Antifreeze Glycoproteins from Arctic Fish*

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Four species of fish in the Barents Sea of the Arctic, north of Norway and Russia, were caught and examined for the presence of antifreeze proteins in their blood sera. Only one species of Arctic fish, the polar cod (Boreogadus saida), was found to contain an antifreeze protein. Antifreeze glycoproteins were isolated and characterized from the blood serum of the Arctic fish, and their structure and function were compared with the antifreeze glycoprotein from the Antarctic fish, Trematomus borchgrevinki. The composition and sequence of the active fraction were similar, if not identical, in both species, although there were differences in the number of multiple molecular forms present. The active glycoproteins and smaller related glycopeptides contained alanine, threonine, and disaccharide. Although the distributions of different lengths of the peptides varied between the species, the active antifreeze glycoproteins from both polar regions had the identical fundamental structure consisting of repeating units of the glycotripeptide, Ala-Ala-Thr, with all the threonine residues glycosidically α-linked to a β, 1 → 3 galactosyl-N-acetylgalactosamine. The smaller glycopeptides also contained proline residues following some of the threonine residues, but the positions were different in the Arctic and Antarctic materials. Also similar were the NH₂- and COOH-terminal residues, the inhibition of hemagglutination by osage orange seed lectin, and the inhibition of antifreeze activity by bovine serum albumin.

In a series of articles from 1953 through 1962, Scholander and co-investigators (1–3) reported that the blood sera of Arctic fish had lower freezing temperatures than did the blood sera of fish not adapted to the cold. Substances in the fraction soluble in trichloroacetic acid were shown to lower the freezing temperature. The original observation of Scholander that an antifreeze-like substance existed in northern polar fish has been more recently confirmed by Scholander and Møgert (4) and Hargens (5) who found it in the saffron cod, Eleginus gracilis, and an antifreeze substance has been reported in the winter flounder (Pseudopleuronectes americanus) (6–8), all off North American shores.

DeVries and Wohlschlag (9) first reported the existence of an antifreeze glycoprotein in Antarctic fish. The antifreeze glycoproteins from two very different Antarctic fishes, Trematomus borchgrevinki and Dissostichus mawsoni, have very similar, if not identical, structures (10–16). Antifreeze glycoprotein is a collective name for a family of at least eight closely related glycoproteins and glycopeptides. They were named glycoproteins or glycopeptides 1 to 8 according to their relative migrations on gel electrophoresis. Glycoproteins 1 to 5 (the larger ones) have similar antifreeze activity. The active proteins have molecular weights of 10,500 to 30,000, although on a weight basis, they depress the freezing temperature of water to a greater extent than does NaCl. They also do not significantly affect the melting point. Active glycoproteins contain only two types of amino acids, alanine and threonine. The fundamental structure is that of a glycotripeptide composed of two alanine residues followed by a threonine residue with the sugars glycosidically linked to the threonine residues. The disaccharide is galactosyl-N-acetylgalactosamine with a β, 1 → 3 internal linkage. This fundamental unit is repeated approximately 17 to 50 times in the active molecules. One or two alanine residues are added at the COOH-terminal of each polymer.

Glycopeptides 6 to 8 (the smaller components) have little antifreeze activity alone. They also differ by containing proline residues following some of the threonine residues (13).

Much less information has been available for the structures of the Arctic antifreeze proteins as compared to those of the Antarctic ones. It has been shown that the glycoprotein of E. gracilis has a composition qualitatively similar to the antifreeze glycoproteins from the Antarctic species, but definitive structures have not been reported (6, 8, 17). The winter flounder antifreeze protein, however, has been reported to be very different by having no carbohydrates, but it does have a nearly identical percentage of alanine in the peptide (approximately 66%). Carboxyl groups were present in the form of both aspartic and glutamic acids (7, 8).

We are presently reporting on the freezing characteristics of fish sera from different parts of the Arctic area, the Barents Sea north of Norway, and the Arctic Ocean, north of the Russian islands, Novaya Zemlya, at approximately 62°E and 78°N. One species, the polar cod (Boreogadus saida), was found to have proteins so similar to the Antarctic samples in structure and composition that we have also called it antifreeze glycoprotein and glycopeptide. Certain characteristics were, however, notably different.

RESULTS

Compositions and Freezing Characteristics of Sera—The identified ninhydrin-reacting constituents in the blood sera of six pooled samples from Mallotus villosus and B. saida caught in the Barents Sea are reported in Table 1M. The average of the total constituents of the serum of M. villosus is 1.32 ± 0.29 mmol/ml, 78M-174, cite author(s) and include a check or money order for $1.95 per set of photocopies.
was approximately 61% that of *B. saida*, but most of the constituents were present in approximately similar percentages. Only one of the constituents was appreciably greater in the *B. saida* serum: phosphoserine, which was nearly 40 times higher than in the serum of *M. villosus*. Also, the concentrations of constituents such as lysine and arginine were different, being two and three times greater, respectively. However, the values should be interpreted with caution, since the diet and physiological condition of the fishes may have a large influence on the concentrations of such substances in the blood.

Only one of the four species of fish investigated, *B. saida*, contained an antifreeze protein in its serum as evidenced by a freezing temperature less than -1.9°C before dialysis and less than -0.6°C after dialysis (Table IIIM).

**Purification and Properties of Antifreeze Glycoproteins from Serum of *B. saida*—**All pooled samples of sera from *M. villosus* and *B. saida* were analyzed by disc gel and SDS-gel electrophoresis stained with Coomassie brilliant blue and on slab gels which were stained for carbohydrate with the a-naphthol-H$_2$SO$_4$ method (10, 19). Only the *B. saida* serum showed the presence of major carbohydrate-rich components. These components stained and migrated to the approximately same extent as did antifreeze glycoproteins in the serum of *T. borchgrevinki*.

The isolation of the antifreeze glycoprotein from the *B. saida* serum on DEAE-cellulose columns using the same procedure as previously described (10) gave an elution profile very similar to that reported for *T. borchgrevinki*. However, significant differences were found in the electrophoretic patterns of the isolated preparations. The electrophoretic migrations of the active antifreeze glycoprotein fraction from *B. saida* showed a different proportion of the components 1 to 5 than the ones from the *T. borchgrevinki* (Figs. 1A and 2). The less active components 6, 7, and 8, however, migrated electrophoretically to the identical locations regardless of the species of fish.

The electrophoretic patterns of an initial separation of antifreeze glycoprotein from *B. saida* serum with DEAE-cellulose is seen in Fig. 1B. Each of these initial fractions were then rechromatographed or further fractionated by preparative electrophoresis to yield purified samples of active antifreeze glycoprotein fractions 3, 4, and 5, and smaller fractions 7 and 8.

Antifreeze samples analyzed by electrophoresis in the absence of borate in the buffer system resulted in very poor resolution and no distinct bands such as those observed in Figs. 1 and 2.

**Composition and Structures of Antifreeze Glycoproteins—**Comparisons of analyses for amino acids and carbohydrates in active and smaller antifreeze glycoprotein preparations from *T. borchgrevinki* and *B. saida* showed similar compositions for corresponding fractions (Table IIIIM).

The dansylation procedure for the NH$_2$-terminal residue was used on the active antifreeze glycoprotein and glycopeptide 8 from both *B. saida* and *T. borchgrevinki*. The derivatives from both species were identical (alanine) for the active forms by polyamide thin layer chromatography and for the glycopeptide 8 fraction by silica gel thin layer chromatography. The COOH-terminal residues were demonstrated by hydrazinolysis and treatment with carboxypeptidases to be alanine for both *T. borchgrevinki* and *B. saida* antifreeze glycopeptides 7 and 8.

Automated sequenator analyses of the purified preparations for the first 12 residues of active antifreeze glycoprotein prep-

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5 The abbreviations used are: SDS, sodium dodecyl sulfate (lauryl sulfate); DFP, diisopropyl fluorophosphate; dansyl, 5-dimethylaminonapthalene-1-sulfonyl.
The proline residues always followed threonine residues in the glycopeptides 8 of both species, but the glycopeptide 8 of \textit{B. saida} had proline residues in only two positions, the 4th and the 10th, while glycopeptide 8 of \textit{T. borchgrevinki} never had proline in position 4 and was a heterogeneous mixture with proline residues in positions 7, 10, and 13 (13).

The galactose and galactosamine in glycopeptides 7 and 8 of \textit{B. saida} and \textit{T. borchgrevinki} were present in the same amounts as the threonine (Table IIM). Galactose was the only hexose present by thin layer chromatography and was quantitated in the samples using galactose as the standard. Galactosamine was identified and quantitated by the column chromatography and further identified by thin layer chromatography as the only amino sugar present. The \( \beta \) elimination results for glycopeptides 8 from the two species are presented in Table IVM. There is a parallel decrease of threonine along with a comparable increase in the amount of \( \alpha \)-amino butyric acid, both of which appear to plateau after 15 h of reaction at 45°C. Fig. 4M shows the formation of chromogens when \( \text{N-acetylgalactosamine} \) and glycopeptides 8 from the two species are treated with alkali. All three appear to reach a maximum after 3.5 h. When samples at various time intervals were treated by \( \text{I}_{2}/\text{KI} \) and the subsequent reagents, a maximum absorption after approximately 9 min was observed with each aliquot.

Active antifreeze glycoprotein, and glycopeptides 7 and 8 from the two species of fish were first digested with elastase and then treated with \( \beta \)-galactosidase. In all cases, galactose was the only free sugar observed by thin layer chromatography.

\textbf{Antifreeze and Antilectin Activities} \hspace{1cm} Active antifreeze glycoprotein fractions from \textit{T. borchgrevinki} and \textit{B. saida} lowered the freezing temperature similarly (Fig. 5M). As previously reported (10), antifreeze glycoprotein functioned additively with \( \text{NaCl} \). Active antifreeze glycoprotein of the two species also similarly inhibited hemagglutination by osage orange lectin. Essentially no inhibition was given by either of the smaller antifreeze glycopeptides 8, in agreement with previous reports for the antifreeze glycopeptide 8 of \textit{T. borchgrevinki} (33–35).

The antifreeze and antilectin activities of the antifreeze glycopeptides in both species were inhibited similarly by the addition of borate and inactivated similarly by hydrolysis with elastase. Results from elastase hydrolysis are seen in Fig. 1A and Table VM. The active antifreeze glycoprotein from both species still retained some freezing temperature depressing activity after 24 h of hydrolysis. This was decreased by extending the incubation to 48 h. Comparison of electrophoretic patterns of active antifreeze glycoprotein after the two incubation periods also indicated that some residual native protein was still present after the 24-h incubation time. The gel patterns showed in all cases that the Sephadex C-10 Fraction 2 (when collected) produced faster migrating spots than the corresponding Fraction 1. The same relative migrations were found when the incubation period was increased from 24 to 48 h. The active antifreeze glycoprotein from both species gave similar or identical migration spots after digestion. The smaller fractions 7 and 8 from both species also migrated similarly before hydrolysis but migrated differently after hydrolysis.

\textbf{DISCUSSION}

Of the four species investigated for freezing temperature, only the \textit{B. saida} (Table IIM) retained a significant depression (33.6%) of the freezing temperature in its serum after dialysis. \textit{B. saida} was shown to contain a nondialyzable “antifreeze.” The other fish were therefore similar to the human, trout, and salmon, in all of which all the substances lowering the freezing temperatures also dialyzed out (36). Conductivity measurements on the undialyzed serum (Table IIM) were approximately one-fourth higher for the \textit{B. saida} serum than for the others. Estimates of the freezing temperature depressions caused by conducting substances calculated as \textit{NaCl} are also listed in Table IIM.

The dialyzable factors lowering the freezing temperature would appear to be mainly \textit{NaCl}, other salts, and low molecular weight organic compounds such as amino acids, sugars, and urea (3, 37–40). The assumption that the freezing temperature calculated from the conductance was mainly due to \textit{NaCl} was based upon the very large amount of \textit{NaCl} present in Antarctic fish sera (37). The amount of potassium was only 0.64% of the total molarity of sodium, potassium, and chloride, determined from the average of two groups of four fish sera analyzed which were caught in the area of the Signy and Saint Georgia Islands. Data on two species of fish caught in the cold waters (\(-1.7°C\)) by Labrador also indicated that the predominant ions were \textit{NaCl} (3).

Not only did the \textit{B. saida} serum contain antifreeze glycoprotein but also the highest amount of \textit{NaCl}, 14.5 g/liter. This value is very similar to the amount calculated from the data on antifreeze glycoprotein-containing Antarctic fish (40). The lower freezing temperature of the serum of \textit{B. saida} is in agreement with the general occurrence of this species in more northern latitudes.

The distributions and values for the amounts of amino acids in the serum of \textit{M. villosus} were in the general ranges of those for either ocean or freshwater chum salmon (\textit{Oncorhynchus keta}) (38) or for trout (39). The values for \textit{B. saida} were significantly higher, but the distributions were not different in any ways that could be interpreted to influence the freezing temperature.

The antifreeze glycoproteins from the Arctic \textit{B. saida} and the Antarctic \textit{D. mawsoni} and \textit{T. borchgrevinki} were similar.
in most all respects. The exceptions were the relative amounts and migration of the different sized active components (Numbers 1 to 5), the much higher amount of proline in the smaller glycopeptide 7 of B. saida, and the positions of the proline residues in the sequences in the smallest existing component, glycopeptide 8. Glycopeptide 8 from B. saida was also mainly homogeneous, whereas that from T. borchgrevinki was a mixture of at least three glycopeptides as shown in Fig. 3 (13). The differences in the proline residues may prove significant in understanding the biosynthesis and function of these smaller components.

The close similarities between the structures and functions of antifreeze glycoprotein from B. saida and the Antarctic D. mawsoni and T. borchgrevinki, as well as possibly the Arctic saffron cod (E. gracilis) (8), suggest evolutionary homology. The similarities and the dissimilarities of these with the "hydrophobic," non-glycoprotein antifreeze proteins from some other Arctic species (7, 8, 16), however, cannot be so simply interpreted. The non-glycoprotein antifreeze protein from the winter flounder (P. americanus), for example, has the identical relative amount of alanine in the protein as does the antifreeze glycoprotein (two out of every three amino acids), but it apparently has mixtures of other amino acids which substitute for the functions of the carbohydrate of antifreeze glycoprotein (7, 8, 15, 16). The similarities and dissimilarities between these interesting antifreeze proteins suggest the need for further studies of other species of fish from ice saltwater environments.

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Note Added in Proof—In recent results of our laboratory (D. T. Osuga, Y. Yeh, F. Ward, and R. E. Feeney, unpublished data, 1978), an additional similarity has been found between the characteristics of antifreeze glycoproteins from T. borchgrevinki and B. saida. When the smallest component glycopeptide 8 (termed inactive because it has no antifreeze activity when tested alone) from either species was added together with smaller amounts of the larger active glycoproteins 1 to 5 of either species, a potentiation of the antifreeze activity of the larger one was observed.

REFERENCES

The references are on p. 5342.
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EXPERIMENTAL PROCEDURES

Materials—Active (5) and inactive (7 and 8) antifreeze glycoproteins from the fish serum of Thalassemus bulleri were prepared according to the method described in the experiment (2).

The Arctic fish for the experiments were caught by gill nets from a marine research vessel in the Sea of Okhotsk, Japan, during September-October of 1972. The nets were placed about 10 km from shore, and the water temperature was about 4°C. The fish were immediately removed from the nets and placed in large holding tanks. There was extensive and continuous pumping of fresh cold water through the tanks until all fish had been caught. Each fish was kept alive in a small tank, under an ice-cold surface layer of liquid water, and carried to the laboratory where they were immediately killed by immersion in absolute alcohol. The antifreeze glycoproteins from the polar cod were isolated in a manner similar to that described for the Antarctic glycoprotein from Diaphus et (3). Thawed serum was centrifuged at 4°C for 20 min to separate the glycoprotein fraction, followed by a 10% sucrose gradient in a 1-ml column. The active fractions were then identified by preparative gel electrophoresis (4) and used for further analysis.

For the preparation of the antifreeze glycoproteins, the fish were caught in the same manner as described above. The serum was then collected and pooled, and the antifreeze glycoproteins were isolated as described above.

The antifreeze glycoproteins from the pool cod were isolated in a manner similar to that described for the Antarctic glycoprotein from Diaphus et (3). Thawed serum was centrifuged at 4°C for 20 min to separate the glycoprotein fraction, followed by a 10% sucrose gradient in a 1-ml column. The active fractions were then identified by preparative gel electrophoresis (4) and used for further analysis.

Conductivity Measurements—An ice bath was prepared by mixing 9 g of sodium azide and 40 ml of 0.1 M sodium hydroxide. The solution was cooled to 0°C and mixed with 1.2 ml of 0.1 M carbonate buffer (pH 9.0) and 4.8 ml of 1 M sodium chloride. The mixture was then stirred for 10 min and filtered through a 0.2-μm-pore-size cellulose acetate filter. The conductivity of the solution was measured using a conductivity meter (Eico, Inc., Tokyo, Japan).

The antifreeze glycoproteins were then isolated and analyzed by gel electrophoresis in 0.1 M sodium carbonate buffer (pH 9.0) using 0.4 M acrylamide and 0.06% sodium dodecyl sulfate. The gels were stained with Coomassie Blue R-250 and the bands were visualized using a UV transilluminator.

Determination of Antifreeze Activity—The antifreeze activity of the glycoproteins was determined by a turbidimetric method using a 10% suspension of ice crystals in the presence of the glycoproteins. The samples were incubated at 4°C and the degree of turbidity was measured at 650 nm using a spectrophotometer (Varian, Model Cary 100, Palo Alto, CA).

The antifreeze activity was then determined by incubating the samples with ice crystals at 4°C and measuring the rate of crystal growth using a polarimeter (Perkin-Elmer, Model 241, Norwalk, CT). The antifreeze activity was expressed as the percent inhibition of crystal growth compared to the control (no glycoproteins).

Reference

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Table 1

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