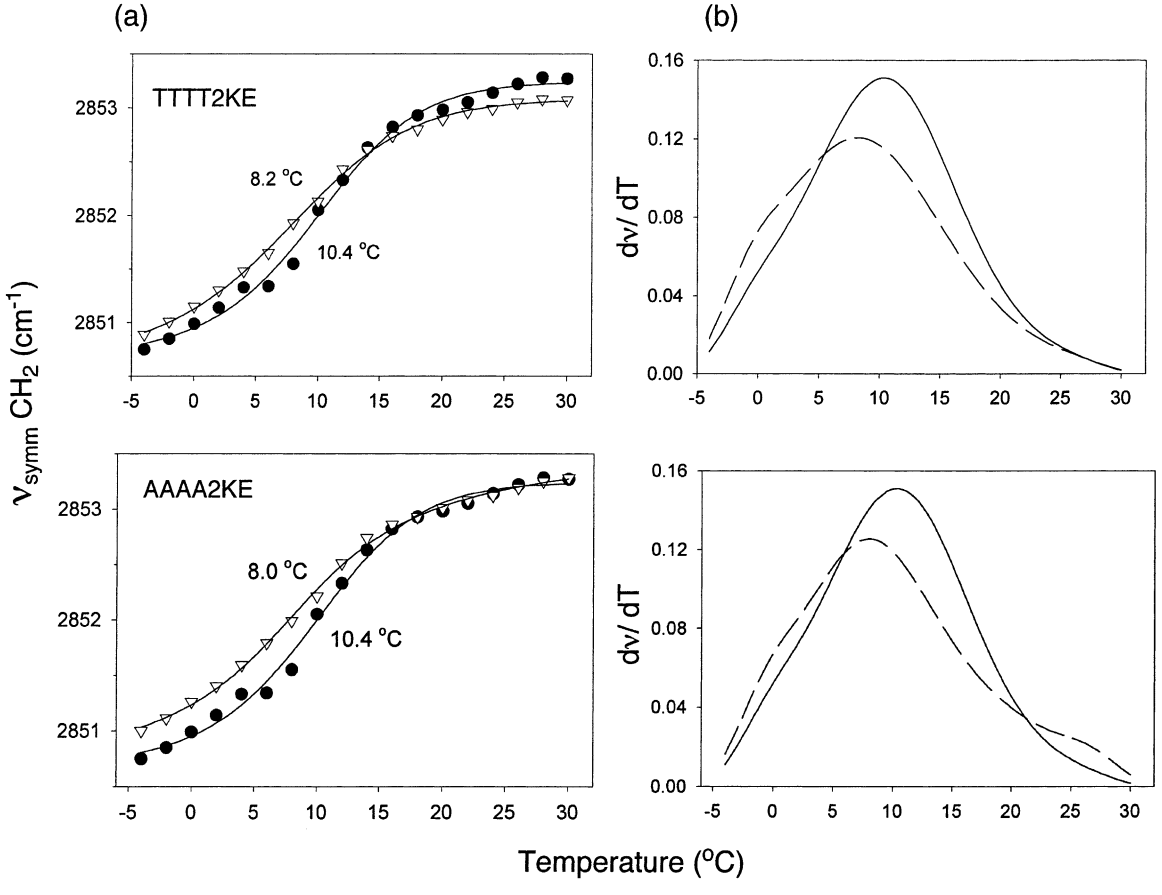

Fig. 1. a: The symmetric CH_2 stretch frequency of the lipid acyl chains as a function of temperature during cooling in the presence (triangles) and absence (circles) of TTTT2KE (upper panels) and AAAA2KE (lower panels) in a 1:1 peptide:lipid mixture; numbers indicate the phase transition temperatures. b: First derivative analysis of the fit of the phase transition curves in panel a; solid line represents the liposomes alone and the dashed line the liposomes in the presence of peptide.

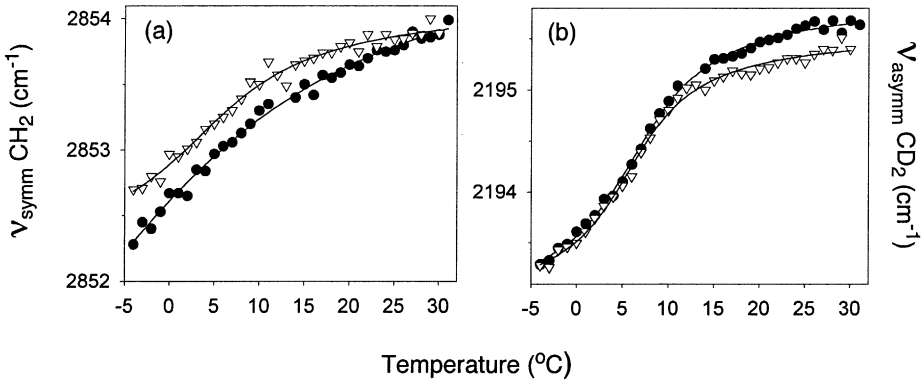

Fig. 2. The symmetric CH_2 stretch of DGDG (a) and the asymmetric CD_2 stretch of d54 DMPC (b) as a function of temperature during cooling in the presence (triangles), and absence (circles) of TTTT in a 1:1 peptide:lipid mixture.

rows as the liposomes pass through their phase transition (T_m), centered around 10°C. This value is usually extracted from the first derivative of the phase transitions, where the differences in T_m and the differences in overall excursion of the two phase transitions are more apparent.

To assess the effect of the peptides XXXX2KE on the lipid bilayer, FTIR spectra were recorded on samples containing a 1:1 (w/w) ratio of peptide to lipid. This ratio was chosen based on previous studies with TTTT, in which the peptide concentration was varied [14,16]. At lower concentrations of peptide (1:10 or 1:5 protein:lipid ratios), there was no detectable shift in the wavenumber of the CH_2 symmetric shift. Optimal, reproducible changes in the FTIR spectra were obtained using a 1:1 ratio of TTTT:lipid. Hence the same peptide:lipid ratio was used in this study.

In order to determine whether the synthetic analogues were preferentially interacting with one of the two lipids present in the lipid bilayers, perdeuterated DMPC (d54 DMPC) was used to differentiate the phase transitions of each lipid [19] in the presence of the peptides. The CD_2 of the d54DMPC has a higher mass than the CH_2 of the DGDG and, thus, its IR vibration is shifted to a lower wavenumber than the CH_2 of the DGDG. This property allows the simultaneous determination of the phase behavior of each lipid in a two lipid system [19]. The T_m of perdeuterated lipids is shifted to a slightly lower temperature than the non-deuterated equivalent. In these experiments the asymmetric CD_2 stretch of the perdeuterated lipid was monitored because it gives a clearer signal in the FTIR spectrum than the symmetric CD_2 stretch.

3.2. Measurement of phase transitions

Fig. 1a shows the symmetric CH_2 stretch frequency of the lipid acyl chains in the binary lipid system plotted as a function of temperature from 30°C to −5°C in the presence and absence of TTTT2KE. The presence of the peptide decreased the overall CH_2 wavenumber and reduced the phase transition (T_m) of the liposomes from 10.4°C to 8.2°C. Fig. 1b shows the derivative analysis of the fit of the T_m curves, which allow the T_m values and the magnitude of the wavenumber changes to be seen more clearly. The reduction in the T_m of the liposomes is the opposite effect of the wild-type AFP, TTTT which *increases* the T_m of the liposomes by 2.5°C [14]. In addition, TTTT2KE affects the T_m in a manner similar to that of cholesterol, in that it decreases fluidity above the T_m and increases fluidity below T_m [20] (Fig. 1b).

Similar experiments were performed with VVVV2KE, AAAA2KE and IIII2KE. In the case of VVVV2KE, the T_m of the liposomes was decreased in the presence of the synthetic analogue by 3.4°C (data not shown) but VVVV2KE had no effect on the symmetric CH_2 stretching frequency above the T_m and did not greatly affect the overall wavenumber excursion of the transition. AAAA2KE decreased the T_m of the DGDG:DMPC liposomes by 2.4°C (Fig. 1a). This analogue decreased the overall wavenumber excursion but, similar to the results with VVVV2KE, it had no effect on the frequency of the symmetric CH_2 stretch above the T_m (Fig. 1b, lower panels). In contrast, the Ile-substituted analogue, IIII2KE had no significant effect on the phase transition of the lipid mixture (data not shown).

3.3. Studies with perdeuterated lipids

Fig. 2 shows the symmetric CH_2 stretch of DGDG and the asymmetric CD_2 stretch of d54 DMPC plotted as a function of temperature in the presence and absence of TTTT. These curves show that TTTT increased the frequency of the DGDG symmetric CH_2 stretch in the mixture, but decreased the frequency of the d54 DMPC asymmetric CD_2 stretch above the T_m. However, at −4°C, the frequency of the CH_2 stretching was still above 2852.5 cm^{-1}, a wavenumber characteristic of the liquid crystalline phase, suggesting that the DGDG in this mixture had not completed its phase transition. In order to determine whether this result indicated that the

Table 1
Polypeptide sequences highlighting mutations of Thr (bold), and additional salt bridges (italicized)

	1	2	13	24	35
TTTT	D	**T**ASDAAAAAAL	**T**AANAKAAAEL	**T**AANAAAAAAA	**T**AR
TTTT2KE	D	**T**ASDA*K*AAA*E*L	**T**AANAKAAAEL	**T**AANA*K*AAA*E*A	**T**ARCONH$_2$
VVVV2KE	D	**V**ASDA*K*AAA*E*L	**V**AANAKAAAEL	**V**AANA*K*AAA*E*A	**V**ARCONH$_2$
AAAA2KE	D	**A**ASDA*K*AAA*E*L	**A**AANAKAAAEL	**A**AANA*K*AAA*E*A	**A**ARCONH$_2$
IIII2KE	D	**I**ASDA*K*AAA*E*L	**I**AANAKAAAEL	**I**AANA*K*AAA*E*A	**I**ARCONH$_2$

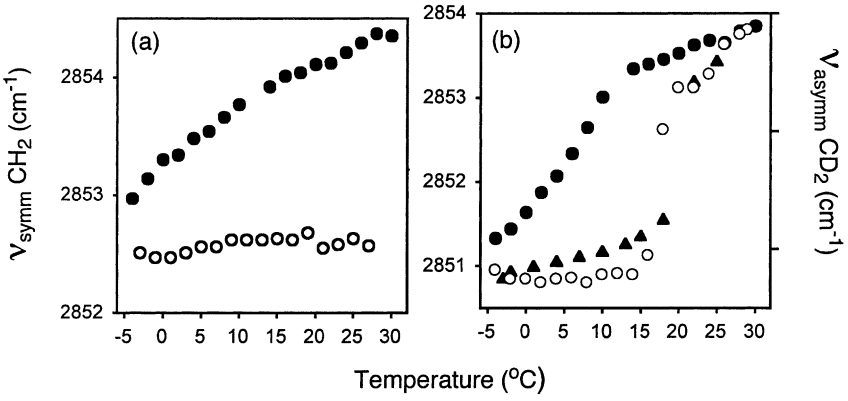

Fig. 3. Comparison of the phase behavior of DGDG and d54 DMPC as single components (open circles) or in the 1:1 lipid mixture (closed circles) as a function of temperature. CH_2 stretch of DGDG (a), CD_2 stretch of d54 DMPC and the CH_2 stretch of DMPC (b); closed triangles represent the phase behavior of isolated DMPC.

two phases were separated, the CH_2 and CD_2 stretch of each lipid was monitored separately and these results were compared with those from the lipid mixture. Two phase transitions will result if a two lipid mixture is laterally phase separated, and these two transitions will be close to the T_m of each isolated lipid.

Fig. 3 shows the change in the CH_2 stretch of DGDG (Fig. 3a), and the CD_2 stretch of d54 DMPC and the CH_2 stretch of DMPC (Fig. 3b) as a function of temperature. In agreement with the reported T_m for DGDG of around −50°C [21], the CH_2 stretch of isolated DGDG did not change as the sample was cooled from 30 to −5°C. In contrast, the CH_2 stretch from the DGDG in the lipid mixture decreased by nearly 1.5 cm^{-1} (Fig. 3a). For comparison, the phase transitions of the isolated DMPC (22°C) and d54 DMPC (17°C) were at higher temperatures than that of the d54 DMPC in the mixture with DGDG (∼6°C; Fig. 3b). These measurements indicate that the lipids were not completely phase separated, although it appears that a portion of the DGDG had phase separated in the mixture since it was still in the liquid crystalline phase at −4°C. In order to compare how the two lipids were acting in the mixture, both transitions were plotted together. The CH_2 and CD_2 stretching vibration frequencies decreased at the same rate until 8°C, when the rate of change of the d54 DMPC stretching frequency increased (data not shown) consistent with similar thermotropic behavior of the two lipids in the mixture.

When DGDG:d54 DMPC liposomes were prepared in the presence of VVVV2KE the frequency of both the CH_2 and CD_2 stretching vibration decreased, indicating that the peptide decreased the phase transition temperature of both lipids (Fig. 4). This is in agreement with the results from the non-deuterated lipid mixture, in which VVVV2KE decreased the T_m of the overall mixture (Fig. 1) and show that the synthetic peptide had no preference for one lipid over the other. The liposomes were affected in a slightly different manner by AAAA2KE and TTTT2KE. The DGDG CH_2 stretching frequency increased in the presence of AAAA2KE, consistent with a decrease in overall T_m; however, there was no effect on the CD_2 stretching frequency of the d54 DMPC (Fig. 4). Similarly, TTTT2KE increased the DGDG CH_2 stretching frequency, but did not affect the d54 DMPC CD_2 phase behavior (data not shown). This indicates a preference by AAAAKE and TTTT2KE for the DGDG in this lipid mixture.

4. Discussion

In this study FTIR spectroscopy has been used to study the interaction of four synthetic analogues of the type I AFP from the winter flounder with model membrane systems. This technique is a highly sensitive probe for monitoring how proteins affect lipid bilayers as they pass through their thermotropic phase transitions. The study of the interactions between AFPs and AFGPs and membranes is of significant interest due to the potential applications of these compounds as cryoprotectants and ice growth inhibitors in agriculture, biotechnology, medicine and the frozen food industry [22–24] where protein/membrane interactions have been implicated, but are not understood at the molecular level.

Table 2 summarizes structural and ice growth inhibition data as well as the effect of the four synthetic analogues XXXX2KE on the phase behavior of 1:1 DGDG:DMPC liposomes. In contrast to the wild-type AFP, TTTT which *increases* the T_m of the liposomes, three of the analogues decreased the T_m of the liposomes. The different results obtained with TTTT and TTTT2KE show that the two additional salt bridges have a significant effect. Fig. 5 shows a

Table 2
Summary of solution conformation data, ice growth inhibition properties and effect on thermotropic membrane phase transitions of polypeptide sequences

Polypeptide	ΔT_m	Preferential interaction with DGDG	Helicity % (22°C)	Helicity % (0°C)	Hysteresis % (2 mM)
TTTT	+2.5	no [31]	50 [27]	100 [27]	100 [32]
TTTT2KE	−2.2	yes	94 [8]	100 [8]	100 [7]
VVVV2KE	−3.4	no	70 [7]	100 [7]	100 [7]
AAAA2KE	−2.4	yes	90 [8]	100 [8]	17 [7]
IIII2KE	0	not determined	80 [10]	100 [10]	0 [10]

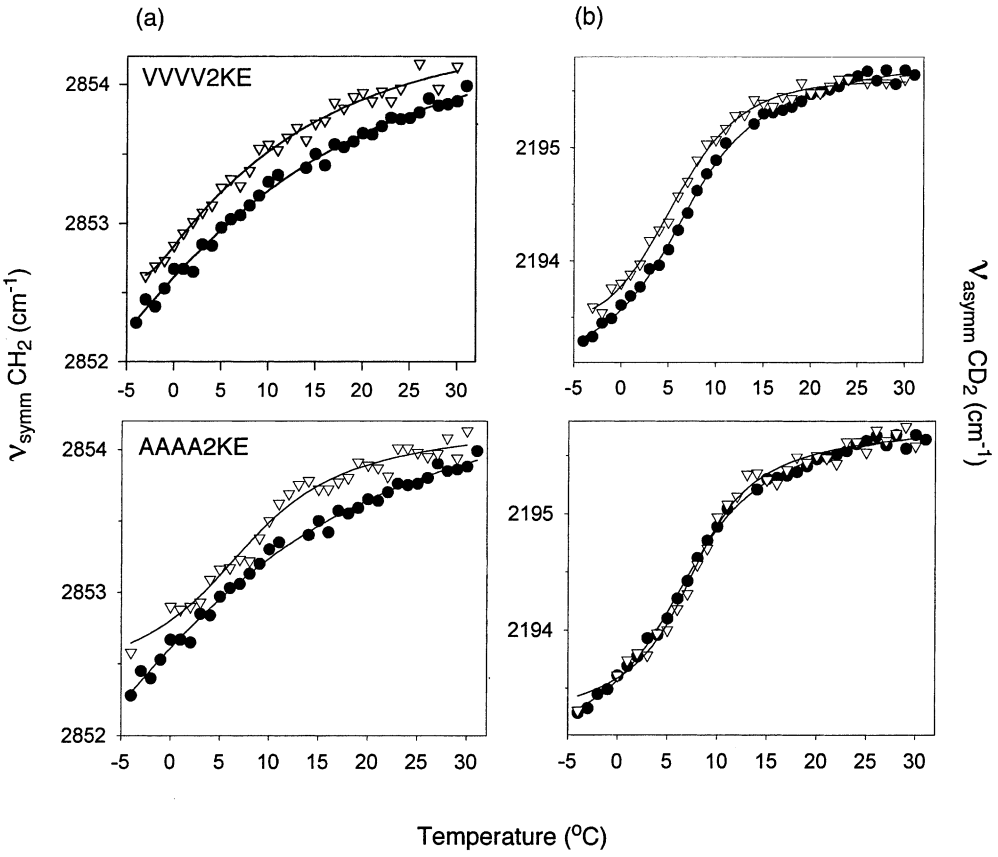

Fig. 4. Graphs showing the effect of peptides on acyl chain mobility of lipids in the DGDG:d54 DMPC liposomes. The symmetric CH_2 stretch of the DGDG (a) and the asymmetric CD_2 stretch of d54 DMPC (b) as a function of temperature in the presence (triangles) and absence (circles) of VVVV2KE (upper panels) and AAAA2KE (lower panels) in a 1:1 peptide:lipid mixture.

computer generated image of the synthetic analogues XXXX2KE highlighting the two additional salt bridges (Lys 7, Glu 11; Lys 29, Glu 33) on the face opposite to that containing the four residues labeled X [25]. These salt bridges stabilize the helical conformation of TTTT2KE at room temperature [26] (Table 1) and also change the biphasic folding pattern of TTTT [27] to a linear dependence of α-helical content with temperature for TTTT2KE [25]. These changes in helicity and folding dynamics do not affect the thermal hysteresis properties, which are measured below 0°C where all peptides are 100% helical, but may be significant in the studies with membranes as each of the synthetic analogues is introduced to the liposome solution at room temperature. In addition, the two salt bridges replace four Ala residues on the face

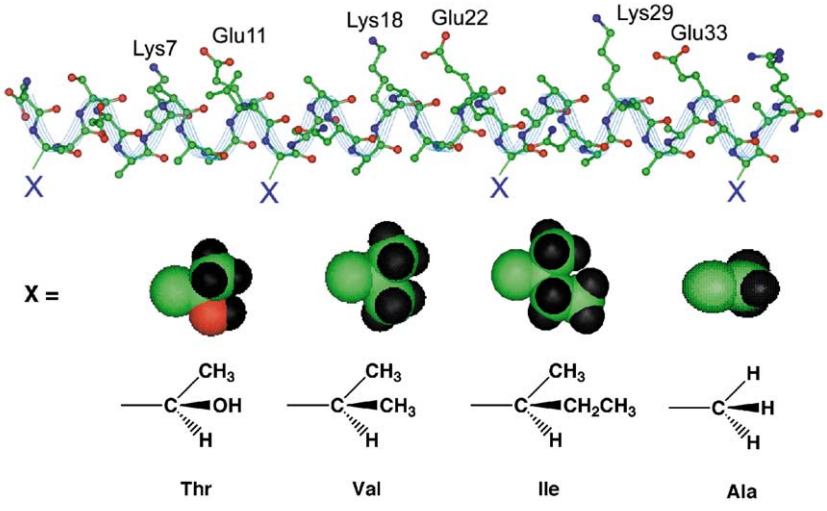

Fig. 5. Computer-generated image of XXXX2KE in an α-helical conformation highlighting the additional salt bridges and the alignment of the four X residues on one face of the helix.

of the helix opposite to the X residues (Fig. 5) and hence this face is more hydrophilic in TTTT2KE than in TTTT. Both the overall solution conformation, and the hydrophilic nature of the inserted salt bridges may contribute to the different effects on T_m shown by these two peptides.

All of the synthetic analogues are significantly more structured at room temperature compared with TTTT. The structural flexibility of TTTT may be an important requirement for increasing T_m and stabilizing membranes against phase transition-induced leakage. This hypothesis is based on recent studies that have shown that the presence of membranes strongly inhibits the folding of TTTT [16]. In the presence of DGDG:DMPC liposomes, TTTT was less than 60% α-helical at 3°C, compared with 80% α-helical in the absence of membranes [16]. Since the synthetic analogues are more highly structured at room temperature before chilling commences, the peptides are almost certainly completely α-helical before they interact with the liposomes, and thus may lack the structural flexibility required to stabilize the membranes. Thus, the increased order of analogues TTTT2KE, VVVV2KE and AAAA2KE may be a key factor that could lead to the destabilization of the membranes that is more typical of the effects of other α-helical peptides on membranes [28–30]. Alternatively, the modified hydrophilic character of one face of the helix may influence the relative orientation and interaction of the synthetic peptides compared with the wild-type TTTT and lead to a decrease in T_m and, possibly, defects in the membrane.

The small, but reproducible differences in the T_m induced by the synthetic analogues (Table 2) are consistent with either a different partitioning coefficient for each of the peptides, or insertion of each peptide into the membrane to a different depth. We propose that the interactions between the proteins and the membranes occur through the hydrophobic face of the α-helix. Substitution of Thr for Val increases the hydrophobicity of one face of the helix (see Fig. 5) and also increases the effect of the protein on T_m. Substitution of the hydrophilic hydroxyl group of Thr by a hydrogen atom in Ala does not significantly change the effect on T_m, consistent with the fact that this modification would not change the hydrophobicity of the protein surface significantly. In the case of the Ile analogue, where the Thr hydroxyl group is replaced by a hydrophobic ethyl group, it is likely that the more bulky side-chain interferes with protein–membrane interaction and thereby abolishes the effect on T_m. This behavior of the four synthetic analogues parallels the ice growth inhibition properties of these compounds, which we have also proposed is directly related to the hydrophobic face of the helix being oriented towards the ice at the ice/water interface [2,8].

VVVV2KE had no preference for either lipid when the T_m of DGDG:d54 DMPC liposomes was determined (Fig. 4), while AAAA2KE and TTTT2KE preferentially affected the DGDG in this mixture. The interaction of TTTT with membranes depends critically on the presence of the highly unsaturated DGDG in the membrane [16]. It is unlikely that the interaction is with the DGDG headgroup, since binding is abolished after hydrogenation of this lipid [16]. It is therefore likely that the preferential interactions of AAAA2KE and TTTT2KE with DGDG are also mediated through the highly unsaturated fatty acyl chains and not through the headgroups. The different behavior of VVVV2KE is more difficult to explain but possibly arises due to the fact that this analogue is sufficiently hydrophobic to interact also with the saturated fatty acyl chains of DMPC. Clearly, small changes in the overall size and hydrophobicity of the mutations at positions 2, 13, 24 and 35 lead to significant differences in the effects those peptides have on the physical behavior and the stability of membranes.

In conclusion, the results of this study support the hypothesis that the interaction between type I AFPs and model bilayers involves the hydrophobic face of the protein. This is the same hydrophobic face that is oriented towards the ice at the ice/water interface and confers the 'antifreeze' properties on these compounds. Introduction of bulky side-chains on the hydrophobic face impedes interaction with the bilayer. The results are consistent with the overall conformation of the peptide playing a significant role in the lipid phase transition properties, although the increased hydrophilic character of one face of the helix may also be important. Further studies on analogues that lack the additional salt bridges, but retain a hydrophobic face containing sterically non-demanding mutations, including those that have been reported in analogues that retain antifreeze activity, are required to establish the structural requirements for membrane stabilization by synthetic AFPs.

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