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Artificial antifreeze polypeptides: α-helical peptides with KAAK motifs have antifreeze and ice crystal morphology modifying properties

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Abstract Antifreeze polypeptides from fish are generally thought to inhibit ice crystal growth by specific adsorption onto ice surfaces and preventing addition of water molecules to the ice lattice. Recent studies have suggested that this adsorption results from hydrogen bonding through the side chains of polar amino acids as well as hydrophobic interactions between the non-polar domains on the ice-binding side of antifreeze polypeptides and the clathrate-like surfaces of ice. In order to better understand the activity of one of the antifreeze polypeptide families, namely the α-helical type I antifreeze polypeptides, four α-helical peptides having sequences not directly analogous to those of known antifreeze polypeptides and containing only positively charged and non-polar side chains were synthesized. Two peptides with regularly spaced lysine residues, GAAKAAKAAAAAAAKAA-KAAAAAAAKAAKAAGGY-NH_2 and GAALKAAKA-AAAAALKAAKAAAAAALKAAKAAGGY-NH_2, showed antifreeze activity, albeit weaker than seen in natural antifreeze polypeptides, by the criteria of freezing point depression (thermal hysteresis) and ice crystal modification to a hexagonal trapezohedron. Peptides with irregular spacing of Lys residues were completely inactive. Up to now, lysine residues have not been generally associated with antifreeze activity, though they have been implicated in some antifreeze polypeptides. This work also shows that lysine residues in themselves, when properly positioned on an α-helical polyalanine scaffold, have all the requisite properties needed for such an activity.

Key words: Antifreeze polypeptide; α-Helix; Winter flounder; Sculpin; Amphipathic helix

1. Introduction

Antifreeze polypeptides (AFPs) are secreted by a variety of fishes that inhabit ice-laden waters to protect them from freezing [1–5]. It is thought that AFPs exert their effect in a non-colligative manner by binding to the surface of incipient ice crystals and inhibiting their growth. Characteristically, fish AFPs exhibit thermal hysteresis – depression of the freezing point of water below the equilibrium melting point of ice – and cause a change in ice crystal morphology from hexagonal plates, seen for water in the absence of AFPs, to hexagonal bipyramids or similar shapes. Four classes of AFPs have been reported: type I, alanine-rich amphipathic α-helixes (M_r ca. 3300–5000); type II (M_r ca. 14 000), cystine-rich globular proteins related to certain lectins; type III (M_r ca. 6500), compact β-sheet structures and type IV (M_r ca. 12 000), thought to be a four helix bundle. Of these, the small helical type I AFP from the winter flounder (WF) has been most intensively studied [6–9]. The WF-AFP is characterized by having several regularly spaced threonine and asparagine residues arranged along one side of the helix where the polar side chains of them can potentially hydrogen bond to the ice lattice. From ice etching studies, Knight et al. [10] deduced that WF-AFP binds to the $\{20\bar{2}1\}$ hexagonal bipyramidal surfaces of ice along the direction $[\bar{1}102]$. Detailed hydrogen bonding models for binding have been proposed by Wen and Laursen [11] and Sicheri and Yang [12]. However, the unique importance of hydrogen bonding was recently brought into question by two studies which showed that replacement of Thr with Val did not greatly diminish the antifreeze activity of WF-AFP [13,14]. A subsequent study with a series of analogs in which Thr was systematically substituted with L-Ser, L-Val and L-*allo*Thr has suggested that both the hydrogen bonding and hydrophobic properties of the Thr side chain play specific roles in antifreeze activity [15].

The list of new, naturally occurring AFPs is growing, with the recent inclusion of new AFPs from insects [16,17], a plant [18] and a bacterium [19]. However, there are no reported artificial AFPs, except for analogs of natural ones. In the present study, we synthesized some simple helical peptides having regularly spaced, positively charged residues and found that these peptides exhibited properties characteristic of typical AFPs, even though their sequences are significantly different from those of any of the known natural AFPs.

2. Materials and methods

2.1. Peptide syntheses and purification

Peptides were synthesized on a Milligen/Biosearch Model 9050 Peptide Synthesizer as peptide amides, using Fmoc-L-amino acids activated by *O*-(7-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate. After synthesis, the crude peptides were purified by reversed phase-high performance liquid chromatography (RP-HPLC) on a Vydac C_{18} preparative column, using water/acetonitrile gradients containing 0.05% trifluoroacetic acid. The molecular masses were confirmed on a PerSeptive Biosystems Voyager-DE matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer.

2.2. Circular dichroism (CD) studies

CD spectra were taken in 0.1 M ammonium bicarbonate buffer (pH ~8) using a 1 mm path length quartz cell on an Aviv model 62DS spectrometer. Concentrations of AFP were determined by measuring the tyrosine absorbance of aliquots of stock solutions diluted in 6.0 M guanidine hydrochloride solutions using $\varepsilon_{275} = 1450\ M^{-1}\ cm^{-1}$ [20]. Potassium phosphate buffers were used for studies of the effect of pH on helicity (ellipticity). The same solutions, but more concentrated, were used for activity studies (see below).

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Abbreviations: AFP, antifreeze polypeptide; WF, winter flounder; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; HPLC, high performance liquid chromatography; RP, Reversed phase

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2.3. Antifreeze activity measurement

Antifreeze activity, defined as thermal hysteresis, was measured as described previously [6–8] in 0.1 M ammonium bicarbonate (pH ~8) using a nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA) with a temperature-controlled sample holder mounted on a microscope stage, in conjunction with a video cassette recorder. Crystal dimensions were measured directly on the video monitor screen.

3. Results and discussion

A common structural characteristic of most of the types I–IV AFPs is a flattened surface containing a preponderance of amino acids (Asn, Gln, Ser, Thr, Asp) potentially capable of forming hydrogen bonds with an ice surface [3]. However, compared with the regularly spaced Thr and Asn/Asp residues seen in WF-AFP, the polar residues in the type I helical AFPs from various species of sculpin form less distinctive patterns and tend to have more positively charged residues [1,4]. For example, the shorthorn sculpin AFP, SS-8, Ac-MNGETPAQKAARLAAAAALAAKTAADAAAKAAAKA-AAIAAAAASA, has five Lys and Arg residues on its putative ice-binding face, in addition to a nearly equal number of neutral, polar or acidic residues. In addition, most type I AFPs have one or two hydrophobic Leu or Ile residues on the ice-binding face that may contribute to ice-binding through hydrophobic interactions [15].

In order to produce a simpler model for structure-function studies of AFPs and as part of an effort to characterize the ice-binding residues of the lysine-rich sculpin AFPs, we designed and synthesized four peptides, designated poly-AK, Ac-poly-AK, AKAAK and LKAAK, containing positively charged, but no neutral, polar amino acids (except for a C-terminal Tyr residue):

poly-AK:
GAAAAAA**K**AAAAA**K**AAAAA**K**A**K**AAAAA**K**A**K**AGGY-NH_2
Ac-poly-AK:
Ac-GAAAAAA**K**AAAAA**K**AAAAA**K**A**K**AAAAA**K**A**K**AGGY-NH_2
AKAAK:
GAA**K**AA**K**AAAAAAA**K**AA**K**AAAAAAA**K**AA**K**AAGGY-NH_2
LKAAK:
GAA**LK**AA**K**AAAAAA**LK**AA**K**AAAAAA**LK**AA**K**AAGGY-NH_2
WF-AFP:
D**T**AS**D**AAAAAA**LT**AA**N**AKAAAE**LT**AA**N**AAAAAAATAR-NH_2

Like the type I AFPs and similar model peptides [21,22], all of the above peptides are rich in helix-favoring Ala residues. Six Lys residues in each peptide provide water solubility to the otherwise insoluble polyalanine chain. Glycine was incorporated at both the N- and C-termini since it frequently occurs at the helix boundary positions [23,24]. The single tyrosine residue at the C-terminus was introduced to facilitate accurate measurement of the peptide concentration by spectrophotometric measurement of the phenolic group absorbance.

In poly-AK, Lys residues are irregularly distributed around the helix rod (Fig. 1). Ac-poly-AK differs from poly-AK only in that its N-terminus is acetylated. AKAAK and LKAAK were modeled roughly after the WF-AFP (rather than a sculpin AFP) by replacing the regularly spaced TAAN/D ice-binding motifs of WF-AFP by KAAK. As seen in Fig. 1, the potential ice-binding motifs (KAAK) are oriented along one side of the helix to give peptides expected to be amphipathic, as is WF-AFP. The C-terminal tyrosine is represented as being on the opposite side of the helix to reduce possible interactions that might prevent adsorption to ice, although the C-terminus is probably unstructured in any case due to the two Gly residues. Assuming that the peptides are nearly 100% helical, the ice putative binding units repeat every 11 residues, with a repeat distance of 16.5 Å, the same spacing as between the threonine residues in WF-AFP, and thus should be able to match the 16.7 Å spacing along the directions $<\bar{1}102>$ on the $\{20\bar{2}1\}$ surfaces of ice [10,11]. Each peptide was designed with three potential ice-binding units since we found earlier (unpublished) that a similar peptide containing only two ice-binding units had no antifreeze activity. In peptide LKAAK, Leu is located in the analogous position to Leu in WF-AFP. Leu was omitted in peptide AKAAK in order to assess the role of the hydrophobic isobutyl side chain on antifreeze activity.

Peptide synthesis was performed by standard solid phase methods using Fmoc chemistry. Analysis and purification of peptides were performed by RP-HPLC. Peptide purity was determined by analytical RP-HPLC and MALDI-TOF mass spectrometry. CD spectra taken in 0.1 M ammonium bicarbonate (pH ~8) at 1°C show the two minima at 222 and 208 nm that are characteristic for an α-helix (Fig. 2). Mean residue ellipticity values at 222 nm indicated that all of these peptides are about 70% helical, values that are lower than

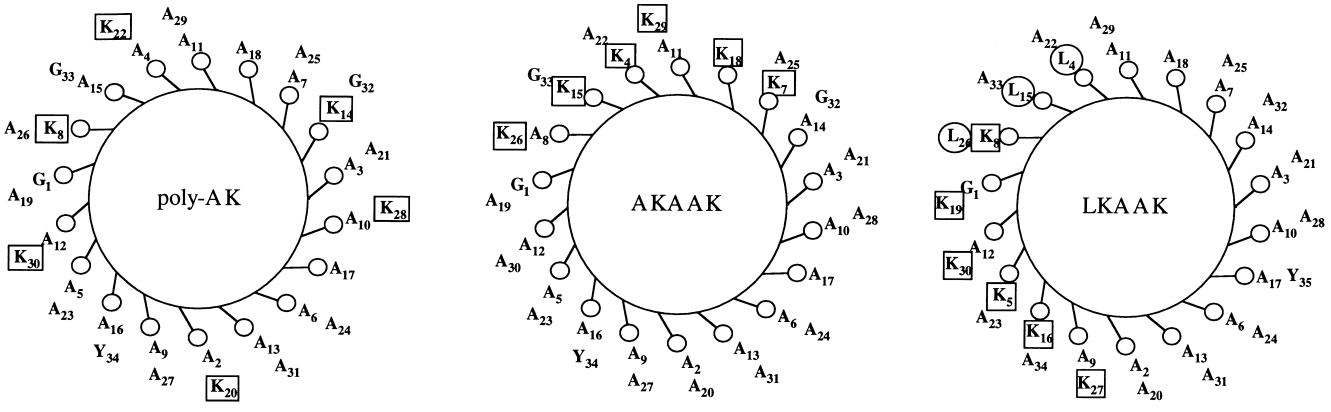

Fig. 1. Helical wheel representations of poly-AK, AKAAK and LKAAK, looking down the helix axis from the C-terminus. Note that in poly-AK, Lys residues are distributed around the helix rod, whereas in AKAAK and LKAAK, they are regularly spaced on one side of the helix rod, making each peptide amphipathic.

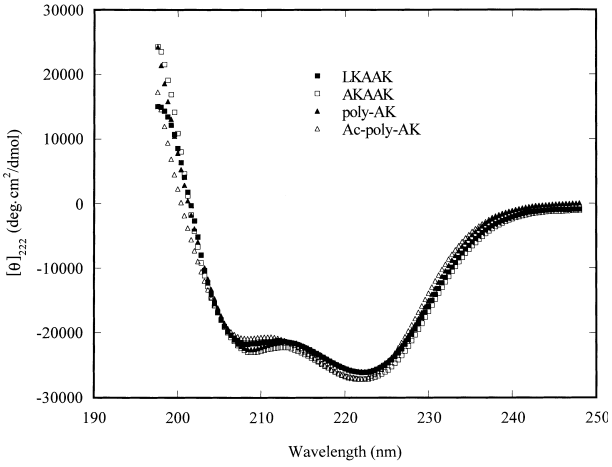

Fig. 2. CD spectra of LKAAK and AKAAK taken in 0.1 M ammonium bicarbonate (pH ~8) at 1°C. The helicity, calculated from mean residue ellipticity values at 222 nm, is about 70% for all peptides.

seen for WF-AFP [7], but similar to that for the sculpin AFP, SS-8 (unpublished observations). The similar helicities of poly-AK and Ac-poly-AK suggested that acetylation at the N-terminus has little effect on the secondary structures of the peptides.

Antifreeze activity was measured as previously described [6–8]. While poly-AK and Ac-poly-AK are completely inactive, both AKAAK and LKAAK show an antifreeze activity that is qualitatively similar to that of wild-type AFPs [1,7], i.e. a non-linear dependence of activity on the concentration (Fig. 3) and the ability to completely arrest ice crystal growth in the thermal hysteresis region for at least 2 h (data not shown), unlike some analogs which only retard growth [9,13,15]. However, the activity of these peptides, on a molar basis, is around 50 times lower than that of the wild-type WF-AFP. There is little effect of the pH on either helicity or antifreeze activity, at least for LKAAK, the only analog studied, over the pH range 2–13 (Fig. 4). The lack of an effect on activity at around pH

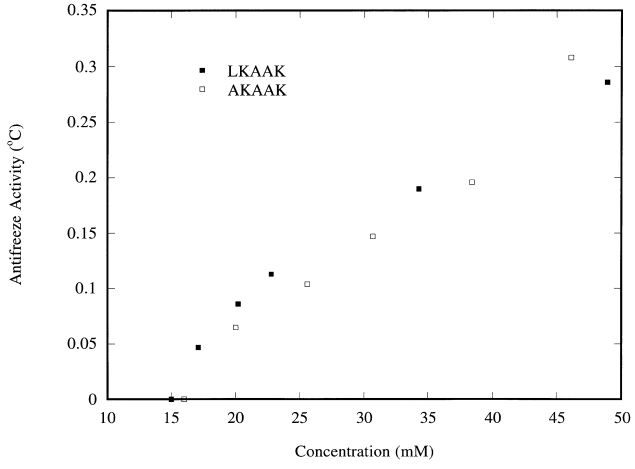

Fig. 3. Antifreeze activity (thermal hysteresis) as a function of the concentration. Assay solutions contained 0.1 M ammonium bicarbonate buffer (pH ~8). Activity is defined as the difference between the equilibrium melting point of ice and the non-equilibrium freezing point.

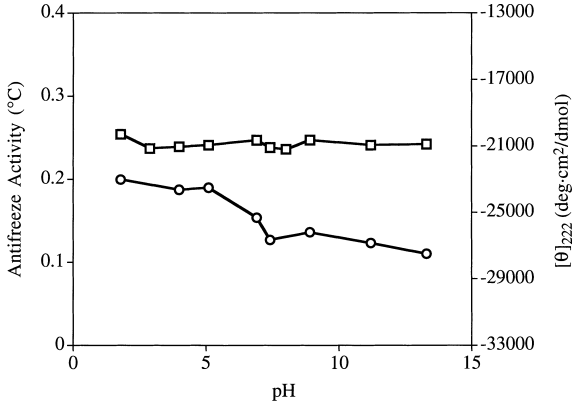

Fig. 4. Helicity (circles) and antifreeze activity (squares) of LKAAK as a function of the pH. Antifreeze activity measurements were made on 40 nM AFP solutions in phosphate buffers. The same solutions were used (after dilution) for CD measurements.

10.5, the approximate pK_a of a lysine amino group, is a little surprising. It implies that the protonated and unprotonated amino groups interact equally well with the ice surface, presumably by hydrogen bonding. The small effect of the pH on helicity implies that the lysine ammonium groups are not close enough to interact significantly, which is consistent with results for similarly spaced carboxyl groups (unpublished observations). The slight decrease in helicity below pH 7 may be due to protonation of the α-amino group, but this requires further study. In any event, the effect is below the physiologically relevant pH range.

Both AKAAK and LKAAK cause ice crystals to assume a shape similar to the hexagonal bipyramidal habit characteristic for native AFPs, except, here, the shape is that of a hexagonal trapezohedron (Fig. 4) rather than a true bipyramid. A second difference seen (Fig. 5) with these peptides is that the *c*/*a*-axis, ranging from about 5/1 to 9/1 as the temperature is lowered, is considerably larger than the ratio of 3.3/1 seen for the WF-AFP. A similar phenomenon has been observed for a type III AFP [25] and for the shorthorn sculpin AFP, SS-8 (unpublished observations), except that these two native AFPs cause ice crystals to form hexagonal bipyramids. Since there is good evidence that the WF-AFP binds to the $\{20\bar{2}1\}$ surfaces of ice [10], we conclude that LKAAK and AKAAK must bind either to another (higher order) crystallographic plane or by a different mechanism, such as the step growth inhibition mechanism originally proposed by DeVries [26] and adapted by DeLuca et al. [25], to explain the observed ice crystal morphologies seen with type III AFPs.

All of the well-studied types I, II and III, and even type IV AFP, are richly endowed with surface hydroxyl, carboxyl and carboxamide groups (e.g. from Thr, Ser, Asn, Gln, Asp) which have been demonstrated or postulated to be important for antifreeze activity by hydrogen bonding to ice surfaces. However, the overriding importance of hydrogen bonds has been called into question recently by several studies [13–15] which indicate that the hydrophobic methyl group of Thr may play a key role in antifreeze activity through van der Waals interaction with the ice surface. In addition, an earlier study [9] showed that deletion of the two hydrophobic Leu residues from WF-AFP caused a one-third loss of activity and a recent molecular simulation analysis [27] suggested that hydrophobic interactions of certain Ala methyl groups may be important.

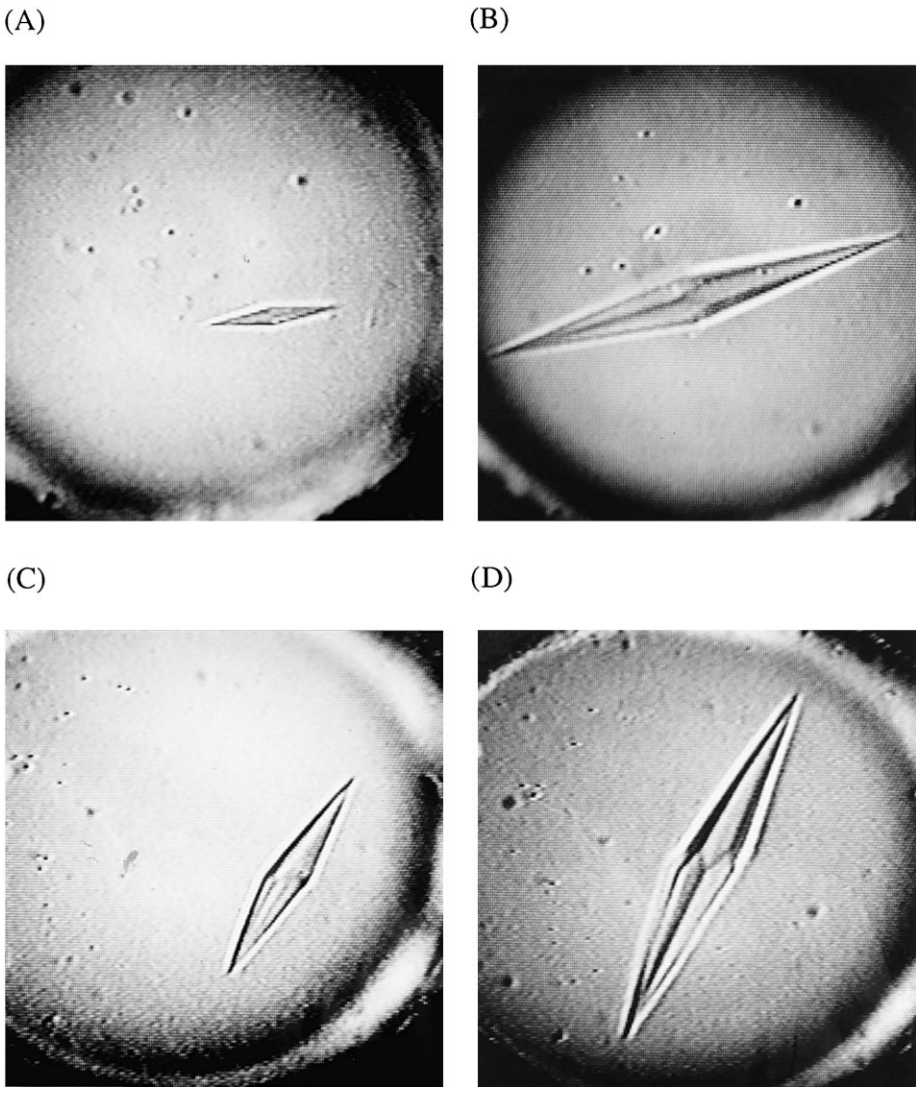

Fig. 5. Photographs showing the effects of LKAAK and AKAAK on ice crystal morphology. (A) An ice crystal grown in a 23 mM LKAAK solution within the hysteresis gap, the *c*/*a* ratio is 5.7. (B) The same crystal as (A) showing a hexagonal trapezohedral shape after the temperature was lowered below the non-equilibrium freezing point, the *c*/*a* ratio has increased to 8.5. (C) An ice crystal grown in a 31 mM AKAAK within the thermal hysteresis gap, the *c*/*a* ratio is 5.4. (D) The same crystal as (C) after the temperature was lowered below the non-equilibrium freezing point, the *c*/*a* ratio is 8.5.

In the present case, neither LKAAK nor AKAAK has any of the neutral, polar or acidic amino acids (Asn, Gln, Ser, Thr, Asp, Glu) seen in most native AFPs, only three regularly spaced Lys pairs. However, it is seen by the activity of LKAAK and AKAAK that the Lys residues in themselves provide the basic elements needed for interaction with ice, presumably the ε-ammonium groups, which can form hydrogen bonds and four methylene groups which may participate in hydrophobic interactions. That lysine side chain ammonium groups can interact with ice surfaces has been predicted by Wierzbicki et al. [28] based on molecular modeling studies with the lysine-rich shorthorn sculpin AFP. The fact that AKAAK lacks the three hydrophobic Leu residues seen in LKAAK but has the same activity suggests a diminished role for leucine, at least in this case.

The artificial AFPs, AKAAK and LKAAK, are a sort of hybrid combining the spacing of WF-AFP and the preponderance of basic residues seen in sculpin type I AFPs. The type I AFPs from sculpin differ from the prototypic WF-AFP by having several positively charged residues (usually Lys) on the putative ice-binding face, for example as in the shorthorn sculpin AFP, SS-8, shown above. Little is known about which residues are essential for activity in the sculpin AFPs, but the mode of binding must be different from that of the WF-AFP. Not only are the *c*/*a*-axis ratios of ice crystals much higher for the sculpin AFPs compared with WF, but also ice etching experiments show different patterns, which Knight et al. [10] interpret as resulting from binding to the $\{20\bar{2}1\}$ hexagonal bipyramidal surfaces of ice for WF-AFP and to the $\{2\bar{1}\bar{1}0\}$ secondary prism faces for a sculpin AFP. The ice morphology modifying properties of AKAAK and LKAAK are different from those of either the WF or the sculpin AFPs, suggesting that these peptides bind to ice surfaces other than the hexagonal bipyramidal or secondary prism planes. Ice etching data are not yet available for AKAAK and LKAAK, but binding by a step inhibition

mechanism, as discussed by DeLuca et al. [25], could provide an explanation for both the longer and variable *c*/*a*-axis ratios.

The complete lack of either thermal hysteresis or ice modifying effects by Ac-poly-AK and poly-AK (an isomer of AKAAK), which have an irregular distribution of Lys residues, demonstrates that the activity of AKAAK and of LKAAK is sequence dependent and not simply due to a bulk effect of a cationic polypeptide. Both AKAAK and LKAAK have three 11 residue repeat sequences which place the Lys side chains on one side of the helix, as is the case for the WF-AFP. The mechanism of interaction of these peptides is not known, but it seems likely that the hydrocarbon moiety of the lysine side chain lies back against the hydrophobic surface of the polyalanine helix, thereby minimizing exposure to solvent to create more or less rigid, regularly spaced ice-binding motifs that can participate in both hydrogen bonding and hydrophobic interaction with the ice surface. As we have postulated [15] for the WF-AFP, both types of interaction may be essential, with hydrophobic effects providing the driving force for interaction of the peptide with the ice surface [13,14,27,29] and hydrogen bonding providing specificity or 'locking' of the peptide in place. Although details of the peptide-ice interaction remain to be elucidated, this work demonstrates that rather simple peptide structures can have ice growth and crystal modification activities and, further, that the observed activity is due to specific structural features of the peptides, rather than bulk properties alone.

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