Structure of an Antifreeze Polypeptide Precursor from the Sea Raven, *Hemitripterus americanus*

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The cystine-rich antifreeze polypeptides (AFP) from sea raven were fractionated by reverse-phase high performance liquid chromatography into several components, with SR2 (M, 17,000) as the major AFP. Sea raven AFP cDNA clones were isolated from a liver cDNA library using a synthetic oligonucleotide, and the identity of one of the clones, C2-1, was confirmed by hybridization selection and cell-free translation. C2-1 encodes a pre-AFP of 195 amino acids with no evidence of any profragments. Comparison of the deduced amino acid sequence with partial peptide sequences from SR2 showed substitutions in at least four amino acid positions, suggesting that C2-1 cDNA codes for a minor component. Both the primary and the predicted secondary structures of sea raven AFP are completely different from those of other fish AFP. This further confirms that sea raven AFP belongs to a different class of antifreezes. The high frequency of reverse turns and the presence of paired hydrophilic amino acids in these structures are striking features of the protein and may contribute to their antifreeze action.

Fish inhabiting the northern and polar seas encounter temperatures as low as −1.8 °C (1). To survive, many of these fish produce serum antifreeze proteins or polypeptides which lower the serum-freezing temperature below that of the surrounding seawater (1, 2). The antifreeze polypeptides (AFP) from various species living in the coastal waters of Newfoundland have been characterized. These include AFP from the winter flounder (3–6), shorthorn sculpin (7), ocean pout (8), and sea raven (9). Although they exhibit the same biological function, many studies have revealed structural and chemical diversities among these AFP. AFP from the winter flounder and shorthorn sculpin are rich in alanine (60 mol %) and α-helix (5, 10, 11). The sequence data from both AFP indicate the presence of a triplicated eleven-residue unit of threonine-$\alpha_3$-polar amino acid-$X_5$, where $X$ is a nonpolar amino acid, mostly alanine (5, 7). By comparison, ocean pout AFP is not alanine-rich; it contains no significant α-helix or β-structure, and its sequence does not indicate the presence of any repeating amino acid unit (8). Sea raven AFP represents yet a different type of AFP. It contains average amounts of alanine; and to date, it is the only fish AFP which contains a significant amount of half-cystine. The role of cystine appears to be important as the activity of sea raven AFP is sensitive to sulfhydryl reagents. Circular dichroism studies indicate the presence of β-structure and little α-helical content (9). Antibodies to sea raven AFP do not cross-react with either winter flounder or shorthorn sculpin AFP. This immunological specificity is consistent with sea raven AFP being a different type of antifreeze protein (9).

In order to further understand the structure-function relationship in antifreeze polypeptides, we have determined the structure of a precursor to sea raven AFP.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Microheterogeneity and Size of Sea Raven AFP—We have demonstrated earlier that sea raven AFP can be fractionated into two major and three minor components by reverse-phase HPLC (12). However, slightly different profiles were obtained from AFP isolated from different pools of fish sera (Fig. 1). The variation was demonstrated in the relative amounts of each component. Nonetheless, SR2 was the major component. The sea raven AFP components showed similar molecular weights, as estimated by SDS-PAGE (Fig. 2). Sephadex G-75-purified AFP and all five AFP components purified by reverse-phase HPLC were analyzed with or without reduction and alkylation. The estimated molecular weight of nonreduced AFP was about 14,000 and was 17,000 for the reduced and alkylation. These results are similar to the range of 14,500–16,000 for the AFP, as previously estimated from gel filtration chromatography by Slaughter et al. (9).

The amino acid composition of SR2 is presented in Table 1. There is a significant level of half-cystine, an amino acid not found in any other fish AFP. Although present in modest amounts, alanine is still the most abundant amino acid, and its level is similar to that found in ocean pout AFP (29). This is in contrast to the 60 mol % of alanine in AFP from winter flounder and shorthorn sculpin (4, 7). The amino acid compositions of the other sea raven AFP components are similar.
to that of SR2, as is the case with the composition of the components from other fish AFP (4, 7, 29).

The number of cysteine residues in SR2 was estimated. SR2 was reduced and alkylated with varying ratios of charged and neutral alkylating agents, followed by electrophoretic separation based on charge differences (Fig. 3). The number \( n \) of cysteine residues was determined by counting the number of bands \( (n + 1) \). A total of 12 bands corresponding to 11 cysteine residues is shown (lane 6).

Specificity of Antibodies to SR2—Polyclonal antibodies were produced against the major component SR2 and were used to check the immunological relationship of SR2 with the other sea raven AFP and AFP from other fish. Results from the immunoblot (Fig. 4) show that antibodies against SR2 cross-react with the other sea raven AFP, thus establishing their immunological similarity. These antibodies do not react with AFP from winter flounder, shorthorn sculpin, or ocean pout, indicating the immunological or structural differences between sea raven AFP and AFP from these other fish.

Identification of the Biosynthetic Precursor—RNA enriched for the 10–11 S mRNA species was recovered from a sucrose density gradient (Fig. 5, lane 4) and translated in a rabbit reticulocyte lysate cell-free translation system. The translation products were immunoprecipitated with antibodies to SR2. The reduced and alkylated immunoprecipitates and the reduced and \(^{[3]H}\)idoacetate-alkylated SR2 were subjected to SDS-PAGE followed by fluorography. Immunoprecipitation of the translation products gave one predominant 20,000-dalton and one minor 14,000-dalton product (Fig. 6A, lane 1). Before the immunoprecipitation, the 14,000-dalton product was the major species (see Fig. 6B, lane 2). Immunoprecipitation resulted in an enrichment of the larger species. In the presence of nonlabeled SR2, a significant decrease in the recovery of the larger product provides evidence for its competition with SR2 for antibodies (Fig. 6A, lane 2) and thus establishes its identity as the biosynthetic precursor for sea raven AFP. This precursor is about 20,000 daltons, whereas the mature AFP (Fig. 6A, lane 3) is about 17,000 daltons. The difference of about 3,000 daltons could be accounted for by the presence of a signal peptide on the precursor. It does not appear to contain an additional prosequence.

Isolation and Sequence of a Sea Raven AFP cDNA—A mixed 17-mer synthetic oligonucleotide was derived from the amino acid sequence of a tryptic peptide of SR2. This probe was used to screen clones from a cDNA library enriched for the liver poly(A)\(^+\) 10–11 S RNA. In the initial screening, the cDNA probe was used as primer-extension of the synthetic oligonucleotide. Positives from this first screening were re-screened by colony hybridization to \(^{32}P\) end-labeled synthetic oligonucleotide. Use of the primer-extended cDNA probe enabled high stringency washes, resulting in lower density gradient (Fig. 4). The insert in clone C2-1 (874 base pairs) was sequenced by the dideoxy chain terminating procedure (Fig. 7, Appendix). The reading frame was established by matching with chymotryptic, tryptic, and thermolysin peptide sequences from SR2. However, there are at least four amino acid substitutions between the deduced sequence and the partial peptide sequences. The DNA sequence in C2-1 encodes a 195-residue preprotein with a molecular weight of 20,009, which is in complete agreement with the size of the biosynthetic precursor identified from the cell-free translation studies. There are 11 base pairs in the 5′-untranslated region and 270 base pairs in the 3′-untranslated region. The highly conserved polyadenylation signal AATAAA is not found in this 3′-untranslated region.

The predicted secondary structure for the deduced amino acid sequence is shown in Fig. 8. The presence of a limited amount of \( \beta \)-structure and little \( \alpha \)-helix is in agreement with the earlier circular dichroism studies of sea raven AFP (9). No homology was found to exist between the sea raven AFP sequence and sequences listed in the MicroGenie Nucleotide and Protein Sequence Data Bank.

**DISCUSSION**

We have confirmed our earlier observations (12) of the microheterogeneity in sea raven AFP both by reverse-phase HPLC and cDNA sequence analysis (e.g. the amino acid substitutions between C2-1 and SR2). As with the other AFP studied, the source of the microheterogeneity may include post-translational modification and the expression of multigene families (5, 6, 8, 30). Furthermore, the variability of sea raven AFP may be related to a recent observation made by Fletcher et al. (31) that phenotypic variations of plasma AFP levels are present in the sea raven. In that study, they found that significant levels of AFP were present during the summer and that 40–50% of the fish did not increase their AFP levels during the winter. Population variation as a cause was ruled out as sea ravens from other geographical locations also demonstrated the same result. The authors postulated that since sea ravens do not normally encounter ice-laden seawater, AFP may not be essential to their survival, and thus, considerable genetic polymorphism may have developed. Re-
cent evidence from genomic Southern blots indicates the presence of more than 40 AFP genes in the sea raven genome. Thus, it is possible that one of the reasons for the observed individual variability may be the differential expression of such polymorphic genes.

The N terminus of the AFP precursor contains a stretch of residues typical of a signal peptide. Based on observations of signal peptidase cleavage sites in 39 proteins (32), cleavage may occur after serine at position 29 or after threonine at position 30. These positions correspond to the end of a hydrophobic stretch of residues as seen on a hydrophilicity plot of the precursor (Fig. 9). Since the N terminus of the mature AFP is blocked, further studies will be needed to identify the cleavage site. However, proteolytic processing at the above positions would generate a mature polypeptide of about 17,000 daltons, which is in complete agreement with the molecular mass for sea raven AFP as estimated by SDS-PAGE in this study. The sea raven AFP precursor is therefore similar to that of the ocean pout AFP. Both of these precursors contain only the signal peptide with no evidence of a propeptide as demonstrated in flounder prepro-AFP.

The presence of substitutions in at least four amino acid positions between the deduced sequence of the cDNA and the peptide sequences of the major component SR2 suggests that this cDNA encodes a minor component of sea raven AFP. The amino acid composition of one of the minor components, SR3, resembles closely the composition from C2-1 (Table 1). Thus, C2-1 cDNA may code for SR3. In the winter flounder, sequencing of the two major components has revealed three amino acid substitutions in otherwise identical sequences (5, 33). Similarly, the sequences of two ocean pout AFP components have shown differences in at least two amino acid positions. The amino acid substitutions in these fish AFP do not appear to affect antifreeze activity (8, 33).

Comparison of the amino acid composition of SR2 and of C2-1 shows that they are similar (Table 1). The number of cysteine residues in SR2, as estimated by reduction and alkylation, agrees with the number predicted for C2-1. This is not surprising, as the number of cysteine residues is expected to be conserved despite the presence of some amino acid substitutions.

Examination of the predicted secondary structure (Fig. 8) shows a high frequency of reverse turns, with the turns accounting for about 37% of the protein. Reverse turns occur in proteins with an average frequency of about 19% (34) and have been implied with roles in receptor binding, antibody recognition, and post-translational modifications (i.e., phosphorylation, glycosylation) (35). Due to their intrinsically polar nature and general occurrence at the surface of proteins (35), the reverse turns in sea raven AFP may be involved in forming hydrogen bonds with water. In addition, the presence of paired hydrophilic amino acids (Asp-Asp at positions 51–52, 160–161, and 179–180) on some of these turns may be important to the AFP-ice interaction. One way of investigating the importance of these turns would be to perform fragment deletion or site-specific mutagenesis in these regions and analyze the effect on AFP activity. Such studies (including chemical modifications) to elucidate the structure-function relationship of sea raven AFP are currently underway in our laboratory.

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FIG. 7. Sequence of a sea raven AFP precursor deduced from its cDNA. Half-arrows indicate sequences indetical with SR2, whereas residues in parentheses below the sequence indicate amino acid substitutions. The arrow above the nucleotide sequence indicates the predicted signal peptidase cleavage site.

\[ \text{5' CACACACATG -1} \]

**APPENDIX**

**Supplementary Materials**

Structure of a Sea Raven Antifreeze Polypeptide from the Sea Raven, *Halicoreus magnus*.

By Nancy J. P. Koff, Tom F. Slough and Choy L. New

**EXPERIMENTAL PROCEDURES**

**Materials**

Solvents used in high performance liquid chromatography were obtained from Cecil Chemicals Inc., New York, New York. Deuterium-brominated and deuterium-thrombophobides (M1 universal primer (TGCAGCCATTGAG3'), Blochemics, Inc. SDS-10% polyacrylamide gel electrophoresis (SDS-PAGE) was performed at room temperature. The molecular weight of each protein was determined using a gel filtration column at room temperature. The molecular weight of each protein was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and SDS-PAGE was performed at room temperature.

**Isolation of Sea Raven AFP**

Sea raven AFP was isolated from pooled (1 kg) and stored at 4°C for 1 year. Gel filtration chromatography was performed using a Superose 6 column. Molecular weight estimates were based on the elution positions of the markers used. The protein was then dialyzed against 10 mM Tris-buffered saline (pH 7.4), and then lyophilized.

**Amino Acid Analysis**

Amino acid analysis was performed using the Waters Prep System. All peptides were derivatized with an excess of phenylisothiocyanate and separated by reverse-phase HPLC using a Waters Prep column and the recommended gradient system. To detect the cysteic acid derivatives, samples were oxidized with performic acid prior to hydrolysis.

**Determination of the Number of Cysteines in SR2**

The number of cysteine residues in SR2 was determined according to Gerasimov (13). The determination is based on reduction of the protein, followed by alkylation with mixtures of varying ratios of thiourea and dithiothreitol. The protein was then electrophoretically separated based on the presence of different numbers of acidic carboxylic acid groups. SR2 (10 mg) was incubated with 20 mM of either 0.1 M NaOH, 10 mM Tris-buffered saline (pH 7.4), 0.25 M sodium dithiothreitol (pH 8.0) with 1 M KI, or 1.1.2.3 and 1.9 ratios of the dithiothreitol to dithiothreitol. Alkylation occurred for 15 min, and the samples were then analyzed. A total of 1000 micrograms of protein was subjected to electrophoresis for 2.5 hr in a 10% polyacrylamide gel using a high pH discontinuous system. The gel was stained with 0.1% (w/v) Coomassie Brilliant Blue. The mobility of SR2 (100 mg) and 10% (w/v) dithiothreitol was observed, followed by densitometry in 7.5% acrylamide and 5% acrylamide.

**Immunization of AFP**

The major AFP component, SR2, purified by reverse-phase HPLC was used to produce polyclonal antibodies. 0.1 mg of SR2 was dissolved in 0.5 ml of 0.9% NaCl, and then emulsified with 5 ml of incomplete Freund’s adjuvant. Rabbits (1 kg, New Zealand White) were immunized intramuscularly at 7-day intervals. Following the first appearance of antibody as detected by the ring test, booster injections (SR2 in equal volumes of 0.9% NaCl and Freund’s incomplete adjuvant) were given. The rabbits were bled 7 days later when the ring test was positive. The anti-SR2 antibodies were used in an immunoblot. 5 μg each of 17-mer, all reverse-phase HPLC-purified AFP, and AFP from another species, shrimp, shrimp, and shrimp were electrophoresed on a 0-22.5% acrylamide gradient gel. The protein bands and immunoreactive bands were detected by autoradiography according to Koff et al.

**Isolation of Sea Raven mRNA**

Sea raven poly(A) mRNA was isolated using the procedure described by Davis and New (19). Total cellular RNA was isolated from 10 g of liver using phenol/chloroform extractions and precipitation. The mRNA was enriched for poly(A) mRNA by repeated chromatography on an oligo(dT)-cellulose column. Further enrichment involved precipitation through 4 x 5% polyethylene glycol (PEG) in 1 M urea, and then precipitation with 1 M NaCl in 8 M urea/methyl mercuric hydroxide. The fractions enriched for the poly(A) mRNA were pooled, ethanol-precipitated, and resuspended.

**Structure of Sea Raven Antifreeze Polyptide**

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Identification of the Antifreeze Precursor

Sea raven poly(A) mRNA was translated using a mRNA-dependent rabbit reticulocyte lysate cell-free translation kit. 0.5-1.5 μg of mRNA and 5x synthesized L-methionine (1000 C/mill) were used with the recommended reaction conditions, in a volume of 30 μl, and incubated at 37°C for 1 hr. Translation products were immunoprecipitated with antibodies to SR2. Following translation, samples were diluted with an equal volume of water, and two volumes of phosphate buffered saline (PBS). 50 mM sodium phosphate pH 7.2, 1.8 M NaCl, 0.5% BSA, 150 M NaO, 2 Triton X-100, 1 mg/ml L-methionine. 3 - 5 μg of IRS2 was added to some samples to compete with antibody. 1 μL of anti-SR2 serum was added, and incubated at 37°C for 1 hr, followed by an overnight incubation at 4°C. Goat anti-rabbit IgG antibody (10 μl) was added, and the samples were incubated overnight at 4°C. The immunoprecipitates were pelleted and washed four times with ice-cold PBS. Prior to SDS-PAGE, they were reduced and alkylated as described by Schede et al (28), with some modification. The immunoprecipitates were resuspended in 10 μl of reducing buffer (50 mM SDS, 60 mM Tris pH 6.8, 1% glycerol, 0.1 M dithiothreitol, 0.14% bromophenol blue), boiled for 3 min, and incubated at 37°C for 30 min. 1 μl of 1 M iodoacetate (prepared prior to use) was added, followed by incubation at room temperature for 30 min. 1 μl of 0.5% N-acryl was added to react this the buffer pH, and the samples were subjected to SDS-PAGE. The translation products were detected by fluorography using EN3HANCE.

Construction of cDNA Clones

A cDNA library was made according to the procedure of Heidecker and Menges (21), using the plasmid vector pUC 9. The cDNA was synthesized from sea raven poly(A) mRNA enriched for the 10-11 fraction.

Isolation of Sea Raven Antifreeze Poly(A) Clones

An amino acid sequence of 16 residues was obtained from a tryptic peptide of SR2, SR2 (9) (C. L. Hew et al, unpublished result). The sequence Ala Ala Cys Cys Met Glu gave the least degeneracy and was used to derive the complimentary sequence of a mixed synthetic 17-mer oligonucleotide. Following custom synthesis, it was purified by polyacrylamide/urea gel electrophoresis.

The cDNA clones were prepared for hybridization on nitrocellulose filters as described by Maniatis et al (22), and further treated to reduce background, according to Woods (23). The first screening used a cDNA probe made by primer-extension, with sea raven poly(A) mRNA as the template and the synthetic oligonucleotide as the probe. Synthesis of this probe was essentially as described by Houghton et al (24), except that the probe was annealed to the mRNA in the presence of 0.1 M NaCl, at 42°C for 1 hr. Positives from the first screening were re-screened by hybridization at 42°C with the synthetic probe, which was end-labeled using [32P]-ATP (3000 Ci/mill) and T4-Polynucleotide kinase as described by Woods (23). The filters were washed with excess 6X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium phosphate, pH 7) and 0.5% sodium pyrophosphate for 10 min at room temperature, three times at 42°C for 20 min each time, and finally once at 56°C for 10 min.

One of the positives, clone C2-1, was chosen for further analysis by hybridization selection. C2-1, plasmid DNA was prepared, and 20 μg was fixed onto a nitrocellulose filter (23). The mRNA corresponding to the C2-1 cDNA was purified by hybridization selection according to Ricciardi and Palka (25). Hybridization occurred at 47°C for 4 hr. The mRNA isolated was translated immediately in a rabbit reticulocyte lysate cell-free translation system. The translation products were immunoprecipitated with anti-SR2 serum, reduced and alkylated, and subjected to SDS-PAGE and fluorography as described above.

DNA Sequence Determination

DNA sequencing was performed by the dinesty chain-termination method (26, 27). Restriction fragments from each a sea A 3A and an A11 digest of the cDNA were subcloned into the M13 mp8 and mp19 vectors.

Oligonucleotide Analysis

DNA and protein sequence analysis including the hydrophobicity plot, were performed using the software program DNA Sequencer II from Testion, West Lebanon, New Hampshire. The secondary structure was predicted for the amino acid sequence deduced from the sea raven AP cDNA. This prediction was performed on an Apple II computer using the program made by Dr. P. C. Huang, Department of Biochemistry, the Johns Hopkins University, Md. The program was based on the Chou and Fasman predictive method (28). Using the DNA/Protein sequence analysis software and Microgenie, from International Biotechnologies, Inc. New Haven, C, and Beckman Instruments, respectively, the nucleotides and amino acid sequence was used to search for homology with sequences listed in the National Biomedical Research Foundation (NBIFS) Protein Data Bank. The data Bank contained translated sequences from NID's Genetic Sequence Data Bank (GenBank), and protein sequences from the National Biomedical Research Foundation (NBIFS) Protein Data Bank.

Data Bank analysis: a proline at residue 10 and asp at residues 2, 3, 4, 5, 6 are conserved, and are similar to the corresponding residues in the amino acid sequence of the sea raven antifreeze protein. The sea raven antifreeze protein is a member of the serpin family of proteins, and is similar to the rabbit antifreeze protein.
Structure of Sea Raven Antifreeze Polypeptide

Fig. 5: Amino acid composition of SR2 and C2-1 cDNA sequence. For SR2, yield of amino acids is in pmol, and the figures in brackets represent calculated numbers of amino acid residues. For C2-1, the figure represents the number of amino acid residues.

Table 1: Amino acid composition of SR2 and C2-1 cDNA sequence. For SR2, yield of amino acids is in pmol, and the figures in brackets represent calculated numbers of amino acid residues. For C2-1, the figure represents the number of amino acid residues.