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IN VIVO BIOSYNTHESIS OF THE ANTIFREEZE PROTEIN IN THE WINTER FLOUNDER - EVIDENCE FOR A LARGER PRECURSOR

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SUMMARY

Biosynthesis of the winter flounder's antifreeze protein was examined by the in vivo incorporation of tritiated alanine into 10% trichloroacetic acid-soluble serum proteins. Two radioactive components of 15,000 and 10,000 daltons were observed. The latter species corresponded to authentic flounder antifreeze protein. Pulse chase experiments, tryptic cleavage and amino acid analysis of the 15,000 daltons component suggest flounder antifreeze protein is synthesized via the larger protein.

INTRODUCTION

The winter flounder, Pseudopleuronectus americanus, which inhabits in the Northern Atlantic coast of United States and Canada contains one principal "antifreeze" protein (AFP) in its serum during the winter (1,2,3). Flounder AFP is a small polypeptide of 10,000 daltons and consists of only eight amino acids with alanine accounting for approximately 60% of its total amino acid residues (1,2). Its primary structure (4) and secondary structure (5) differ significantly from that of the Antarctic antifreeze glycoproteins (6).

Flounder AFP is absent in the summer and appears only in the winter when the seawater temperature is low (1,2,7). Because of its seasonal appearance and its regulation by environmental factors such as temperature, photoperiod (8) and the endocrines (9), the flounder AFP provides an interesting

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Abbreviations - AFP (Antifreeze protein)

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and important experimental system to study the control of specific gene expression. The present paper describes the in vivo biosynthesis of the flounder AFP during the winter months of November to January. Active synthesis of AFP was demonstrated by the incorporation of tritiated alanine. In addition, our results indicate the presence of a larger polypeptide which was more rapidly labelled with tritiated alanine than the flounder AFP. Enzymic digestion and amino acid analysis of this larger component showed close structural similarity with the authentic AFP. The possibility that flounder AFP is synthesized via a larger protein is discussed.

MATERIALS AND METHODS

Collection of Materials

The winter flounder were collected in Chapel's Cove, Newfoundland and were kept in ambient seawater. For the collection of plasma samples, the blood was kept in a heparinized tube for 10 minutes before low speed centrifugation. The osmolality of the sample (0.2 ml) was determined with a freezing point osmometer (Model 3D, Advanced Instruments Ltd., Needham Heights, Mass. 02134, U.S.A.). This instrument measures freezing point of liquids; 1 milli Osm/Kg corresponds to 1.856 milli degree C^{o}.

In Vivo Experiments

Only male winter flounder were used in our study in order to avoid any possible complication and contamination arising from vitellogenesis in the female.

The winter flounder (400-600 gm) was injected intravenously via a caudal blood vessel with 50-250 μCi of ^{3}H alanine (L-Alanine 3-[^{3}H], specific activity 30-50 Ci/mmole, New England Nuclear, Boston, Mass., U.S.A.). The fish were bled twice at different time interval and the blood allowed to clot at $4^{o}C$ for 3-4 hours. The serum was precipitated with 10% trichloroacetic acid. Acid-soluble materials were desalted on a Sephadex G25 column (1.5 x 40 cm) in 0.05 M NaCl. Protein fractions in the void volume were lyophilized and chromatographed on a Sephadex G75 column (1.6 x 86 cm) in 0.05 M NH_4HCO_3. Radioactivity of each fraction was determined by counting an aliquot in 10 ml of Aquasol 2 (New England Nuclear) in a Packard Liquid Scintillation Spectrometer, Model 3375. The recovery of the AFP from the serum was determined as 85% using tritiated AFP prepared from tritium gas labelling.

Amino Acid Analysis

Samples for amino acid analysis were hydrolyzed in 6 N HCl at $110^{o}C$ for 18 hours. Analysis were performed on a Beckman Spinco 121 M Amino Acid Analyzer.

Succinylation of Flounder's Antifreeze Proteins

Purified flounder proteins in 1 ml of 0.05 M phosphate buffer, pH 7.5 was added to a scintillation vial containing 50 μCi of succinic anhydride [1,4-^{14}C] (specific activity 2-10 mCi/mmole, New England Nuclear) for 15 minutes at 4°C with stirring. The mixture was then diluted with 9 ml of cold phosphate buffer. Solid succinic anhydride (25 mg) in aliquots were added and the pH of the solution maintained between 7-9 with N NaOH. After 1 hour, the reaction mixture was dialyzed against 2 changes of 2 liter of 0.05 M NH_4HCO_3 and lyophilized. The molecular weight of succinylated components was determined on a Sephadex G-75 column in 8 M urea, 0.1 M tris-glycine buffer, pH 8.6.

RESULTS AND DISCUSSION

Fig. 1 shows the osmolality of the flounder's plasma at different times of the year. These measurements represent the monthly average of 4 years (June, 1972 - May, 1976) results with approximately 10-30 fish per point. The osmolality of the fish serum increases fom 330 mOsm in October to more than 600 mOsm in January and February when the monthly average temperature of the seawater is at its lowest of -1°C. The osmolality starts to decline when the seawater warms up and reaches a minimum value of 320-330 mOsm again in June and July. The increase in osmolality in the flounder's serum in the winter has been shown earlier to be due mainly to the presence of the "antifreeze" macromolecules (1, 2,7). The sharp increase in osmolality occurs in mid October to December and would indicate active synthesis and accumulation of the anitifreeze protein. All our *in vivo* experiments were performed within these periods.

In our present study the identification of the *in vivo* biosynthetic products of AFP in the serum depends on the following criteria: (i) its solubility in 10% trichloroacetic acid. (Flounder AFP is soluble in the acid and the recovery was estimated at 85%), (ii) incorporation of tritiated alanine into the polypeptide, and (iii) a molecular weight of 10,000 daltons. Radioactive alanine was incorporated into AFP-like serum protein within 24 hours. The presence of a radioactive component that fulfilled the above criteria was demonstrated in most of these experiments. However, in addition to this component, there was a larger component of molecular

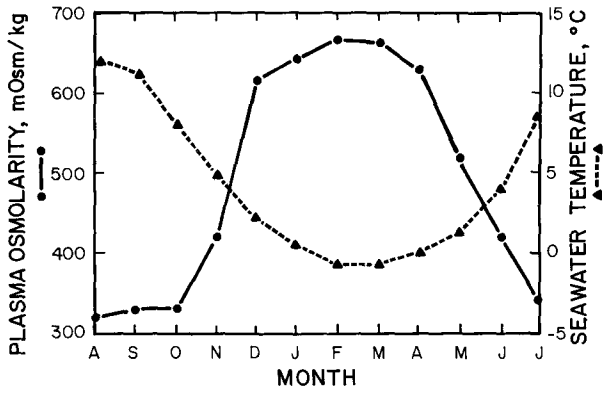
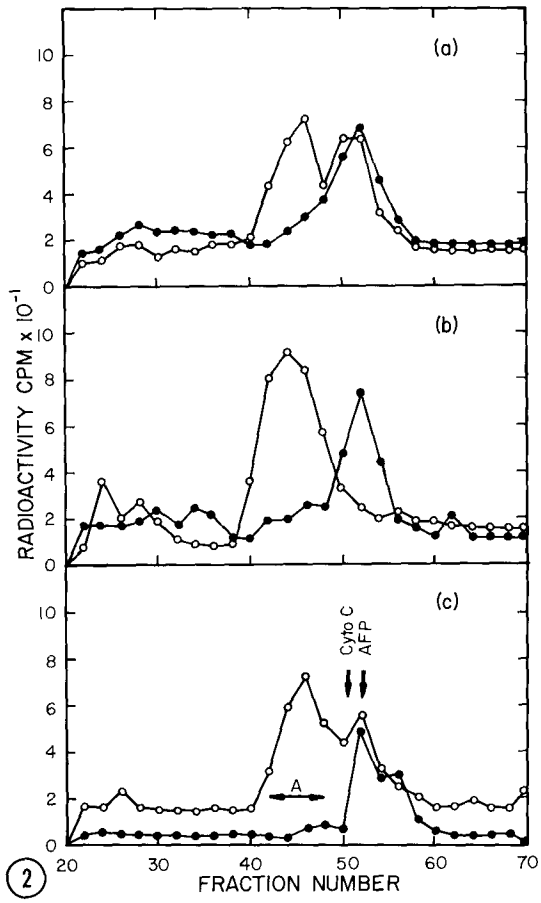


Fig. 1. The seasonal variation of the plasma osmolality of the winter flounder Pseudopleuronectus americanus. The broken line (x---x) indicates the average monthly water temperature (June 72 - May 76).

weight 15,000 daltons in the serum, which in many cases was the predominant radioactive species (Fig. 2).

To investigate the biosynthetic relationship between these two components, time chase experiments were performed. The flounder was bled twice; 24 hours and 48 hours after the initial injection of the tritiated alanine. In one experiment, both components were present initially at 24 hours. However after 48 hours, only the 10,000 daltons component was demonstrated (Fig. 2A). In another experiment, the serum contained only the larger radioactive component after 24 hours and when it was examined 48 hours later, the radioactivity occurred exclusively in the 10,000 dalton position on the column (Fig. 2B). Furthermore the radioactivity in the larger component was converted to peptides of approximately 10,000 dalton and smaller molecular weights by tryptic digestion (Fig. 2C).

Though the purity of the 10,000 dalton component can be demonstrated readily as flounder AFP, (1) the chemical nature of the 15,000 daltons component required further characterization. Due to the difficulty in raising specific antibodies against the flounder AFP, we were unable to demonstrate their structural similarity using immunological methods. To provide additional evidence for their structural similarity, we have



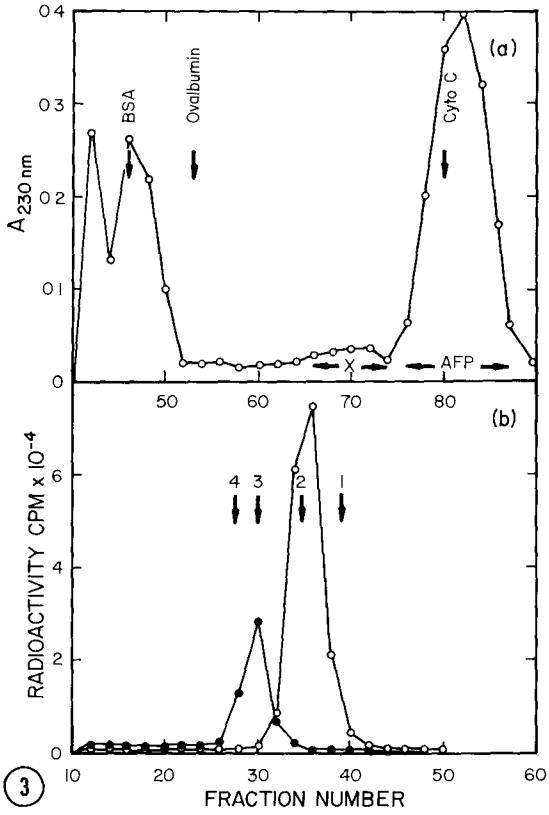


Fig. 2. a. In vivo incorporation studies. The animal was injected with 100 μCi of ^{3}H-alanine. Blood was withdrawn 24 hr. (o——o) and 48 hr. (•——•) later. AFP was isolated as described in methods. Acid-soluble materials were chromatographed on a Sephadex G75 column (2.2 x 90 cm) in 0.1M NH_4HCO_3. Fraction size of 2.2ml was collected. Aliquots were taken for radioactivity determination. b. In vivo incoporation studies. Another animal was injected with 50 μCi of ^{3}H alanine. Blood was withdrawn 24 hr. (o——o) and 48 hr. (•——•) later. c. Enzymic cleavage experiment. The larger component (15,000 dalton) as indicated by A in the column from an in vivo experiment (o——o) was pooled, digested with trypsin and rechromatographed on the column. Radioactivity after digestion (•——•).

Fig. 3. a. Isolation of the 15,000 dalton component from flounder serum in Sephadex G75 chromatography. Trichloroacetic soluble material from the serum was applied on the column (2.6 x 92 cm) in 1M NH_4HCO_3. Fraction size was 3.9ml. Material indicated as "x" was pooled and rechromatographed. b. Molecular weight estimation of succinylated AFP (o——o) and succinylated x (•——•) an Sephadex G75 column in 8M urea, 0.1 M tris glycine pH 8.6. Column size was 2.2 x 90 cm and fraction size was 2.2ml. The protein markers are 1. insulin (6,000) 2. cytochrome C (11,700) 3. myoglobin (17,000) 4. chymotrypsinogen (25,000).

TABLE 1: Amino Acid Composition of the "large" Flounder AFP

	15,000 component dalton		AFP
Amino Acid	Yield in μmoles	No. of Residues	No. of Residues*
Asp	0.70	18.5	14
Thr	0.47	12	12
Ser	0.23	6	4
Pro	0.39	10	0
Glu	0.21	5.5	2
Gly	0.08	2.2	0
Ala	3.82	100	76
Val	0.08	2.1	0
Leu	0.27	7.1	7
Tyr	0.04	1.0	0
Phe	0.04	1.0	0
Lys	0.19	5.0	4
Arg	0.13	3.4	2
Total amino acids		171	121
Molecular weight		15,500	10,457

* Reference # 1

attempted to isolate and purify the 15,000 dalton components by re-examining the elution profiles from Sephadex G75 chromatography used for the isolation of flounder AFP (1) as shown in Fig. 3A . There was indeed a minor shoulder peak with a corresponding molecular weight. The amino acid composition of this component after rechromatography is shown in Table 1. The composition of the 15,000 daltons component is very similar to that of the AFP. In addition, it contains extra amino acids, notably glycine and proline which were absent in the flounder AFP. The presence of additional amino acids is consistent with its being a larger precursor. The molecular weights of the succinylated derivatives of the larger component and the ATP as determined by the 8 M urea Sephadex G75 column were 17,000 and 11,000 daltons respectively. After subtracting the contribution of the succinic acid added to the polypeptides (5 lysine and one N-terminal NH_2 for the

larger component and 4 lysine and one N-terminal NH_2 for AFP), the corrected molecular weights i.e. 16,460 and 10,600 daltons agreed with the amino acid analysis and the molecular weights obtained from the unmodified components in G75 column in 0.1 M NH_4HCO_3.

Our present study would indicate that the flounder AFP might be synthesized via a larger protein. These results agree with our in vitro studies of the flounder liver which showed the presence of a similar component of 15,000 daltons (10). These observations on the presence of possible precursor proteins will be important to our understanding on the synthesis, secretion of the antifreeze protein and its control mechanism.

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