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A natural variant of Type I antifreeze protein with four ice-binding repeats is a particularly potent antifreeze

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Abstract

A 4.3-kDa variant of Type I antifreeze protein (AFP9) was purified from winter flounder serum by size exclusion chromatography and reversed-phase HPLC. By the criteria of mass, amino acid composition, and N-terminal sequences of tryptic peptides, this variant is the posttranslationally modified product of the previously characterized AFP gene 21a. It has 52 amino acids and contains four 11-amino acid repeats, one more than the major serum AFP components. The larger protein is completely α-helical at 0 °C, with a melting temperature of 18 °C. It is considerably more active as an antifreeze than the three-repeat winter flounder AFP and the four-repeat yellowtail flounder AFP, both on a molar and a mg/mL basis. Several structural features of the four-repeat winter flounder AFP, including its larger size, additional ice-binding residues, and differences in ice-binding motifs might contribute to its greater activity. Its abundance in flounder serum, together with its potency as an antifreeze, suggest that AFP9 makes a significant contribution to the overall freezing point depression of the host.

Keywords: α-helix; ice-binding motifs; serum freezing point depression

Type I AFPs are the alanine-rich, amphipathic, α-helical antifreezes found in righteye flounders and sculpins (Davies & Hew, 1990). Those present in flounder serum have a particularly well-defined repetitive structure based on an 11-amino acid unit Thr-X-X-Asx-X-X-X-X-X-X-X, where X is generally alanine (DeVries & Lin, 1977). Their continuous helical structure is consistent with secondary structure predictions and has been confirmed by CD (Ananthanarayanan & Hew, 1977; Raymond et al., 1977; Wen & Laursen, 1992a) and by X-ray crystallography (Yang et al., 1988). The helix is stabilized at both ends by remarkably cohesive cap structures (Sicheri & Yang, 1995), by terminal charge groups that reinforce the helix dipole, and, in some Type I AFPs, by intrachain salt bridges (Davies & Hew, 1990).

Fish AFPs are thought to function by an adsorption–inhibition mechanism (Raymond & DeVries, 1977). By binding specifically to the surface of ice, they force the ice between the bound AFPs to grow with a curvature that results in a lowering of the nonequilibrium freezing point (Knight et al., 1991). Furthermore, Knight et al. (1991) have shown that winter flounder AFPs bind selectively to the $\{20\bar{2}1\}$ bipyramidal plane in the direction $<01\bar{1}2>$. They postulated a 16.5 Å spacing between the i, $i+11$ threonines, forming a match to the 16.7 repeat spacing between accessible oxygens along the $<01\bar{1}2>$ direction of the $\{20\bar{2}1\}$ binding plane.

The role of the hydrogen bonding residues in the $i+3$ positions relative to the threonines is less well established. In the model of Wen and Laursen (1992b), the Asp or Asn in the $i+3$ positions interact with a rank of ice lattice oxygens that has the same spacing but is slightly recessed. The model of Sicheri and Yang (1995) has these residues hydrogen bonding to adjacent oxygen atoms in the same rank as those bound by the threonines, whereas the modeling of Madura et al. (1994) suggests that the asparagines fit within polyhedra cages on the ice surface. In yet another modeling study, the Asp and Asn in the + 3 positions do not feature directly in the binding of AFP to ice (Chou, 1992). In the yellowtail flounder, a close relative of the winter flounder, a longer protein with four 11-amino acid repeats is the predominant serum AFP (Scott et al., 1987). This antifreeze had a lower activity than the three-repeat winter flounder AFP on a mg/mL basis, but was comparable in activity on a molar basis. Notable was the absence of the supporting $i+3$ ice-binding

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residue in three of the four repeats, which might help explain the diminished activity.

In winter flounder, the dominance of the three-repeat sequences such as HPLC-6 and -8 (Fourney et al., 1984) can be attributed to the tandem amplification and homogenization of their genes (Scott et al., 1985; Davies, 1992). However, a four-repeat AFP sequence has been seen at the DNA level, both as a genomic clone (Davies et al., 1984; Gauthier et al., 1990) and as a cDNA clone (Gourlie et al., 1984). In this report, we have reexamined the profile of serum AFPs in winter flounder and have found a component that matches the genomic sequence 21a. This component is exceptionally active, and its comparison with the four-repeat yellowtail flounder AFP and the three-repeat winter flounder AFPs helps to identify those structural features that are important for antifreeze activity in the Type I AFPs.

Results

Purification of a Type I AFP variant (AFP9)

When serum from winter flounder collected in the winter months was subjected to protracted size-exclusion chromatography on Sephadex G-75, a series of A_{230nm}-absorbing peaks eluted after the massive void peak (Fig. 1A). Peak fractions 105 and 119 had thermal hysteresis activities of 100 and 110 mOsm, respectively. Eluate in the void peak shoulder at fraction 89 and in the total volume peak at fraction 135 showed only 20 and 10 mOsm of thermal hysteresis, respectively. Reversed-phase chromatography of pooled fractions 111–127 produced a protein profile typical of that seen by Fourney et al. (1984), in which the major serum AFPs, HPLC-6 and -8, predominate (not shown). This profile also contained a small quantity of a component with antifreeze activity that bound tightly to the C18 column and eluted under the same conditions as HPLC-9 in the original study (Fourney et al., 1984). Reversed-phase chromatography of the preceding Sephadex G-75 peak (fractions 97–110) showed the presence of one major component, which eluted late in the profile at 74% B in a comparable position to HPLC 9 (Fig. 1B). Rechromatography of this major component, which is referred to here as AFP9, on an analytical C18 reversed-phase column confirmed its purity and integrity (Fig. 1B, inset).

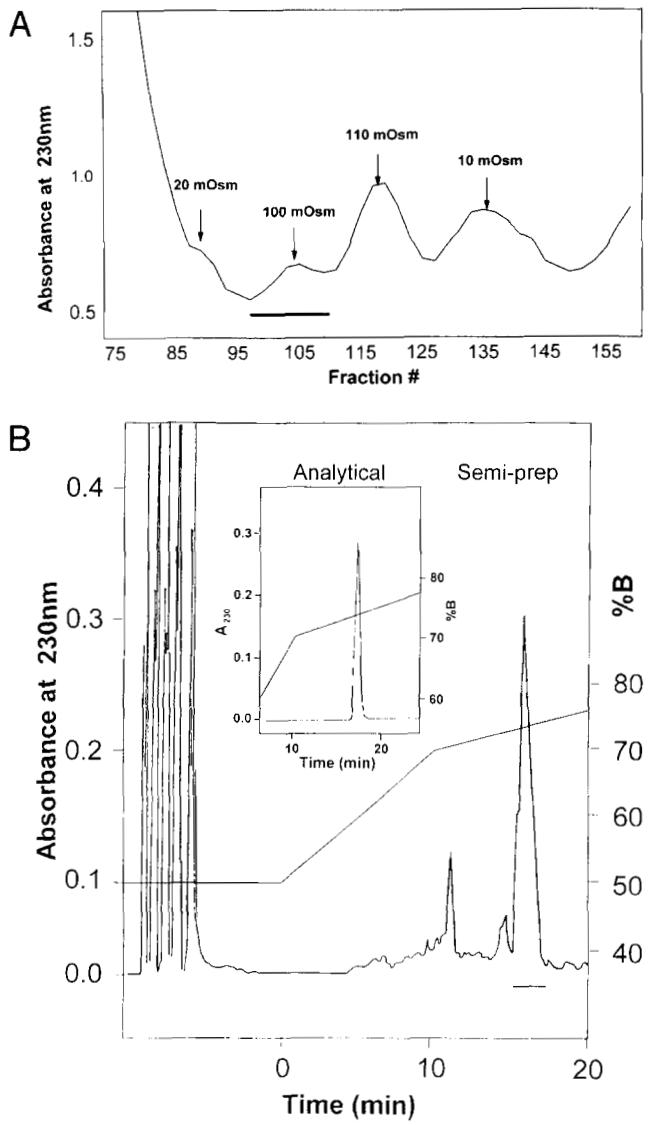

Fig. 1. Purification of AFP. **A:** Size exclusion chromatography of winter flounder serum was performed on Sephadex G-75 as described in Materials and methods. Only the low molecular weight end of the elution profile (post-void peak) is displayed. Thermal hysteresis values (mOsm) for selected fractions are shown. Fractions covered by the horizontal bar (97–110) were pooled for further purification. **B:** Reversed-phase HPLC chromatography of AFP on semi-preparative and analytical (inset) C18 columns. The protein profile was monitored by absorbance at 230 nm. Fractions pooled from the semi-preparative run are indicated by the horizontal line under the major peak. %B refers to the content of acetonitrile in 0.05% TFA used as the eluting solution.

Identification of AFP9 as the product of gene 21a

Several lines of evidence indicate that AFP9 is the product of the winter flounder AFP gene 21a. The molecular weight of AFP9 was determined by electrospray mass spectrometry to be 4,281.7 ± 0.7. This agrees closely with the value of 4,283.7 computed for the gene product of 21a if the putative preproAFP were to be processed in the same fashion as the HPLC-6 and -8 precursors (Fig. 2), which includes removal of the C-terminal glycine during amidation of the penultimate residue (Arg) (Hew et al., 1986). Amino acid analysis was performed on AFP9 in such a way as to accurately measure the molar ratio of the less abundant amino acids (Table 1). As a result, the number of Ala and Thr (the two most abundant amino acids) per mol AFP9 could not be computed because their signals were off-scale. The molar ratio for Arg, Asx, Leu, Lys, Ser, and Val was close to the ratio predicted for 21a (1:3:1:1:1:1), with the exception of Ser, which is typically underestimated. Glycine was absent from the composition, which is consistent with the loss of this residue during C-terminal amidation.

Tryptic cleavage of AFP9

The predicted sequence of AFP9 has a single basic residue, lysine 30. Cleavage of the protein with trypsin provided two other criteria for identifying it as the gene product of 21a. One was the location of the lysyl residue in the sequence, as determined by mass spectrometry of the tryptic cleavage products. The other

```
                                                  10
Asp-Thr-Ala-Ser-Asp-Ala-Ala-Ala-Ala-Ala-

                                                  20
Ala-Ala-Thr-Ala-Ala-Thr-Ala-Ala-Ala-Ala-

                                                  30
Ala-Ala-Ala-Thr-Ala-Val-Thr-Ala-Ala-Lys-

                                                  40
Ala-Ala-Ala-Leu-Thr-Ala-Ala-Asn-Ala-Ala-

                                                  50
Ala-Ala-Ala-Ala-Ala-Thr-Ala-Ala-Ala-Ala-

Ala-Arg-Gly
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Fig. 2. Amino acid sequence of the 21a gene product. The sequence predicted for the mature protein is displayed. Shading of the C-terminal Gly signifies its removal during amidation of Arg 52. The single basic residue (Lys 30) is shown in bold, and the underlining indicates the N-terminal sequences determined on the two tryptic peptides.

was limited N-terminal sequencing of the two tryptic peptides. This was deemed to be more informative than extensive sequencing from the N-terminus alone because the latter would lead into an alanine-rich region. The progress of tryptic digestion was monitored by measuring the thermal hysteresis activity of AFP9. At time zero, the stock AFP9 solution was fully active in the ammonium bicarbonate digestion buffer (Table 2). The activity was reduced to 5% after 30 min, and was completely eliminated by 60 min. At this point there was not even any influence of the digest on ice crystal morphology, indicating that the digestion had gone to completion and that the tryptic peptides themselves were inactive as antifreezes. When the tryptic digest was analyzed by reversed-phase HPLC, only two peptides were apparent (Fig. 3). These had molecular weights of 1,811.48 ± 0.14 and 2,489.21 ± 0.07, which agree closely with their predicted molecular weights of 1,812.0 and 2,490.7, respectively. There was no sign of any undigested AFP9. These two peptides were analyzed by N-terminal Edman degradation for six cycles. The larger peptide gave a unique sequence of DTASDA, which confirmed its identification as the N-terminal peptide of the processed 21a gene product. The smaller peptide began with the sequence AAALTA, which matches the internal sequence immediately following Lys 30.

Table 1. *Amino acid composition of AFP9*

Amino acid	21a[a]	AFP9[b]
Ala	37	Off-scale
Arg	1	0.94
Asx	3	2.76
Gly[c]	1	00.0
Leu	1	0.98
Lys	1	0.98
Ser	1	0.54
Thr	7	Off-scale
Val	1	1.1

[a] Number of amino acids predicted in the gene product of 21a.
[b] Number of amino acids per mole of protein determined by amino acid analysis.
[c] Eliminated during C-terminal amidation.

Table 2. *Trypsin digestion*

Time (min)	mOsm
0	220
30	10
60	0

Secondary structure

The far UV CD spectra of AFP9 were characteristic of an α-helical structure (Fig. 4A). Ellipticity minima were observed near 208 nm and 222 nm, and a corresponding absorption maximum was evident near 190 nm. At 0 °C, the ellipticity value at 222 nm was 3.97×10^{-4} deg·cm^2·dmol^{-1}, which suggests a helical content of 100% based on theoretical calculations (Chen et al., 1974).

The α-helical content of this peptide was sensitive to temperature change (Fig. 4B). Maximum helical content was only observed near 0 °C, and the helicity of the peptide was markedly reduced by increasing the temperature. The peptide was completely denatured at 60 °C, as indicated by the CD spectrum, which resembled that of a random coil structure (not shown). The thermal denaturation profile was reversible in this temperature range, with the loss of less than 10% of the initial helicity when the AFP9 solution was cooled back to 0 °C. The estimated T_m was ~18 °C.

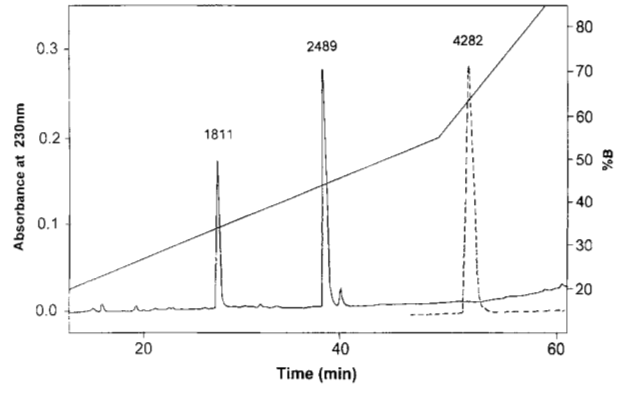

Fig. 3. Reversed-phase HPLC chromatography of the tryptic digest of AFP9. Digestion products (300 μg) in 1 mL of 0.1% TFA were loaded onto a C18 analytical column and eluted using a biphasic linear gradient of acetonitrile in 0.1% TFA increasing from 10% to 55% in 45 min and to 100% over a further 15 min. The protein profile (solid tracing) was monitored by absorbance at 230 nm, and molecular weights displayed above each peak were determined by electrospray mass spectrometry. The dotted line represents the elution profile of undigested AFP9 under similar chromatography conditions. %B refers to the content of acetonitrile in 0.1% TFA used as the eluting solution.

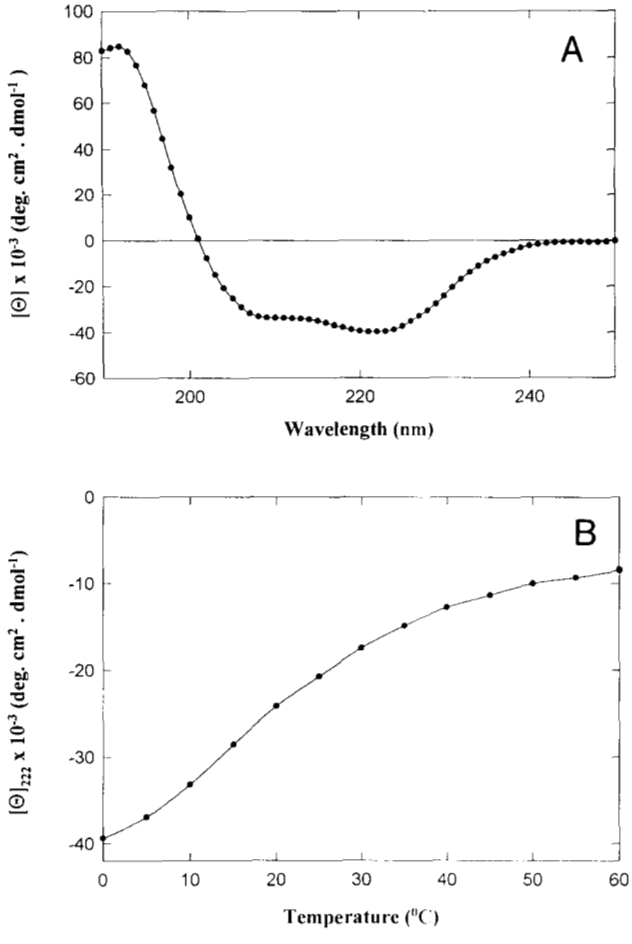

Fig. 4. A: Far-UV CD spectra of AFP9. The CD wave-scan (190–250 nm) was recorded at 0 °C. Protein concentration was 1.0 mg/mL. **B:** Thermal denaturation profile of AFP9. For the temperature denaturation study, change in helicity as a function of temperature was monitored at 222 nm. Protein concentration was 0.61 mg/mL. The buffer used for both studies was 10 mM Na phosphate, pH 7.4, containing 140 mM NaCl and 2.7 mM KCl.

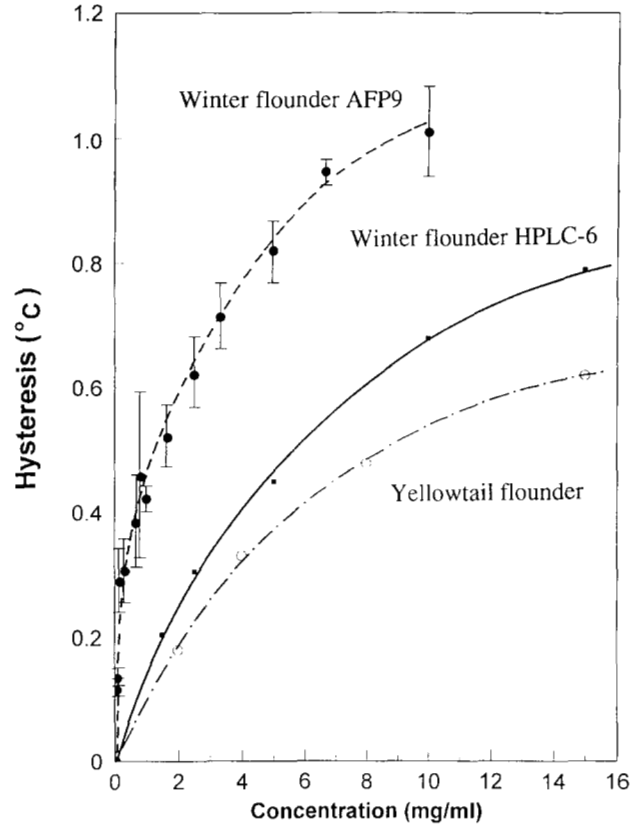

Fig. 5. Thermal hysteresis activity as a function of AFP concentration. Activity curves for winter flounder HPLC-6 and yellowtail flounder AFP were taken from Scott et al. (1987). The curve for AFP9 was based on serial dilutions of a stock 10 mg/mL solution. Each point is the average of at least three determinations. Standard deviations are shown by the vertical bars.

Antifreeze activity

The thermal hysteresis activity of AFP9 was measured over a range of concentrations from 0 to 10 mg/mL (Fig. 5). It was considerably more active than both the three-repeat AFP from winter flounder (HPLC-6), and the four-repeat AFP from yellowtail flounder, showing 1.0 °C of thermal hysteresis at 10 mg/mL compared with 0.68 °C and 0.56 °C for the other two AFPs, respectively. The superior activity of AFP9 was particularly evident at low concentrations and was even more pronounced if the two winter flounder AFPs were compared on a molar basis. The ice crystal morphology seen in the presence of AFP9 was that of a hexagonal bipyramid (not shown).

Discussion

The identification of AFP9 as the 21a gene product is consistent with all the evidence presented. AFP9 is a perfect match to the sequence of 21a (Gauthier et al., 1990), which was originally derived from a genomic library of *Eco*R I-generated winter flounder DNA fragments (Davies et al., 1984). A protein encoded by the slightly different four-repeat cDNA sequence (IIA7) reported by Gourlie et al. (1984) would have an additional alanine in place of the single valine. This is a difference that would have been detected by any one of several analyses used (reversed-phase HPLC, mass spectrometry, and amino acid analysis) had a second isoform of this sequence been present in flounder serum. Both the serum sample, which was pooled from several fish, and the genomic library, which came from an individual flounder collected over a decade bv earlier, were from Newfoundland waters, whereas the cDNA clone was derived from Long Island fish. It is quite possible, therefore, that allelic variants of this AFP sequence exist within or between the two populations sampled.

The larger size of AFP9 (4.3 kDa) compared with HPLC-6 and -8 (3.3 kDa) is consistent with its elution before the three-repeat AFPs during Sephadex G-75 size exclusion chromatography, and with its increased retention time during C18 reversed-phase chromatography. It was noted previously that the adsorption of recombinant Type I AFPs of various lengths to C18 and C4 HPLC columns increased dramatically with each additional 11-amino acid repeat (Warren et al., 1993). The retarded elution position of AFP9 from the C18 column matches that of the HPLC-9 component described by Fourney et al.

(1984). Despite some quantitative differences in their amino acid compositions, which could be attributed to the difficulty of quantifying amino acid ratios that vary over 10- to 30-fold, the two proteins are probably identical.

One of the significant findings of this study, which was revealed by the protracted size-exclusion chromatography, was that AFP9 is moderately abundant. This, together with its higher specific activity as an antifreeze, suggests that AFP9 makes a significant contribution to the overall freezing point depression of flounder serum. Only one AFP9 gene (21a) has been found to date and there is no evidence of tandem repetition as has been observed for the three-repeat AFP genes in winter flounder. However, the AFP9 gene does contain a good match to the liver-specific enhancer located in the intron of the three-repeat AFP genes, which is critical for high level expression (C. Hew, pers. comm.).

AFP9 appears to be as completely α-helical as HPLC-6 and -8 at 0 °C despite the additional 11-amino acid repeat, a longer C-terminus, and the absence of intrachain salt bridges. These structural features might explain the lower T_m value for AFP9 compared with the three-repeat winter flounder AFPs. AFP9 has the potential to form internally hydrogen bonded N- and C-cap structures (Presta & Rose, 1988), identical to those seen in the X-ray structure of HPLC-6, which are thought to be major determinants of the overall helical structure (Sicheri & Yang, 1995). In this regard, it is interesting to note that both of the AFP9 tryptic peptides were completely lacking in antifreeze activity, even though the larger one retained six putative ice-binding residues, the N-cap structure, and had a C-terminal basic residue (Lys 30) to help stabilize the helix dipole (Bodkin & Goodfellow, 1995). CD analysis revealed that this peptide had lost its helicity and therefore the critical spacing of the ice-binding residues. Presumably, the C-terminal amide plays a crucial role in the internally hydrogen bonded C-cap structure.

There are a number of possible reasons why AFP9 is more active than the three-repeat AFPs. One is that AFP9 has an increased affinity for ice stemming from the additional two potential ice-binding residues, bringing the total to nine. Sicheri and Yang (1995) have emphasized the frequent pairing in Type I AFPs of ice-binding residues with i, $i + 3$ spacing, which are usually Thr-Asn or Thr-Asp (Fig. 6), to the extent that they have labeled a single such residue as an incomplete ice-binding motif. The Thr-Thr pairs in AFP9 (Fig. 6) constitute an arrangement not seen in any of the other Type I AFPs (Sicheri & Yang, 1995), especially because they lack a supporting residue with, $i + 4$ spacing, which Sicheri and Yang identify as part of the "ice-binding motif." Because there are two of these pairs in AFP9, accounting for four of the nine ice-binding residues, it is likely that they are both active in binding the protein to ice because of the overall greater activity of AFP9 compared with the three-repeat AFPs. If both threonines in a pair are bound to the ice, it is unlikely that the threonines in the $i + 3$ position could hydrogen bond to the same three oxygen atoms of the ice lattice as do the longer $i + 3$ Asx residues in the model of Sicheri and Yang. It is quite possible that the threonine pairs make an alternative, better fit to the ice lattice than the Thr-Asx pairs.

A partial explanation for the superior antifreeze activity of AFP9 lies in its larger size. It has been reported that the longer antifreeze glycoprotein molecules (AFGP 1-5) are more active than the shorter ones (AFGP 6-8) (Mulvihill et al., 1980), even though the very smallest AFGP has potentially eight hydroxyl

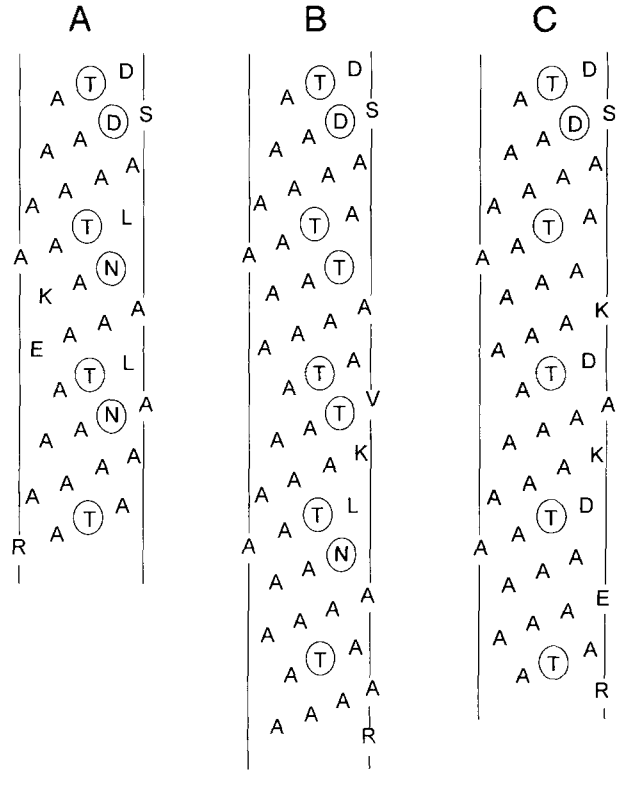

Fig. 6. Helical net representations of Type I AFPs. **A:** HPLC-6 from winter flounder. **B:** AFP9 from winter flounder. **C:** Yellowtail flounder AFP. Putative ice-binding residues are circled.

groups with which to bind to ice (Knight et al., 1993). The fact that HPLC-6 and the four-repeat yellowtail flounder AFP have comparable activities on a molar basis, despite the latter having fewer ice-binding residues, argues for some effect of size. Thus, a comparison of the two four-repeat AFPs emphasizes the importance of the $i + 3$ ice-binding residue in optimizing antifreeze activity, and suggests that Thr-X-X-Thr might be a particularly effective arrangement of ice-binding residues.

Although the evolutionary history of Type I AFPs is far from clear, it appears to have involved recent and extensive gene amplification that has seen three-repeat AFPs predominate in winter flounder at the expense of four-repeat AFPs (Scott et al., 1985) and vice versa in the closely related yellowtail flounder (Scott et al., 1987). With this background, it is quite possible that gene amplification met the need for more antifreeze activity and overrode the slow evolution of the optimal Type I AFP, elements of which might exist in AFP9.

Materials and methods

AFP purification

Pooled serum (25 mL) from winter flounder collected in the winter months was diluted with an equal volume of 0.1 M NH_4HCO_3, clarified by centrifugation at 10,000 × g for 10 min, and chromatographed through a Sephadex G-75 column (50 × 1,000 mm) eluted with 0.1 M NH_4HCO_3 at a flow rate of 1.5 mL/min. Fractions (10 mL) were monitored for absorbance at

230 nm and thermal hysteresis. Selected fractions were pooled and lyophilized twice to remove NH_4HCO_3. The freeze-dried material was dissolved in 0.05% TFA and applied to a C18 reversed-phase semi-preparative HPLC column (Vydac 10 × 250 mm). The column was equilibrated in 50% acetonitrile containing 0.05% TFA. AFP was eluted using a biphasic linear gradient of acetonitrile in 0.05% TFA increasing from 50% to 70% in 10 min (2% acetonitrile/min) and to 80% over a further 20 min (0.5% acetonitrile/min) at a flow rate of 3 mL/min. An aliquot (1/15th) of the pooled fractions from the semi-preparative chromatography was chromatographed on a C18 analytical HPLC column using the same solvent system and elution gradient.

Trypsin digestion

AFP9 (300 μg) in a final volume of 300 μL of 0.1 M NH_4HCO_3 containing 0.1 mM $CaCl_2$ was digested with trypsin (12 μg) for 1.5 h at 37 °C. The digest was supplemented with an additional aliquot (12 μg) of trypsin after 1 h, and was sampled for thermal hysteresis activity at 30-min intervals.

CD spectroscopy

CD spectra were recorded on a Jasco J-500C spectropolarimeter (Easton, Maryland) linked to an IBM PS/2 computer running the Jasco DP-500/PS/2 system software (version 1.33a) through the Jasco IF 500 II interface. The temperature of the cuvette holder was controlled by a Lauda model RS water bath using a 50/50 water/polyethylene glycol mixture as the coolant. The spectropolarimeter was calibrated with an aqueous solution of recrystallized *d*-10-(+)-camphorsulfonic acid at 290.5 nm. Results were expressed as mean residue molar elipticity [Θ] ($deg \cdot cm^2 \cdot dmol^{-1}$) calculated from the equation:

$$[\Theta] = ([\Theta]_{obs} \times MRW)/(10 \times l \times c),$$

where $[\Theta]_{obs}$ is the observed elipticity expressed in millidegrees, MRW is the mean residue molecular weight (molecular weight of the peptide divided by the number of amino acids), l is the optical pathlength in cm (0.05 cm), and c is the final peptide concentration in mg/mL. For wave *i* scan (0 °C), CD spectra were the average of four scans obtained by collecting data at 0.1-nm intervals from 250 to 190 nm. For the temperature denaturation run (0–60 °C), changes in the helical content of the sample were monitored at 222 nm. At each temperature, the reported [Θ] was the average of 32 samplings. The peptide concentration was 1.0 mg/mL for the wave-scan and 0.61 mg/mL for the temperature denaturation study. Buffer was 10 mM Na phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4, (PBS) for both studies.

Mass spectrometry, amino acid analysis, and peptide sequencing

The mass of the samples was measured by electrospray mass spectrometry using a Fison VG Quattro mass spectrometer (VG Biotech, Cheshire, England). For amino acid analysis, peptides were hydrolyzed in 6 N HCl at 160 °C for 1.5 h in sealed evacuated tubes. Norleucine was added to the samples as an internal standard. Hydrolyzed material was analyzed in a Beckman Model 6300 amino acid analyzer (San Ramon, California). N-terminal peptide sequencing was performed on a Hewlettt Packard G1005A protein sequencer (Palo Alto, California) using the Routine 3.0 chemistry.

Thermal hysteresis measurements

Thermal hysteresis, defined as the temperature difference (°C) between the freezing and melting points of an AFP solution, was measured using a nanoliter osmometer (Clifton Technical Physics, Hartford, New York) by the method of Chakrabartty and Hew (1991). All measurements were made in 0.1 M NH_4HCO_3, pH 7.9.

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