Type I Shorthorn Sculpin Antifreeze Protein
RECOMBINANT SYNTHESIS, SOLUTION CONFORMATION, AND ICE GROWTH INHIBITION STUDIES*

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A number of structurally diverse classes of “antifreeze” proteins that allow fish to survive in sub-zero ice-laden waters have been isolated from the blood plasma of cold water teleosts. However, despite receiving a great deal of attention, the one or more mechanisms through which these proteins act are not fully understood. In this report we have synthesized a type I antifreeze polypeptide (AFP) from the shorthorn sculpin Myoxocephalus scorpius using recombinant methods. Construction of a synthetic gene with optimized codon usage and expression as a glutathione S-transferase fusion protein followed by purification yielded milligram amounts of polypeptide with two extra residues appended to the N-terminus. Circular dichroism and NMR experiments, including residual dipolar coupling measurements on a 15N-labeled recombinant polypeptide, show that the polypeptides are α-helical with the first four residues being more flexible than the remainder of the sequence. Both the recombinant and synthetic polypeptides modify ice growth, forming faceted crystals just below the freezing point, but display negligible thermal hysteresis. Acetylation of Lys-10, Lys-20, and Lys-21 as well as the N-terminus of the recombinant polypeptide gave a derivative that displays both thermal hysteresis (0.4 °C at 15 mg/ml) and ice crystal faceting. These results confirm that the N-terminus of wild-type polypeptide is functionally important and support our previously proposed mechanism for all type I proteins, in which the hydrophobic face is oriented toward the ice at the ice/water interface.

The type I AFPs† found in the blood of cold water teleosts (1–3) have attracted significant interest, due to the potential applications of such compounds in biotechnology and medicine (4–9). These compounds are alanine-rich α-helical proteins of 33–47 residues in length (for reviews see Refs. 10–13). Although 14 type I proteins have been identified in nature (summarized in Ref. 12), almost all studies to date have centered on HPLC6, the 37-residue protein from the winter flounder (see Table I below) (14). This protein is characterized as an AFP, because it modifies both the rate and shape of ice crystal growth and displays thermal hysteresis, i.e. a positive difference between the ice growth temperature and the equilibrium melting temperature of ice.

During the last decade, significant progress has been made in elucidating the structural features of HPLC6 that are required to give antifreeze activity. Structure-activity studies have identified the importance of the Thr residues at positions 2, 13, 24, and 35 plus surrounding residues, for ice growth inhibition activity. Although the Thr residues were assumed to be involved in hydrogen-bonding interactions with ice for many years (15–19), more recent mutations have identified the hydrophobicity provided by the γ-methyl group of Thr as a key factor related to the ability to inhibit ice growth (20–24). Hydrogen-bonding and other roles for the surrounding residues have also been considered (24–29). However, a model that explains the selective interaction of HPLC6 with the [2 0 2 1] interface has not emerged (for a full description of the different ice interfaces see the previous review (12)). Recent computational studies on the nature of the ice/water interface have allowed the first real simulations of the interaction of HPLC6 with the fluid interface to be carried out (30). These studies support experimental data on mutants (20–24) that have shown that hydrogen bonding involving the hydroxyl groups of the fourThr residues is not the primary reason for the interaction of HPLC with the ice/water interfacial region.

In contrast to HPLC6, type I AFPs from the grubby sculpin (Myoxocephalus arenaeus) (31), the shortnose sculpin (Myoxocephalus scorpius) (3, 32), and the Arctic sculpin (Myoxocephalus scorpioides) (33) have been much less studied. These proteins differ from both HPLC6 and sequences from the right-eye flounders in the N-terminal region of the sequence. The N-terminal residues of the sculpin proteins (e.g. SS3, SS8 in Table I) include established helix-breaking residues followed by an 11-residue repeat unit that is similar to the Thr-X2-Asx-X7 repeat present in the other type I proteins (12).

Two proteins, XSS3 and XSS8 (Table I), have been isolated from the blood serum of the shortnose sculpin (3). It has been estimated using CD spectroscopy that XSS8, the major component, is more helical than XSS3, and it has been proposed that there are two different structural and possibly functional do-

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The abbreviations used are: AFP, antifreeze polypeptide; SS, shortnose sculpin; HPLC, high performance liquid chromatography; CD, circular dichroism; GSH, glutathione; GST, glutathione S-transferase; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; DQF-COSY, double-quantum filtered correlation spectroscopy; HSQC, homonuclear single quantum coherence.

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mains, the nine-residue N terminus and the alanine-rich helical region between residues 8 and 42 (3). Vacuum phase molecular dynamics studies on SS8, a closely related sequence to XSS8, concluded that the polypeptide conformation resembles an idealized α-helix, except for a short section of the N-terminal region (34). This study also proposed that SS8 acts via insertion of the side chains of the Arg and Lys residues into the ice lattice. However, no experimental or condensed-phase computational evidence exists to support these conclusions. Of particular significance is the fact that the sculpin proteins accumulate specifically at a diﬀerent ice plane than the winter flounder protein HPLC6 (15). The origin of this diﬀerence in specificity for ice growth inhibition by the type I AFPs in the present study was explored through a microscale overexpression study in a New Brunswick Scientiﬁc BioFlow III Fermentor using the protocol of Cai et al. (37). Three-liter cultures were grown on a minimal medium, in which the only source of nitrogen was ammonia chloride. For the production of 15N-labeled SS8, 15NH4Cl (0.75 g) was added to the medium when the dissolved oxygen content of the medium increased sharply (indicating exhaustion of the ammonium chloride). Following a second increase in dissolved oxygen content, 3.75 g of 15NH4Cl was added, overexpression was induced, and the culture was allowed to grow for a further 5 h before harvesting as described above.

**HPLC Purification**—All recombinant samples were puriﬁed by reverse-phase HPLC using an Alltech Altima 5-μm C18 column. A linear AB gradient was used (30–45% buffer B over 15 min for rSS8 and 15–60% buffer B over 30 min for 4Ac-rSS8) at a ﬂow rate of 1 ml/min was used, and the eluate was monitored at 215 and 280 nm. The major peaks were collected, lyophilized, and stored at −20 °C. The identity of rSS3 and 15N-rSS8 was conﬁrmed by electrospray mass spectrometry (rSS3: M,calc = 3084 Da; M,exp = 3083 ± 1 Da; 15N-rSS3: M,calc = 3125 Da; M,exp = 3124 ± 2 Da).

**Acetylation**—rSS3–rSS8 (4.0 mg) was dissolved in water (50 μl) and sodium acetate solution (saturated, 50 μl) added. The mixture was stirred at 0 °C, and acetic anhydride (10 μl, 9.4 μmol) was added in five equal amounts over 2 h. The mixture was allowed to react for a further 2 h at 0 °C, and then subjected to reverse-phase HPLC; products were identiﬁed by electrospray mass spectrometry. Samples with three or fewer acetyl groups were subjected to the reaction conditions. Fractions containing 4Ac-rSS3 were combined (M,calc = 3125 Da; M,exp = 3150 ± 5 Da).

**Circular Dichroism Studies**—CD measurements were made using a Jasco J-710 spectropolarimeter equipped with a 0.1-cm water-jacketed cell connected to a NIESLAB RTE-111 water bath. Peptide samples were between 0.1 and 0.5 mg ml−1 in water or 100 mM NH4HCO3 buffered solutions, at pH 8.5. The sample pH was adjusted using 0.1 M NaOH or 0.1 M HCl solutions as required. Variable temperature measurements were made at regular intervals between 2 °C and 50 °C. Sample concentrations for CD and thermal hysteresis measurements were determined by amino acid analysis, which was carried out by laboratory personnel.

**Thermal Hysteresis**—Ice crystals in a Clifton nanoliter osmometer were observed through a microscope and photographed with a digital camera, and time evolution was recorded by a video camera linked to a video recorder (see Ref. 21). Still images were also obtained from the videotape record at regular intervals over a period of ~1 min. The absolute length scale in the video images was determined from previous measurements of the absolute diameter of the holes in the (unchanged) aluminum sample holder (21).

**NMR Spectroscopy**—NMR samples of rSS3, 15N-rSS3, and 4Ac-rSS3 were prepared by dissolving lyophilized peptide in 550 μl of 90% H2O/10% D2O to ﬁnal concentrations of 1 mM. The pH was adjusted to 5.0 using NaOH. The following homonuclear two-dimensional experiments were performed: TOCSY (τc = 35, 70 ms; (39)), DQF-COSY (40), and NOESY (τc = 70, 100, and 250 ms (41)). Three-dimensional HNHA (42) and NOESY-HSQC (43) experiments were used to assign the [15N,1H]HSQC spectrum of rSS3, and the HNHA was used to derive 1H,H coupling constants. Spectral widths were typically 12 ppm for 1H and 30 ppm for 15N. 2,2-Dimethylsilapentane-5-sulfonic acid (20 μM) was added as an internal reference. Spectra were recorded on Bruker DRX600 spectrometers equipped with an HCN triple-resonance probe head. Water suppression was achieved by the use of pulsed ﬁeld gradient and the indirect dimension data were processed using the States-time proportional phase incrementation method (43). Spectra were processed as described previously (44) and were analyzed using the program XEASY (45).

Samples in aligned media for the measurement of residual dipolar couplings were prepared using the method of Ruckert and Otting (46). Briefly, polyoxymethylene 5-octyl ether was dissolved in 90% H2O/10% D2O with a sucrose solution of the same concentration. The mixture was rapidly sealed in a thin-walled high-pressure vessel (21).

**EXPERIMENTAL PROCEDURES**

**Materials**—SS3 prepared by solid-phase peptide synthesis (sSS3) was supplied by Auspep Pty. Ltd. (Melbourne) at >90% purity. Cloning and Overexpression of rSS3—A synthetic gene encoding SS3 was constructed from the following three overlapping primers: 1) AFP1 (GG GGA TTC ATG AAC GCT CCG GCA GCT GCA GCT GAA ACT GCA GCT GAC GCA CTG GCT G), AFP2 (GCA GGA GCA GCA GCT GCC GCT GCC GTT TTT TTT GCC GCA GGT GCA GCG TCA GCC GCC GCC GCT G), and AFP3 (CCG CAG CTC CGG CAG CGG CTG CTC CGG CAG CGG CTG CAG AAT GGT CAG G) using overlap extension (36). The three primers were annealed, and PCR was used to ﬁll the gaps. Two end primers, AFPPRIM1 (GG GGA TCC ATG AAC GCT C) and AFPPRIM2 (CCG AAT TCT CAC TAT TAA GC), were then used to amplify the gene by PCR. The amplified product was inserted into pGEX-2T using the BamHI and EcoRI restriction sites and used to transform *Escherichia coli* (DH5α). Transformant colonies selected for growth on ampicillin were screened for the insert DNA by PCR. Plasmid DNA was puriﬁed from positive clones and sequenced. Plasmid DNA from a single clone identiﬁed as containing the rSS3 gene was used to transform *E. coli* (BL21(DE3)) for protein production.

Overnight cultures from freshly transformed *E. coli* BL21(DE3) cells were used to inoculate Luria broth containing 150 μg ml−1 ampicillin. The cells were grown at 37 °C with shaking (200 rpm), and expression was induced using isopropyl-1-thio-β-D-galactopyranoside (0.4 mM) when the absorbance at 600 nm (A600) was ~0.6. The cells were allowed to grow for a further 4–6 h at 25 °C and were then harvested by centrifugation (15 min, 5000 × g, 4 °C). The cell pellets were resuspended in lysis buffer (20 mM Tris, pH 8.0; 150 mM NaCl; 0.5 mM phenylmethylsulfonyl ﬂuoride; 1.1% (w/v) β-mercaptoethanol) and lysed by either sonication or ﬁve passages through a Ronnie Mini-lab 8.30H homogenizer at 500 kPa.

The lysate was centrifuged (20 min, 15,000 × g, 4 °C), the insoluble fraction was discarded, and the soluble fraction was passed through a column containing 15 ml of glutathione-Sepharose 4B (Amersham Biosciences, Inc.) equilibrated with lysis buffer. The column was washed with 50 ml of wash buffer (50 mM Tris, pH 8.0; 100 mM NaCl; 10% glycerol; 1.4 mM phenylmethylsulfonyl ﬂuoride; 1.4 mM β-mercaptoethanol) and re-equilibrated with 120 ml of thrombin buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 2.5 mM CaCl2). After incubation with thrombin (100 units) for 1 h at 37 °C, the cleaved rSS3 was eluted with thrombin buffer in four 25-ml fractions, snap frozen, and stored at −20 °C. Further scale overexpression was developed and resequenced) propose a mechanism that involves appreciable hydrophobic interactions, with the lysine residues enhancing solubility (35).

The lack of understanding of the one or more mechanisms through which the type I AFPs inhibit ice growth has also hampered the realization of industrial and biotechnological applications (6, 7). Furthermore, the production of these proteins using solid-phase synthesis (which has been used to produce almost all the analogues of HPLC6 reported to date) is unsuitable for the large scale production of structural variants of the wild-type proteins. In this paper, we address both these issues by producing a type I polypeptide from the shorthorn sculpin (rSS3) using recombinant methods. This has allowed complete 1H and 15N resonance assignments and characterization of the solution conformation of rSS3 using heteronuclear NMR experiments. Ice growth inhibition studies on rSS3 and an acetylated derivative have provided important new clues regarding the mechanism of action of these proteins and have allowed a new contribution to the mechanism to be proposed to explain the unique interaction of the sculpin AFPs with ice.
D$_2$O to a final concentration of 5% (w/w), the pH was adjusted to 5.0, and 1-octanol was added in micro liter steps, with vigorous shaking, to a final molar ratio of 0.87 (polyoxyethylene 5-octyl ether:1-octanol). Formation of the $L_a$ phase was detected by the solution becoming instantaneously transparent and opalescent. 15N-Labeled rSS3 was added from a stock solution (1.2 mM, pH 5.0) to a final concentration of 200 µM. All spectra were recorded at 278 K. Measurements of $^1$H$_{HN}$ were made by comparing $^{15}$N-HSQC spectra, recorded without $^{15}$N-decoupling in F2, in aligned and unaligned media.

**RESULTS**

**Overexpression and Purification**—Because type I AFPs are relatively small proteins (3000–5000 Da) that lack a globular shape, they are potentially susceptible to degradation when expressed in heterologous systems such as bacteria. For this reason rSS3 was expressed as a fusion with the 26-kDa C-terminal domain of GST from *Schistosoma japonicum*. This fusion protein was expected to show greater stability in bacteria than rSS3 on its own, and indeed no degradation was observed when expressed in *E. coli*. The fusion protein was present in approximately equal amounts in the insoluble and soluble components at 37 °C, and reduction of the temperature at which the bacterial cells were induced from 37 °C to 25 °C resulted in ~50% soluble fusion protein (data not shown). Cleavage of the bound protein between the GST and SS3 domains using the site-specific protease thrombin resulted in the production of the sculpin sequence with two additional residues (Gly-Ser) appended to the N terminus (rSS3). Fig. 1 shows the affinity purification of rSS3; the presence of a single band in lane 4 indicates that no appreciable breakdown of the SS3 portion of the fusion protein was observed. The yield from 4 liters of bacterial culture was 7.5 mg of rSS3, at >90% purity.

**The Solution Conformation of SS3**—To characterize the solution conformation of SS3 and to ascertain whether the addition of two non-native residues onto the N terminus had a significant effect on the solution properties of the protein, we used a combination of circular dichroism and NMR methods. CD spectra of rSS3 and ss3 recorded over a range of temperatures (Fig. 2) indicated that both the synthetic and recombinant versions of SS3 are $\alpha$-helical and that the helicity increased substantially as the temperature was lowered. At 2 °C, rSS3 was estimated to be 40% $\alpha$-helical. This value is comparable to the previously reported literature estimate of 45% for XSS3 (3).

The 600-MHz $^1$H NMR spectrum of rSS3 was assigned using a combination of three-dimensional HNHA and NOESY-HSQC experiments, together with DQF-COSY, TOCSY, and NOESY spectra, all recorded at 5 °C. Despite the high degree of signal overlap resulting from a combination of the 25 alanine residues and the helical conformation, assignments were made for every resonance in the spectrum (Fig. 3). This result contrasts with the difficulties experienced in previous NMR studies of the naturally occurring type I AFP, HPLC8 (47, 48). In the current study, the assignment process was assisted by the availability of uniformly $^{15}$N-labeled polypeptide $^{15}$N-rSS3, emphasizing the utility of protein production in a recombinant system.

Analysis of the NOESY spectra, in particular the amide-amide region (Fig. 4), revealed many of the characteristic connectivities expected for an $\alpha$-helix. A summary of medium range NOEs, $H^\alpha$ chemical shift data, and $^1J_{HN}$ scalar coupling values is presented in Fig. 5. For $H^\alpha$ chemical shifts, a series of negative values for $\delta(H^\alpha)_{\text{rSS3}} - \delta(H^\alpha)_{\text{coil}}$ are indicative of helical structure. The values were negative for all residues apart from Gly-(1)-Met-1, Ala-3, and Leu-15, consistent with most of the polypeptide adopting an $\alpha$-helical conformation. Similarly, values of $J_{HN}$, which are typically <−6 Hz for $\alpha$-helices (49), and NOE patterns support a predominantly $\alpha$-helical conformation, although a number of the expected medium range NOEs could not be unambiguously identified because of resonance overlap.

An issue that often arises when using NMR methods to characterize the solution conformation of rod-shaped proteins is that of whether the overall shape is straight or bent. Most NMR-derived structural constraints are short-range in nature (e.g. NOEs scalar coupling constants), and are therefore poorly suited to defining the relative conformations of the two ends of a rod. However, residual dipolar couplings, measured in weakly aligning media such as dilute solutions of liquid crystals, have recently been shown to be well suited to this task (50–52), and the availability of a recombinant form of SS3 allows these parameters to be measured. We measured residual $^1$H–$^{15}$N dipolar couplings ($^1D_{HN}$) for the backbone amide protons of $^{15}$N-rSS3, and the results are shown at the bottom of Fig. 5. The magnitude of these couplings is an indication of the angle formed between the N–H bond vector and the bulk magnetic field and can, therefore, be used to infer the angular relationship between two N–H bond vectors within a single molecule. The observation that the values of $^1D_{HN}$ are rather uniform across most of the polypeptide is consistent with a conformation in which all N–H bond vectors are oriented in approximately the same direction, as would be expected for an $\alpha$-helix with no significant kink. Notably, the values of $^1D_{HN}$ tail off toward zero toward the N terminus, consistent with the helical
structure in this region being more flexible than the rest of the polypeptide. Further refinement of this data to obtain a full structure would require additional constraints from measurements on doubly labeled (13C,15N) polypeptide.

**Thermal Hysteresis of sSS3 and rSS3**

The thermal hysteresis values for sSS3 and rSS3 were measured by nanoliter osmometry in unbuffered aqueous solutions on 4 mM, 2 mM, and 1 mM polypeptide samples at pH 5; concentrations of each stock solution were determined by amino acid analysis. Fig. 6a shows a single crystal of ice grown from a solution of purified rSS3 at 16 mg/ml. The hexagon bipyramidal crystals are characteristic of kinetic ice growth inhibitors of this type. The behavior of sSS3 (>90% pure) was similar but yielded truncated bipyramidal crystals or “barrels,” again a signature of ice modification. Similar barrels were obtained with an independent sample of rSS3 of lower purity than the sample used to obtain the crystals shown in Fig. 6a. Both sSS3 and rSS3 exhibited negligible hysteresis values of between zero and 0.02 °C at concentrations up to 18 mg/ml (data not shown). The ability to facet growing ice crystals, but not inhibit their growth at all, as shown by
both sSS3 and rSS3, is typical of polypeptides that interact weakly with the broad ice/water interfacial region. The wild-type protein isolated from blood serum (XSS3) has been reported to exhibit measurable thermal hysteresis (3). Although no raw data were presented, XSS3 was reported to give 0.39 degrees hysteresis (3) but also substantial thermal hysteresis (Fig. 8), suggesting that either blockage of the N terminus was retained with rSS3 and sSS3.

**Acetylation of rSS3**—To determine whether a post-translational modification of SS3 may affect its ability to act as an AFP and to test the hypothesis that the Lys/Arg residues are required for activity (34), we chose to acetylate the free amino groups on the polypeptide. Reaction of rSS3 with acetic anhydride under standard conditions, followed by reverse-phase HPLC, yielded 4Ac-rSS3 in which all three lysine residues and the N terminus were derivatized with acetyl groups. The identity of this polypeptide was confirmed by mass spectrometry and NMR spectroscopy. Complete assignments of the $^1$H resonances using two-dimensional NMR methods revealed that there were no significant conformational changes in 4Ac-rSS3; all $^1$H resonances were located less than 0.05 ppm from their position in the unacetylated peptide.

Remarkably, 4Ac-rSS3 exhibits not only faceting of growing ice crystals (Figs. 6 and 7) but also substantial thermal hysteresis (Fig. 8), suggesting that either blockage of the N terminus and/or acetylation of the three lysine residues (Lys-10, Lys-20, Lys-21) is important for activity. The time evolution of an ice crystal is shown for the highest concentration studied in Fig. 7.

**DISCUSSION**

Compared with the type I winter flounder proteins (in particular HPLC6), much less is known about the type I sculpin proteins. There are no x-ray or NMR structures of AFPs from any of the sculpin proteins, and, with the exception of a very recent report on SS8 (35), systematic structure-activity studies to determine the residues that are crucial for activity have not been reported. The sculpin proteins are important, given that SS8 was proposed to interact with the ice surface based on the spacing of Lys-9, Lys-23, Lys-31, and Arg-12 (34); i.e. a completely different mechanism to that proposed for HPLC6 and other synthetic flounder AFPs, including VVVV2KE, in which hydrophobic interactions play a dominant role in the mechanism (21, 23, 24, 29, 30). We have previously noted that a common hydrophobic face is present in all type I proteins, including the sculpins (12), and hence proposed that the type I AFPs act via a common mechanism. Very recently this hypothesis has been confirmed by point mutations made on SS8, which have identified the alanine-rich surface as oriented toward ice, and the charged residues oriented toward liquid water (35).

Despite the presence of a common hydrophobic face in all type I AFPs (12), the sculpins accumulate at a different ice plane than the flounder proteins; the winter flounder sequence HPLC6 (and the synthetic mutant VVVV2KE) accumulates on the 12 equivalent $\{2 \ 0 \ 2 \}$ bipyramidal planes of the ice 1h crystal (15) whereas the shorthorn sculpin (and the synthetic mutant AAAA2KE) accumulates on the six equivalent $\{2 \ 1 \ 0 \}$ planes (34) for a detailed description of the origin and naming of the different ice planes see for example Ref. 12. The origin of this difference is not understood, but an obvious structural difference between the natural flounder and sculpin sequences is the N-terminal region of the polypeptides (Table I).

We chose SS3, as one of the simplest sculpin proteins, to prepare and investigate the solution conformation, particularly of the N-terminal region, and to allow preparation of a simple acetylated derivative that would establish the residues that are required for antifreeze activity. A highly effective expression system for production of the protein as a GST fusion was developed, which yielded milligram quantities of purified rSS3 from a few liters of bacterial culture. After affinity chromatography and reverse-phase HPLC, rSS3 was >99% pure, a level of purity much greater than sSS3, or other related proteins that our laboratory has produced using solid-phase peptide synthesis (21, 23, 53). Thus, in our hands, the expression of rSS3 is superior to solid-phase production of sSS3 in terms of the time required for the synthesis, overall cost, and the purity of the isolated protein. The recombinant system also offers the additional advantages of simple and inexpensive production of mutants (which may be used for mechanistic or biotechnological studies) and the ability to produce isotopically labeled forms of the protein (e.g. $^{15}$N-rSS3 or $^{15}$N,$^{13}$C-rSS3) for detailed structural analysis using NMR spectroscopy.

Although CD measurements are consistent with ~42% helicity at 2 °C, NMR studies (Figs. 3 and 4) provide clear evidence that rSS3 is highly structured and predominantly $\alpha$-helical in conformation at low temperature. Examination of the NMR parameters presented in Fig. 5 suggests that residues 5–33 of rSS3 adopt a stable $\alpha$-helical conformation, whereas residues 1–4 are partially helical. This corresponds to an overall helicity of ~80–90% (taking into account the non-native
It is interesting that there is such a large apparent difference between the estimates of helicity based on CD and NMR data. This is most likely related to the method used to estimate helicity from CD data (38), which is not well suited to proteins containing non-helical secondary structure. Alternatively, there may be some flexibility in the helix (especially near the termini) that reduces the CD value and is not accurately reflected in the NMR parameters we have measured. This issue will be resolved when a full structure determination is carried out using more NMR data.

For comparison, vacuum modeling has predicted that SS8 adopts a fully α-helical conformation (34), whereas more recent molecular dynamics calculations on SS8 have predicted that the N-terminal cap structure folds the N terminus up and away from the Ala-rich surface of the protein (35). Although direct comparisons between the conformation of residues 1–10 of SS3 and SS8 cannot be made, our results suggest that the first 10 residues of SS3 are aligned approximately parallel to the α-helix formed by residues 11–33, with no significant deviation similar to that predicted for SS8 (35). Further structural constraints derived from 15N, 13C-labeled protein (including 13C-X residual dipolar couplings) are required to fully refine the structure.

Although both sSS3 and rSS3 cause faceting of the ice crystals to occur, zero or very low thermal hysteresis values were measured. Thus, both polypeptides behave as ice growth modifiers (12) rather than as true kinetic ice growth inhibitors or AFPs. The measured hysteresis value is significantly lower than that reported from SS3 isolated from sculpin blood serum (3). However, both of the naturally occurring sculpin proteins XSS3 and XSS8 isolated and characterized by Hew et al. (3)
contained an uncharacterized blocked N terminus. In the case of SS8, cleavage of this blocking group reduced the thermal hysteresis (Table I). Thus, the difference between the measured hysteresis values for sculpin XSS3 (0.37 °C) and the synthetic sSS3/rSS3 polypeptides (0.0 °C) is consistent with the requirement of an N-terminal blocking group for activity.

The thermal hysteresis and characteristic ice faceting of 4Ac-rSS3 (Figs. 6–8) show that not only is this derivative a true kinetic ice growth inhibitor, but also that this derivative is more active than rSS3, which exhibited negligible hysteresis. These results conclusively rule out an inserted charge mechanism, similar to that proposed for SS8 involving the protonated lysine amino groups and the arginine side chain (34) and support our earlier hypothesis that the hydrophobic face is important in the mechanism of ice growth in both the winter flounder and sculpin proteins (12). Our hypothesis is also supported by the very recent work by Baardnes et al. (35) on SS8 in which point mutations of the hydrophobic face were seen to switch off antifreeze activity. This study also identified the blocking group as an acetyl group and, although SS3 was not studied, it seems plausible that the blocking group X is the same in both SS3 and SS8.

Fig. 9 shows a helical wheel representation of SS3. Lysine residues that are acetylated in 4Ac-rSS3 are shown in bold on the hydrophilic face which is opposite the hydrophobic face. Similar hydrophobic faces in type I AFPs have previously been noted in reference (12).

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N-terminal group in activity, originally proposed by Hew et al. (3). The N-terminal blocking group may affect several properties of the polypeptide, including overall charge, dipole moment, N-terminal capping network, and solution conformation, all of which need to be considered in further refinement of the mechanism for ice growth inhibition.

In addition, our recent studies have shown that there is significant charge inhomogeneity at the ice/water interface (54, 55). The full charge distribution of SS3, or any polypeptide, interacts with the charge inhomogeneity that occurs naturally at any broad interface such as the ice/water interface. This full charge distribution may be summarized crudely by the dipole moment of the polypeptide. Thus we have proposed that, next in importance to hydrophobicity, the overall charge distribution of the antifreeze activity. Beyond supporting the hydrophobicity of the interaction of the overall charge distribution of the polypeptide with the ice/water interface (55), the complete details of the role of the interaction of the overall charge distribution of the polypeptide, including overall charge, dipole moment, N-terminal capping network, and solution conformation, all of which need to be considered in further refinement of the mechanism for ice growth inhibition.

In summary, this study reports the first recombinant production, solution conformation, and preparation and ice growth inhibition properties of an acetylated derivative of the 33-residue ACP from the shorthorn sculpin. Together with recent independent studies on the 42-residue shorthorn sculpin ACP, SS8 (35), these data confirm our hypothesis (12) that all type I ACPs contain a common hydrophobic face that is required for antifreeze activity. Beyond supporting the hydrophobicity mechanism, the data are consistent both with a proposed role for the N-terminal blocking group (3) and with the proposed role of the interaction of the overall charge distribution of the polypeptide with the ice/water interface (55). The complete details of the accumulation of SS3 at the specific (2 1 0) ice plane require further detailed molecular simulations and experimental studies.

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