Antifreeze Glycoproteins from Antarctic Fish

INACTIVATION BY BORATE*

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Antifreeze glycoprotein, which has previously been shown to be inactive in the presence of borate, migrates electrophoretically as the borate complex, presumably through formation of borate complexes with hydroxyl groups on the sugar side chains. Antifreeze glycoprotein (0.5 mg/ml) has been found to be completely active in the presence of 0.1 M borate at pH 7, but inactive at pH 9. A titration curve of pH versus the antifreeze activity of glycoprotein (5 mg/ml) in 0.1 M borate showed a progressive decrease in antifreeze activity as the pH was increased. Concomitant with decreases in activity were increases in binding of borate. At pH 9.0, nearly 2 mol of borate were complexed per glycoprotein. Ultracentrifuge analyses showed similar molecular weights and laser quasi-elastic light scattering showed similar diffusions at pH 7.0 and 9.0 in borate and in the absence of borate. The binding of borate, rather than a change in conformation, is thus directly related to the loss of antifreeze activity. Alkaline borate also decreased hemagglutinating activity of Osage orange lectin and decreased the inhibition of the activity by the antifreeze glycoproteins.

Glycoproteins from the blood sera of several Antarctic fish function as antifreeze agents (1, 2). These antifreeze glycoproteins consist of repeating units of a glycotripeptide composed of the tripeptide, Ala-Ala-Thr, with all threonines glycosidically linked to the disaccharide, 6-O-galactopyranosyl-(1 → 3)-N-acetyl-D-galactosamine (3, 4). Three active glycoproteins have molecular weights ranging from approximately 11,000 to 23,000 as determined by ultracentrifugation, light scattering, or osmotic pressure, but have molecular weights of only approximately 20 by determination of the freezing temperature of their aqueous solutions (5).

Studies on structural requirements for antifreeze activity have indicated that both the carbohydrate side chains and the polypeptide chain are important (2). Chemical modifications of the carbohydrate residues by acetylation of the hydroxyl groups caused loss of antifreeze activity. Oxidation of the C-6 primary alcohols of the galactose to aldehyde did not cause any loss in antifreeze activity, while positioning a negative charge on position C-6, either by further oxidation of the aldehyde groups to carboxyl groups or by bisulfite addition, caused complete loss of antifreeze activity. Complexing of borate (5) with the carbohydrate moiety also caused loss in antifreeze activity (6, 7). The mechanism by which these glycoproteins function as antifreeze has not been proven, but the functional groups responsible for the activity appear to be the hydroxyl groups of the carbohydrate side chains (2).

In this paper, further studies on the inactivation of the antifreeze activity of the glycoprotein by complexing of borate are described. Associated studies on inactivation of the antilec- tin activity (7, 8) are also described.

EXPERIMENTAL PROCEDURE

Materials—Antifreeze glycoprotein was purified from the blood serum collected from Trematomus borchgeruinki caught in Antarctica as previously described (5). Three types (5) of antifreeze glycoprotein were used in these studies: a mixture of the active glycoproteins 1 to 4; purified active glycoprotein 4; and purified inactive glycoprotein 7. Unless otherwise noted, experiments were done with the mixture of active glycoproteins 1 to 4. Osage orange seeds were purchased from Herbst Brothers Seedmen Inc., New York. D-Galactose oxidase was obtained from Sigma Chemical Co. Other reagents were of analytical or reagent grade.

Preparation of Glycoprotein Polyaldehyde—The primary alcohol groups of the disaccharide part of the antifreeze glycoprotein were enzymatically oxidized to aldehyde groups using D-galactose oxidase. The oxidation and determination of the extent of oxidation of galactose and N-acetylgalactosamine were carried out as described previously (3).

Effect of Borate on Diffusion Coefficients and Molecular Weights—Diffusion coefficients of antifreeze glycoproteins 4 and 7 were determined in the presence and absence of borate using both quasi-elastic light scattering (9) and ultracentrifugation (10). For these determinations, 5 mg/ml solutions of antifreeze glycoprotein in water/0.1 M borate at pH 7.0 and pH 8.6 were used. Determination of Borate Bound to Antifreeze Glycoprotein—The amount of borate bound to antifreeze glycoproteins 1 to 4 at pH 8.0 and 9.0 was determined using a dialysis technique. Glycoprotein solutions (15 mg/ml) were introduced inside dialysis bags and then dialyzed against 2 ml of borate solution (0.075 M) at pH 8.0 and 9.0 at room temperature. Preliminary experiments showed that equilibrium was reached in less than 48 hours. Aliquots from outside the dialysis...
bages were taken after 48 and 72 hours for triplicate borate determinations. Control experiments were done in absence of glycoprotein. The borate was determined spectrophotometrically at λ = 585 nm using the eosin reagent method (11). These determinations were then repeated in order to approach equilibrium from the opposite direction. This was done by placing the glycoprotein in borate inside the dialysis bages and dialyzing against water. All experiments were done in duplicate with triplicate analyses on each aliquot of the solutions.

**Effect of Borate on Antifreeze Activity of Antifreeze Glycoprotein and Its Polyaldehyde**—Solutions of glycoproteins 1 to 4 in 0.1 M borate or 0.1 M phosphate solution (5 mg/ml) in the pH range of 7.0 to 8.8 were used to determine the effect of borate and pH on antifreeze activity. The antifreeze activity was measured with high precision osmometer (Advanced Instruments, Inc., Newton Highlands, Mass.) as previously described (3). The instrument determines the freezing temperature by sensing the heat of fusion on freezing.

Effect of Borate on Lectin and Antilectin Activities—Preparation of partially purified Osage orange lectin as well as the hemagglutination assay was carried out as previously described with sheep red cells (7, 8). Lectin solution (7 mg/ml in 0.9% NaCl) was used to prepare a series of dilutions using 0.9% NaCl solution. One drop of the lectin solution was mixed with 1 drop of 0.5 M borate or 0.5 M phosphate buffer in the pH range of 7.0 to 9.0. Borate solutions in the concentration range of 0.025 to 0.4 M were also used. The hemagglutination assay was then continued as before (7, 8). Dilution of the lectin solution was carried out until an additional 2-fold dilution did not result in macroscopic agglutination. The minimal amount of lectin in milligrams required for hemagglutination was used to represent the lectin activity.

Antilectin activities of antifreeze glycoprotein and its polyaldehyde were determined (7) using a series of dilutions of antifreeze glycoprotein solution (1 mg/ml). One drop from each antifreeze glycoprotein or its polyaldehyde solutions, lectin solution (0.5 mg/ml), and borate or phosphate buffer (0.5 M) in the pH range of 7.0 to 9.0 was incubated for 5 min. Dilutions of antifreeze glycoprotein solution were continued until macroscopic agglutination occurred. The minimal amount of glycoprotein in milligrams required to inhibit hemagglutination was used to represent the antilectin activity of antifreeze glycoprotein solutions.

**RESULTS**

Effect of pH on Borate Inactivation of Antifreeze Activity of Glycoprotein—Previous studies on the effects of borate on the antifreeze glycoproteins were done with alkaline borate buffers for electrophoresis of the complex (5, 12) or with sodium tetaborate (at approximately pH 9.0) for demonstrations of inactivation (6, 7). Since pH might affect both the equilibrium of complexing of borate with carbohydrates and the types of complexes formed (13, 14), the effect of pH on the inactivation of antifreeze glycoproteins by borate was determined (Fig. 1; Table I). There was gradual decrease in antifreeze activity from pH 7.0 to approximately pH 8.4, at which point about one-half of the activity had been lost. This was followed by a sharp decrease from pH 8.4 to pH 8.8. Similar effects were obtained when antifreeze glycoprotein polyaldehyde was used (Table II).

Effect of pH on Amount of Borate Bound to Antifreeze Glycoprotein—The amount of borate bound to the antifreeze glycoprotein was strongly influenced by the pH of the solution between pH 7.0 and pH 9.0. The number of moles of borate bound per disaccharide unit was only about 0.5 at pH 8.0 but approached 2 at pH 9.0 (Table III).

![Fig. 1 (left). Effect of pH on antifreeze activity of antifreeze glycoprotein in the presence of borate. Antifreeze glycoproteins 1 to 4 (5 mg/ml) were measured in 0.1 M phosphate and 0.1 M borate and adjusted to the pH values indicated. The freezing temperatures of the solutions were measured by the sensing of the heat of fusion. The values for the freezing temperatures of control solutions were subtracted from the values for samples containing the antifreeze glycoprotein (see Table I).](http://www.jbc.org/)

![Fig. 2 (center). Effect of pH on hemagglutination by lectin in the presence of borate. Hemagglutinations (7) were done in solutions of approximately 0.1 M phosphate and 0.1 M borate at the indicated pH values. The minimal amount of lectin in micrograms required for hemagglutinations was used to represent lectin activity.](http://www.jbc.org/)

![Fig. 3 (right). Effect of pH on the antifreeze glycoprotein inhibition of hemagglutination by lectin in the presence of borate. Hemagglutinations (7) were done in solutions of approximately 0.1 M phosphate and 0.1 M borate at the indicated pH values. Different amounts of antifreeze glycoprotein were mixed in the appropriate buffers with twice the amount of lectin (70 μg) which was necessary to give hemagglutination at pH 9.0 in the presence of borate. These mixtures were then added to the erythrocytes.](http://www.jbc.org/)
TABLE I

Effect of pH on antifreeze activity of antifreeze glycoprotein in presence of borate

| pH  | Freezing temperature of 0.1 M borate (A) | Freezing temperature of antifreeze glycoprotein in 0.1 M borate (B) | Freezing temperature due to antifreeze glycoprotein (B - A) | Activity (%) |
|-----|----------------------------------------|---------------------------------------------------------------|----------------------------------------------------------|-------------|
| 7.0 | -0.277± | -0.754± | -0.477± | 97.9 |
| 7.4 | -0.273± | -0.738± | -0.465± | 95.5 |
| 7.6 | -0.262± | -0.715± | -0.453± | 93.0 |
| 7.8 | -0.256± | -0.688± | -0.432± | 88.7 |
| 8.0 | -0.249± | -0.655± | -0.403± | 80.7 |
| 8.3 | -0.238± | -0.558± | -0.320± | 65.7 |
| 8.4 | -0.219± | -0.466± | -0.247± | 50.7 |
| 8.5 | -0.214± | -0.367± | -0.133± | 31.4 |
| 8.6 | -0.206± | -0.238± | -0.032± | 6.6 |
| 8.8 | -0.188± | -0.206± | -0.018± | 3.7 |

Table II

Effect of pH on antifreeze activity of polyaldehyde of antifreeze glycoprotein in presence of borate

| pH  | Freezing temperature of 0.1 M borate (A) | Freezing temperature of antifreeze glycoprotein in 0.1 M borate (B) | Freezing temperature due to antifreeze glycoprotein (B - A) | Activity (%) |
|-----|----------------------------------------|---------------------------------------------------------------|----------------------------------------------------------|-------------|
| 7.0 | -0.280± | -0.671± | -0.391± | 97.8 |
| 7.6 | -0.298± | -0.630± | -0.362± | 90.5 |
| 8.2 | -0.229± | -0.460± | -0.240± | 60.8 |
| 8.8 | -0.203± | -0.227± | -0.024± | 8.6 |

* The extent of oxidation is 70% for galactose and 12% for N-acetylgalactosamine. The antifreeze activity of the antifreeze glycoprotein polyaldehyde was 82.1% of the native.

†The freezing temperature of the antifreeze glycoprotein polyaldehyde (5 mg/ml) in water was -0.400±, which was used as 100% antifreeze activity.

Table III

Effect of pH on binding of borate to antifreeze glycoprotein

| pH  | Mol of free borate (x 10^-6) | Mol of bound borate (x 10^-6) | Mol of borate bound per mol of disaccharide in glycoprotein |
|-----|------------------------------|-------------------------------|----------------------------------------------------------|
| A   | B   | A    | B    | A         | B         |
| 8.0 | 14.03 | 13.91 | 0.97 | 1.09 | 0.42 | 0.47 |
| 9.0 | 10.96 | 10.66 | 0.45 | 0.34 | 1.75 | 1.88 |

* A, experiment started with 15.0 x 10^-4 mol of borate and 2.31 x 10^-4 mol of antifreeze glycoprotein 1 to 4 (calculated as a trimer of repeating glycopeptide) in 1 ml of solution inside the dialysis bag which was dialyzed against 2 ml of water.

†B, experiment started with 2.31 x 10^-4 mol of antifreeze glycoprotein 1 to 4 in 1 ml of water inside the dialysis bag which was dialyzed against 15.0 x 10^-4 mol of borate in 2 ml of water.

**DISCUSSION**

Although the complexing of borate to hydroxyl groups with the necessary stereochemical relationships on carbohydrates might be considered a part of classical carbohydrate chemistry, the need for much more quantitative information in such critical areas as equilibria and kinetics of interaction has recently been noted by Acree (15). Even less information is apparently available on the interactions of borate in biological systems and the properties of the complexes. Techniques such as the use of magnetic resonance are now beginning to provide some of this information (14).

When tetraborate salts (e.g., Na₂B₄O₇) are added to solutions of aliphatic organic polyhydroxyl compounds which satisfy certain spatial requirements, the pH of the solution decreases and borate ion is bound to the hydroxyl groups (15). In acidic solutions the product is described as a borate anion complexed to two hydroxyl groups (Fig. 4A). In more basic solutions, the amount of complex increases and one borate, still remaining as an anion, can complex with four hydroxyl groups (Fig. 4B) and thus form cross-linkages (14, 15).

The antifreeze glycoprotein is inactive when two borate anions are complexed per glycotripeptide. We did not show, however, where these borate anions were bound. Inspection of ball and stick models (Dreiding) of the carbohydrate side chain constructed in our laboratory indicated that the borate might bind to the 3,4 or 4,6 hydroxyl groups of the terminal galactose residues and to the 4,6 hydroxyl groups of the N-acetylgalactosamine residues. The C-6 hydroxyl groups of the galactose residues do not appear to be essential for activity as shown by the retention of antifreeze activity by the polyaldehyde antifreeze glycoprotein in which oxidation had occurred at approximately 80% of the C-6 hydroxyl groups of the N acetylgalactosamine residues and only 20% of the C-6 hydroxyl groups of the N acetylgalactosamine residues.
Borate Inactivation of Antifreeze Glycoprotein

The molecular weights are approximately 19,000 and 4,400 for antifreeze glycoprotein 4 and 7, respectively.

| Buffer       | Ultra-centrifugation | Quasi-elastic light scattering |
|--------------|----------------------|--------------------------------|
|              | $v_{max} \times 10^{12}$ | $D_{max} \times 10^7$ |
| Anti-freeze glycoprotein 4 |                        |
| H$_2$O      | 1.48                  | 5.64                          |
| 0.01 M borate at pH 7.0 | 1.43                  | 5.09                          |
| 0.01 M borate at pH 10.0 | 1.44                  | 4.97                          |
| Anti-freeze glycoprotein 7 |                        |
| 0.01 M borate at pH 7.0 | 1.65                  | 11.60                         |
| 0.01 M borate at pH 10.0 | 1.33                  | 4.80                          |
| Anti-freeze glycoprotein 7 |                        |
| 0.01 M borate at pH 7.0 | 1.48                  | 9.30                          |
| 0.01 M borate at pH 10.0 | 1.44                  | 9.10                          |

Possible mechanisms for the lowering of the freezing temperature by the antifreeze glycoprotein have been recently reviewed (2). Those involving interaction of the glycoprotein at an ice-water interface appear among the most plausible possibilities (2, 7, 8, 19–21). The borate complexes could prevent this interaction by simple physical interference with fittings on the surface or by blocking of the hydroxyls, as well as by the insertion of a negative charge. The inactives of the C-6 carboxylic acid derivatives of the galactose residues and the bisulfite adducts of the C-6 polyaldehyde of the galactose residues (3, 7) also indicate that the negative charge could cause inactivation.

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