Antifreeze Proteins from the Sea Raven, *Hemitripterus americanus*

**FURTHER EVIDENCE FOR DIVERSITY AMONG FISH POLYPEPTIDE ANTIFREEZES**

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The antifreeze proteins of the sea raven, *Hemitripterus americanus*, were isolated and compared with other fish antifreeze proteins. The sea raven contains one major protein of molecular weight 14,000–16,000 with little or no carbohydrate. Except for its similar seasonal appearance, the sea raven antifreeze protein differs from other polypeptide antifreeze in its amino acid composition, secondary structure, and immunological specificity. Amino acid analysis of sea raven antifreeze showed that it contains a high amount of half-cystine, hydrophilic amino acids, and only an average amount of alanine. In contrast, all other fish antifreeze proteins contain approximately 60% alanine and no half-cystine residues. Furthermore, the sea raven antifreeze protein is sensitive to sulfhydryl reagents. The antifreeze activity was decreased by 67% in the presence of 0.01 M dithiothreitol. Circular dichroism studies indicated the absence of significant amounts of α-helix and the possible presence of β-structure. Antibodies raised against the antifreeze protein did not cross-react with the known polypeptide antifreeze from the winter flounder and short horn sculpin (Hew, C. L., Fletcher, G. L., and Ananthanarayanan, V. S. (1980) Can. J. Biochem. 58, 377–383). A specific radioligandimmunooassay was developed for the sea raven antifreeze protein and was used to quantitate the protein concentration in the fish. The seasonal profile obtained by radioligandimmunooassay was compatible with the antifreeze activity determined with a freezing point osmometer.

The freezing point of the serum of most teleost fishes inhabiting temperate waters is approximately −0.6 to −0.7°C (1). However, in the polar oceans, the sea water freezes at −1.4 to −2°C. Fishes residing in these oceans must exhibit physiological and biochemical adaptations to prevent them from freezing. A number of extensive investigations have now established that many of these polar species of teleost fish produce serum proteins or glycoproteins which lower the freezing point of the serum below that of the surrounding environment (2, 3).

All of these antifreeze proteins share the following characteristics: 1) their effects on freezing temperatures are noncolligative, that is, they lower the freezing point much more than would be expected on the basis of the osmolality of their solutions; 2) they show thermal hysteresis, that is, the apparent freezing temperature is lower than the melting temperature, with the latter being equivalent to that expected from the osmolality of the antifreeze solution; 3) plots of thermal hysteresis versus antifreeze concentration are convex rather than linear, and 4) freezing point depression due to thermal hysteresis is additive with that due to the colligative properties of the antifreeze solutions (2).

Although earlier studies of fish antifreeze proteins indicated a striking homology amounting to near identity among them, discoveries since then have revealed that they are, in fact, biochemically quite diverse, and early hopes of finding a common set of structure-function relationships shared among all of them now seem much less promising. The data in this paper will underscore these points in particular.

The first fish antifreeze proteins to be studied were all glycoprotein in nature and comprised mainly of an Ala-Ala-Thr repeating tripeptide unit with a galactosyl-β-N-acetylgalactosamine disaccharide attached to the threonyl residue. The second alanyl residue was occasionally replaced by a prolyl residue (2, 4). The best studied of the antifreeze glycoproteins are those from the antarctic fish *Trematomus borchgrevinki*. Eight size classes of serum glycoproteins, numbered 1 to 8 from the largest to the smallest, have been isolated, ranging in molecular weight from 2,600 to 33,000. Among these, only glycoproteins 6 to 8 contained any proline. Physical studies on some of these AFGP§ suggested either an extended conformation (5) or a completely flexible random coil (6).

The level of antifreeze proteins of these antarctic fish in the serum does not vary with the season. This is to be expected since the sea water temperatures remain below freezing all year round.

Another type of antifreeze protein has been isolated from the winter flounder, *Pseudopleuronectus americanus*, which inhabits the Northern Atlantic coast of the United States and Canada (7, 8). The winter flounder contains one major AFP of 10,000 daltons. The flounder AFP is not a glycoprotein, 60% of the residues are alanine, and only eight different amino acids are found in the protein. In addition, flounder AFP exists predominantly in the α-helical conformation at −1°C (9) in contrast to the random coil conformation of the AFGP (5). Flounder AFP levels vary with the season, with peak levels appearing at minimum water temperatures (10, 11). AFP from the shorthorn sculpin *Myoxocephalus scorpius* is similar to that from the winter flounder in terms of the parameters mentioned above (12). Comparison of thermolysin peptides from the AFP of these two species suggests that considerable...
Amino acid sequence homology may be shared between them. A new type of AFP from the sea raven, *Hemitripterus americanus*, an inhabitant of the same waters as the winter flounder and the shorthorn sculpin, is described in this paper. While the AFP from the sea raven is also a nonglycoprotein, we find that it differs markedly from both flounder AFP and the AFP from the antarctic fish in terms of its amino acid composition, secondary structure, sensitivity to sulfhydryl agents, and immunological specificities.

**MATERIALS AND METHODS**

I. Collection of Experimental Animals—Sea ravens were collected from Witless Bay, Newfoundland, and were kept in seawater at seasonally ambient water temperatures. Seasonal samples of sea raven serum were collected from fish held in captivity for periods of up to several months under seasonally ambient conditions of water temperature and photoperiod. Blood was collected from the caudal veins into a centrifuge tube containing a protease inhibitor, phenylmethylsulfonyl fluoride. After clotting and low speed centrifugation, the serum was collected and stored at -20°C. The freezing temperature of the serum was determined using a freezing point osmometer.

II. Contribution of Macromolecular Antifreeze to Serum Freezing Point Depression—Sea raven serum collected during January (4 ml, 65% protein) was applied directly on a Sephadex G-25 column (1.5 × 44 cm) in 0.1 M N H4HCO3 buffer. After lyophilization, individual fractions were redissolved in 0.01 M NH4HCO3, and the antifreeze activity was monitored with the osmometer.

III. Identification and Purification of Sea Raven Antifreeze Protein—The serum (5 ml) was concentrated to 2.5 ml using an Amicon ultrafiltration device with a UM05 membrane before being applied to a Sephadex G-75 column (1.6 × 86 cm) in 0.1 M NH4HCO3, and the "antifreeze" activity was monitored with the osmometer as in the earlier section. "Active" fractions were pooled and chromatographed once on a Sephadex G-75 column and later fractionated on a QAE-Sephadex A-25 column (4.5 × 50 cm) in 2.5 mM Tris-HCl buffer, pH 9.5, using a NaCl gradient. The materials from the QAE-Sephadex were analyzed further on polyacrylamide gel electrophoresis in sodium dodecyl sulfate buffer (16) and 4 M urea Tris-glycine, pH 9.2, using a slab gel apparatus (model 220, Bio-Rad). The major component as seen in a urea-Tris-glycine buffer system was isolated from the gel for amino acid analysis. One guide strip was stained with Coomassie blue, and the rest was eluted directly. The sample was hydrolyzed separately in 6 N HCl and in 1 N mercaptoethanesulfonic acid (17) at 110°C for 24 h. For hexosamine analysis, the sample was hydrolyzed in 4 N HCl for 4 h at 110°C. Amino acids and hexosamine were determined on a Beckman 121 amino acid analyzer. The protein concentration was determined by the Lowry procedure (18) using bovine serum albumin as a standard.

IV. Circular Dichroism Studies—Circular dichroism spectra were measured using a Jasco J-20 spectropolarimeter as described in our earlier publications (9, 12). The CD spectra are expressed in terms of the ellipticity (θ), in degrees cm² dmol⁻¹ using a mean residue weight of 105.4 for the protein.

V. Ouchterlony Immunodiffusion and Radioimmunoassay—The acrylamide band containing the unstained major antifreeze protein from urea-Tris-glycine electrophoresis was homogenized in 5 ml of 0.9% NaCl and then in an equal volume of complete Freund's adjuvant. The rabbits were immunized intramuscularly at 10-day intervals. After the 8th injection, the rabbits were bled and the serum tested for antibody activity by Ouchterlony immunodiffusion. The possible immunological cross-reactions of flounder and shorthorn sculpin AFP with the sea raven AFP antiserum were examined using the same procedure.

Sea raven AFP eluted from polyacrylamide gel was ¹²⁵I-radiolabeled according to the method of Greenwood et al. (15), using Na¹²⁵I as an iodine source and chloramine-T as an oxidizing agent. The reaction was terminated after 30 s by the addition of sodium meta-bisulfite. The labeled protein was separated from unincorporated label on Sephadex G-25. Radioimmunoassays were conducted according to the method of Crim et al. (20) using radiiodinated and nonradiiodinated gel-eluted sea raven AFP, respectively, as standard label and standard competitor. All assays were done using 3 μM KCl, 1% Triton X-100, 0.01 M Tris, pH 7.0, as buffer, and the percentage of inhibition of ¹²⁵I-labeled sea raven AFP immunoprecipitation by nonradioactive competitor was plotted against competitor concentration on logit paper. Serum sea raven AFP concentrations were determined from a standard curve.

VI. Measurement of Thermal Hysteresis of Antifreeze Protein Solutions—Freezing and melting temperatures were measured using a modified osmometric procedure designed to achieve the same advantages as that described by Mulvihill et al. (21); in the present study freezing was initiated by mechanical agitation of supercooled 0.2-ml samples mixed with 50 mg of AgI in plastic air-jacketed osmometer tubes at 0 to -3.5°C. The temperature of the thermistor was recorded (Fisher Recordall, Series 5000). Details of this method will be published elsewhere. Thermal hysteresis was defined as the difference between freezing and melting temperatures.

VII. Reagents—Iodine I-¹³¹ (¹³¹I) was purchased from New England Nuclear; Sephadex G-25, G-75, and QAE-Sephadex (A-25) were purchased from Pharmacia. All other chemicals were reagent grade.

**RESULTS AND DISCUSSION**

The level of AFP in the sea raven was found to vary seasonally in a manner similar to that observed for the flounder and shorthorn sculpin. A typical sea raven's serum had a summer freezing temperature of -0.6°C compared to -1.2°C in the winter, indicating the presence of antifreeze in the winter serum. Using a Sephadex G-25 desalting column to separate the macromolecular antifreeze from the inorganic ions and small organic molecules, the contribution of macromolecular antifreeze to the total freezing point depression in the winter was estimated to be 35-40% (Fig. 1A). This corre-
sponded to a freezing point depression of 0.4–0.5°C due to the antifreeze proteins.

Using Sephadex G-75 filtration, most of the serum proteins from sea raven eluted in the void volume and had little antifreeze activity (Fig. 1B). The “antifreeze” activity was associated with a component of M, 16,000, which coincided with a peak having absorbance at 280 nm. The proteins containing antifreeze activity appeared to be larger than those AFP (M, 10,000) from the flounder and the shorthorn sculpin, which invariably eluted after cytochrome c in this procedure. On QAE-Sephadex chromatography only one major component (A) was detected (Fig. 2). The molecular weight of this component was estimated to be 14,500 by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 3A). Urea-Tris-glycine gel electrophoresis confirmed that component A was slightly contaminated with a faster running minor species (Fig. 3B). The major band showed antifreeze activity. The minor band was not further investigated. The major band from urea-Tris-glycine gel electrophoresis was eluted for amino acid analysis.

The amino acid composition of the sea raven AFP is different from that of the winter flounder and the shorthorn sculpin in several ways (Table I). Although alanine is still the most common amino acid residue present, it is not as predominant as in the flounder and sculpin (12). It occurs at a frequency (14.4 mol %) not very different from that in many other proteins and is less than 25% of the frequency in the flounder, the sculpin, or the glycoprotein antifreeze. Furthermore, the sea raven AFP contains all of the common amino acids in its composition. In comparison, the flounder AFP contains only 8 and the shorthorn sculpin 12 different amino acids. In addition to alanine, nine amino acid residues including aspartic acid, glutamic acid, threonine, serine, proline, glycine, half-cystine, methionine and leucine, occur in excess of 5 mol % in the sea raven AFP. Only three of these, aspartic acid, threonine, and leucine occur in similar amounts in the flounder or sculpin.

### Table I

Amino acid composition of fish polypeptide antifreezes

|               | Sea raven | Winter flounder | Shorthorn sculpin |
|---------------|-----------|-----------------|-------------------|
| Aspartic acid | 0.27      | 3.3             | 0.04              |
| Threonine     | 0.20      | 0.8             | 0.16              |
| Serine        | 0.21      | 6.7             | 0.17              |
| Proline       | 0.17      | 0.0             | 0.03              |
| Glutamic acid | 0.23      | 2.4             | 0.06              |
| Glycine       | 0.21      | 0.0             | 0.03              |
| Alanine       | 0.37      | 0.0             | 0.03              |
| Half-cystine  | 0.19      | 0.0             | 0.03              |
| Valine        | 0.03      | 0.0             | 0.03              |
| Methionine    | 0.14      | 0.0             | 0.03              |
| Isoleucine    | 0.04      | 0.0             | 0.03              |
| Leucine       | 0.16      | 0.0             | 0.03              |
| Tyrosine      | 0.03      | 0.0             | 0.03              |
| Phenylalanine | 0.05      | 0.0             | 0.03              |
| Lysine        | 0.05      | 0.0             | 0.03              |
| Histidine     | 0.06      | 0.0             | 0.03              |
| Tryptophan    | 0.07      | 0.0             | 0.03              |
| Arginine      | 0.06      | 0.0             | 0.03              |
| Hexosamine    | 0.0       | 0.0             | 0.03              |

### Table II

Effect of dithiothreitol on antifreeze activity

All samples were incubated for 4 h at 50°C in 0.01 M NH4HCO3 and 0.01 M dithiothreitol, where applicable, before thermal hysteresis was measured.

|               | Thermal hysteresis |
|---------------|--------------------|
|               | -Ditho-+Dith-| % Inhibition |
|               | threitol| threitol| |
| Winter flounder (1 mg/ml) | -0.21 -0.21 | 0 |
| Sea raven (1.4 mg/ml) | -0.15 -0.05 | 67 |
sculpin AFP's. None of these AFP's contain any hexosamines in their composition. Polyacrylamide gels containing the proteins did not stain with periodate-Schiff reagent. We have tentatively concluded that these polypeptides do not contain any detectable amount of carbohydrate.

The half-cystine residues, unique in their presence in sea raven AFP, appear to be functionally significant. Thermal hysteresis of sea raven AFP was reduced by 67% when measured in the presence of 10 mM dithiothreitol (Table II). In contrast, the winter flounder antifreeze activity was unaffected by this sulfhydryl-reducing agent.

In addition to the differences in the amino acid composition, sea raven AFP has a different conformation compared to the flounder and shorthorn AFP as revealed by circular dichroism. While the AFGP have an extended conformation, both flounder and sculpin AFP have a relatively high helical content (~80%) at -1°C (9, 12). The CD spectrum of the sea raven AFP, on the other hand, does not show the characteristic bands at 208 and 222 nm of the α-helix, but is suggestive of the presence of β structure with a CD band centered around 213 nm (Fig. 4A). The interpretation of the CD spectrum is complicated, however, by the presence of CD bands due to aromatic residues in the region between 250–310 nm (Fig. 4B). The negative bands at 300 and 292 nm are likely to arise from the tryptophan residue(s) while the positive envelope between 250 and 285 nm might represent contribution from the tyrosyl and phenylalanyl residues. In addition, a rather unusual positive CD band centered around 233 nm was also observed in the spectrum. The origin of this band is not clear. The presence of these aromatic CD bands would indicate that the aromatic residues are situated in an asymmetric environment, possibly in the interior regions of the sea raven AFP. It might be recalled that none of the other AFP's have aromatic residues.

The availability of specific antibodies against sea raven AFP has made it feasible to examine the immunological cross-reactivities of different antifreeze proteins. Flounder and sculpin AFP do not cross-react with antisera against sea raven AFP (Fig. 5). This is consistent with the observed differences in the primary and secondary structures of these polypeptides. Essentially the same information was obtained using the radioimmunoassay. In the latter procedure, there was no competition of 131I-labeled sea raven AFP by either flounder or sculpin AFP (Fig. 6).

Our successful development of a radioimmunoassay for sea raven AFP has provided us with a sensitive, specific, and direct method for the quantitation of this polypeptide in the serum. We investigated the onset of AFP in individual sea ravens using the radioimmunoassay (Table III). In one experiment the level of the antifreeze increased from a low value of 0.02 mg/ml in early October to a high value of 5 mg/ml in January, a 250-fold accumulation within a 3-month interval. However, the amount of the antifreeze polypeptide was found to vary considerably from animal to animal. Nonetheless, in most cases, there was a good correlation between the level of......
AFP in the serum and the corresponding freezing point depression, as well as the yield of polypeptide isolated by Sephadex G-75 filtration. In addition, the onset of the increase in serum concentration of the sea raven AFP was similar to that observed by us earlier for the winter flounder and shortnose sculpin (12). A complete seasonal profile of the sea raven AFP is now being carried out in our laboratories.

The thermal hysteresis of the purified sea raven AFP was approximately equivalent to that of theantarctic fish T. borchgrevinki at all concentrations studied and similar to that of the winter flounder at the lower concentrations (Fig. 7). Whether the differences in the activity of these antifreeze proteins at high concentrations are significant remains uncertain, since the optimal conditions for the measurement of activity of any of these AFP have not been determined. The activity profile of T. borchgrevinki shown in Fig. 7 was generated from visual observations of the growth and shrinkage of ice crystals in appropriate solutions as a function of temperature (13).

When AFP's were first isolated and characterized it was initially thought that all fish antifreeze proteins might belong to a single, highly conserved family of proteins. The discovery and characterization of the flounder AFP demonstrated the existence of at least one other family of antifreeze proteins. However, the preponderance of alanine in both types of antifreeze showed that they still shared one compositional feature that would distinguish them from virtually all other proteins and strongly suggested a role for alanine in the antifreeze mechanism (2, 3). The demonstration of antifreeze activity in a synthetic polypeptide containing alanine as a major component (65%) along with aspartic acid (35%) seemed to augment this point (14). The characterization of sea raven AFP, however, shows clearly that the abundance of alanine is not essential. A recent report of a low alanine antifreeze from the larvae of Tenebrio molitor (15) supports this contention. However, the structure and properties of that insect antifreeze have yet to be characterized.

Although sea raven AFP shows profound compositional differences from those of the other fish AFP's, alanine is still its most common amino acid residue. Thrreonine and aspartic acid also occur in significantly high concentration in all of these AFP's. Interactions between these three amino acid residues with water or ice might be argued to play a key role in the generation of thermal hysteresis and provide a unifying mechanistic thread for the action of all of the fish AFP. The differences in secondary structure for the various fish AFP might be construed to mean that whatever steric properties they have in common in their interactions with water or ice are achieved by different conformational means. The role of conformation, if any, as well as the orientation of the AFP molecule during its interaction with the ice lattice at freezing temperatures is not yet understood.

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The onset of serum accumulation of sea raven AFP as determined by radioimmunoassay.

| Date   | Serum AFP level in mg/ml | Freezing point depression in °C |
|--------|--------------------------|---------------------------------|
| Oct. 1 | 0.09                     | -0.60                           |
| Oct. 15| 0.1                      | -0.72                           |
| Oct. 20| 0.6                      | -0.78                           |
| Nov. 16| 1.9                      | -0.90                           |
| Dec. 21| 3.3                      | -1.10                           |
| Jan. 15| 5.0                      | -1.21                           |

Fig. 7. The antifreeze activity of sea raven AFP. Various amounts of AFP from sea raven and winter flounder were dissolved separately in 0.01 M NH₄HCO₃. Thermal hysteresis of sea raven and flounder AFP was monitored by osmometer as described under "Materials and Methods." The dashed line represents the activity of the antifreeze glycoprotein fromantarctic fish from Reference 13. •, flounder AFP; ○, sea raven AFP.