Antifreeze Glycoprotein from an Antarctic Fish

EFFECTS OF CHEMICAL MODIFICATIONS OF CARBOHYDRATE RESIDUES ON ANTIFREEZE AND ANTILECTIN ACTIVITIES

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SUMMARY

Structural requirements for antifreeze and antilectin activities of the antifreeze glycoproteins from the Antarctic fish Trematomus borchgrevinki have been compared. Graded removal of different amounts of the disaccharide side chain by β elimination caused similar reductions in the capacities for depressing the freezing temperature of water (antifreeze activity) and for inhibiting hemagglutination by Osage-orange lectin (antifreeze activity). Both activities were also reduced to nearly similar extents by acetylation of hydroxyls of carbohydrate residues, positioning a negative charge on the C-6 carbons of the carbohydrate, or by formation of borate complexes. But oxidations of the C-6 hydroxyls of the carbohydrate to aldehyde groups caused no reductions of either activity. Structural similarities required for both activities therefore exist.

Antarctic fishes living at ambient water temperatures as low as −1.9° resist freezing partly because of the presence of a unique group of glycoproteins in their serum (1−10). These antifreeze glycoproteins consist of repeating units of the glycotripeptide Ala-Ala-Thr with all the threonine glycosidically linked to a galactosyl-N-acetylgalactosamine disaccharide. The internal linkage is β,1→3 (4) or β,1→4 (5). Three active glycopeptides characterized by physical and chemical methods have molecular weights ranging from 10,500 to 21,000 g as determined by ultracentrifugation, light scattering, or osmotic pressure measurements. The freezing temperature depression is therefore not due to a colligative property. It has been suggested that a probable mechanism may involve action of AFGP at an interface separating solid and liquid during growth of ice crystals (4).

AFGP also inhibited the hemagglutinating activity of a lectin prepared from Osage-orange seeds (6). Glycopeptide fragments of AFGP had only very weak activity for inhibiting the hemagglutination (antilectin activity). Therefore, these preliminary observations indicated that both the antilectin and the antifreeze activities were associated with the properties of the intact molecule.

We are now describing the effects of various treatments and modifications of AFGP on both its antifreeze and antilectin activities. A high degree of similarity has been found in the sensitivities of both activities to the different treatments.

EXPERIMENTAL PROCEDURE

Materials—AFGP was purified from the fish blood serum collected from Trematomus borchgrevinki caught in Antarctica as previously described (8−10). AFGP was employed as a mixture of the Components 1 to 5 which differ only in molecular size (8). Sheep blood was supplied by the Veterinary School of the University of California, Davis. Osage-orange seeds were kindly provided by Dr. Joseph Chuha or purchased from Herbst Brothers Seedsmen Inc., New York. p-Galactose oxidase and sugar standards were obtained from Sigma Chemical Co., and reagent grade pyridine and acetanhydride from Fisher Scientific Co. Other reagents were of analytical or reagent grade.

Preparation of Crude Osage-orange Lectin—Osage-orange seeds were macerated in a Waring Blender and then stirred for 2 hours or overnight at 4° with 0.9% NaCl (10 volumes of solution per 1 g of seeds). The mixture was centrifuged for 35 min at 4° (15,000 × g), filtered, heated for 20 min at 65°, and centrifuged. The clear yellow supernatant was precipitated with 85% saturated ammonium sulfate and filtered, heated for 20 min at 65°, and centrifuged.

This term is used in lieu of the term freezing point depressing glycoprotein previously employed (1, 3, 4) because the temperature of freezing is depressed but the temperature of melting is not depressed (7). The physical definition of the freezing point is the temperature at which solid and liquid phases are in equilibrium (i.e., freezing and melting temperatures are identical) and this term is therefore incorrect for this system.
(NH₄)₂SO₄ and centrifuged. The precipitate was dissolved, dialyzed against water, and lyophilized. Further purifications of this crude product are described in the text.

β Elimination of O-Threonyl-linked Carbohydrates—The β elimination was done by a method adapted from Ballou (11) as follows. A solution containing 0.1 mg/ml of AFGP in 0.1 N NaOH was incubated at 20°. The reaction was monitored by following the absorption at 241 nm. Aliquots taken at different time intervals were neutralized with 0.1 M NaOH was incubated at 20°. The reaction was monitored by following the absorption at 241 nm. Aliquots taken at different time intervals were neutralized with 0.1 M HCl, desalted on a Sephadex G-25 column, and lyophilized.

The β elimination and loss of the sugar moiety were quantified by determining the loss of threonine (9, 12). The β eliminated samples were hydrolyzed with 6 N HCl at 110° for 22 hours according to the method of Moore and Stein (13). Amino acid analyses were done on a Technicon Auto Analyzer, using the the standard 21-hour run (14). Loss of threonine in the β eliminated samples was calculated by comparison with the threonine content in the hydrolysate of the native glycoprotein.

Periodate Oxidation—AFGP was oxidized with sodium periodate. Completion of the oxidation was shown by the disappearance of the galactose in the hydrolysate of the oxidation product as previously reported (9).

Oxidation of AFGP—The primary alcohol groups of the disaccharide part of AFGP were enzymatically oxidized to aldehyde groups, using N-galactose oxidase as described by Vandenheede et al. (4). The newly formed aldehyde groups were then oxidized in carboxyl groups with iodine under mild alkaline conditions (4, 15). The oxidized galactose and galactosamine were determined in the hydrolysate of the two-stage oxidized glycoproteins as described by Vandenheede et al. (4).

Complexing of β-Galactose Oxidase-treated AFGP with Bisulfite—Formation of a bisulfite adduct of β-galactose oxidase-oxidized AFGP was done at a concentration of 5 mg of glycoproteins per ml of 15 mM NaHSO₃ and at pH 3.8 (4, 16). For assays of hemagglutination inhibition, the dilution of the bisulfite adduct was done with a solution of 15 mM sodium bisulfite in 0.9% NaCl.

Acetylation and Borate Complex Formation of AFGP—The acetylation of the AFGP was done at a concentration of 0.25 mg per ml in a 1:1 mixture of pyridine and acetic anhydride. Samples were removed at intervals and lyophilized. The degree of acetylation was determined as described by Komatsu et al. (9). For formation of the borate complex, 5 mg of AFGP were dissolved in 1 ml 0.1 M sodium borate. The hemagglutination inhibition assays of the borate complex were done using 0.1 M sodium borate for the dilution as well as for the control.

Hemagglutination Inhibition Assay—The assay was run according to the method given by Chuba et al. (8, 17). The erythrocyte indicator solution was prepared by washing sheep erythrocytes three times with 0.9% sodium chloride followed by suspension in the saline to a final concentration of 2% (v/v). The crude extract of Osage-orange was diluted with 0.9% sodium chloride (1:8 titer) until an additional 1:8 fold dilution did not result in macroscopic agglutination with the erythrocyte indicator. Units of specific activity were calculated as the number of times required for dilution of a solution of the lectin (10 mg per ml) to give the least detectable agglutination.

In the test for antilectin activity, 1 drop of inhibitor solution (AFGP, its derivatives, or controls) previously diluted with 0.9% sodium chloride was added to 1 drop of 1:8 titer hemagglutinating reagent in separate tubes. The tubes were shaken and incubated for 5 min at room temperature, after which 1 drop of the erythrocyte indicator was added to each tube. The reaction mixture was incubated for 20 min at room temperature followed by centrifugation for 15 s at 1000 × g. The sedimented erythrocytes were gently resuspended (by shaking the tubes) and examined for clumping.

Determination of Antifreeze Activity—These measurements were made as previously described (4) with a high precision osmometer (Advanced Instruments, Inc., Newton Highlands, Mass.). The instrument determines the freezing temperature by sensing the heat of fusion on freezing. All samples were desalted on a Sephadex G-25 column, except the samples of bisulfite adduct. In all instances treated, control, and untreated samples were analyzed in series. Concentrations of samples used for the assay were 5 mg per ml. Calculations of activities were done by comparing lowerings of freezing temperatures against standard curves obtained with concentration of AFGP from 1 to 8 mg per ml.

RESULTS

Partial Purification of Osage-orange Lectin—Heating of the crude extract at 65° resulted in a loss of 95% of soluble protein without any detectable loss of hemagglutination activity. Also, neither dialysis nor freeze drying resulted in any loss of activity. Fractional ammonium sulfate precipitation was unsuccessful as a method for achieving consistently high specific activity, but precipitation at higher concentrations of (NH₄)₂SO₄ was extremely useful for concentrating the crude extracts. Fractionations of the precipitate by ion exchange chromatography on DEAE-cellulose and CM-cellulose were also not consistently successful. Fractionations by particle exclusion on Sephadex G-100 gave the best purifications (Table I). Several Sephadex G-100 experiments yielded specific activities >2000. These preparations however, were still impure by disc gel electrophoresis. Specific activities in routine larger scale preparations were usually 200 to 500.

β Elimination of AFGP—Incubation of AFGP in 0.1 N NaOH at 20° caused a rapid increase in absorbance at 241 nm, which

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>No.</th>
<th>Volume (ml)</th>
<th>Protein yield (mg)</th>
<th>Activity (Specific, Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>15</td>
<td>500</td>
<td>100</td>
<td>35, 17,500</td>
</tr>
<tr>
<td>1</td>
<td>110</td>
<td>14.5</td>
<td>2.8</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>104</td>
<td>35.6</td>
<td>7.0</td>
<td>64, 2,300</td>
</tr>
<tr>
<td>3</td>
<td>116</td>
<td>23.5</td>
<td>4.6</td>
<td>256, 6,000</td>
</tr>
<tr>
<td>4</td>
<td>217</td>
<td>29.6</td>
<td>5.8</td>
<td>256, 7,600</td>
</tr>
<tr>
<td>5</td>
<td>152</td>
<td>22.0</td>
<td>4.3</td>
<td>16, 350</td>
</tr>
<tr>
<td>6</td>
<td>361</td>
<td>385</td>
<td>75.4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Assays and activity values are described in text.
FIG. 1. Increase in absorbance at 241 nm on β elimination of carbohydrate from antifreeze glycoprotein. β Elimination was done with glycoprotein concentrations of 0.1 mg per ml in 0.1 N NaOH at 20° for the times indicated.

TABLE II
β Elimination
Relationship of losses of threonine to absorbance at 241 nm.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>ε&lt;sub&gt;241nm&lt;/sub&gt;</th>
<th>% Loss of threonine</th>
<th>Conversion factor</th>
<th>Calculated β elimination</th>
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</thead>
<tbody>
<tr>
<td>15</td>
<td>0.48</td>
<td>4.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>1.16</td>
<td>11.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>1.99</td>
<td>20.9</td>
<td>10.72</td>
<td>19.4</td>
</tr>
<tr>
<td>90</td>
<td>2.72</td>
<td>27.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>330</td>
<td>5.25</td>
<td>49.5</td>
<td>9.42</td>
<td>52.3</td>
</tr>
<tr>
<td>540</td>
<td>7.03</td>
<td>68.2</td>
<td>9.70</td>
<td>70.0</td>
</tr>
<tr>
<td>720</td>
<td>8.04</td>
<td>80.0</td>
<td>9.95</td>
<td>80.0</td>
</tr>
</tbody>
</table>

* 0.1 mg of glycoprotein per ml of 0.1 N NaOH at 20°.

gradually leveled off (Fig. 1). Samples with different ε<sub>241nm</sub> were used for amino acid analysis to determine the loss of threonine. A factor of 9.96 was used for converting ε<sub>241nm</sub> to percentage of β elimination (Table II). Both antilectin and antifreeze activities decreased as the extent of β elimination increased (Fig. 2).^2

Effect of Various Types of Oxidations on Antilectin and Antifreeze Activities—Periodate oxidation of the galactose residues of the disaccharide moieties caused extensive loss of both activities (Table III). In contrast, when the primary alcohol groups of C-6 of the carbohydrate moieties were oxidized to aldehyde groups with α-galactose oxidase, over 85% of both activities were still present. The extent of oxidation was 15 and 70% for N-acetylgalactosamine and galactose, respectively. However, when the aldehyde groups of this active product were either oxidized to the carboxyl groups or complexed with bisulfite, both the antilectin and antifreeze activities were lost. Only approximately 15% of both activities remained after oxidation to carboxyl groups or on the addition of bisulfite to the aldehyde derivative.

Effect of O-Acetylation of Hydroxyl Groups—The acetylation of the hydroxyl groups of AFGP caused loss of both antilectin and antifreeze activities. There was, however, a disparity in the degree of activity loss which was not observed after the other modifications (Table III).

^2 Side reactions, such as scissions of peptide bonds, are possible under the alkaline conditions of β elimination, but they were not observed at least in the first half of the β elimination process.

FIG. 2. Inactivation of antifreeze and antilectin activities of antifreeze glycoprotein by β elimination of carbohydrate. Antilectin activity was determined by inhibition of Osage-orange lectin as described in the text. Antifreeze activities were determined by measurements of freezing temperatures as described in text. β Elimination was done with glycoprotein concentrations of 0.1 mg per ml in 0.1 N NaOH at 20°. Extent of β elimination was determined according to data of Fig. 1 and Table II.

TABLE III
Comparative effects of different treatments of antifreeze glycoproteins on antifreeze and antilectin activities

<table>
<thead>
<tr>
<th>No.</th>
<th>Reaction</th>
<th>Degree of modification</th>
<th>Activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Antilectin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Antifreeze</td>
</tr>
<tr>
<td>1</td>
<td>Oxidation of C-6 alcohols to aldehydes</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td>70</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>N-Acetylgalactosamine</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>Aldehyde-bisulfite complex^b</td>
<td>(Excess bisulfite)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Oxidation of C-6 to carboxyls^c</td>
<td>Galactose</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>N-Acetylgalactosamine</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>Periodate oxidation</td>
<td>80</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>Borate complex^d</td>
<td>(Excess borate)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Acetylation</td>
<td>80</td>
<td>9</td>
</tr>
<tr>
<td>7</td>
<td>β Elimination</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Condition given in text.
^b Bisulfite was added to oxidation product from No. 1.
^c Oxidation of oxidation product of No. 1.

Effect of Complexing of Hydroxyl Groups with Borate—Both antilectin and antifreeze activities were lost when AFGP was dissolved in 0.1 M borate solution. In all determinations, <10% of the original activity was found. Control experiments with erythrocytes and 0.1 M borate did not agglutinate in antilectin assay.

DISCUSSION

The nearly identical effects resulting from chemical treatments and formation of complexes of AFGP on its antifreeze and antilectin activities might suggest similar structural requirements for these two very different functions. These findings might well
be fortuitous, and any models supporting similar mechanisms would be purely speculative.

One possible mechanism for lowering the freezing temperature of water which involves an interaction at surfaces appears to account for properties that might also be required for interaction with lectin. Such a mechanism for lowering the freezing temperature has been suggested to involve an interface separating solid and liquid during growth of ice crystals (4, 18-20). In this mechanism the methyl groups on the side chains of alanine might form a hydrophobic core or layer and the experimentally essential hydroxy groups of the sugars might provide a very hydrophilic layer (4). In consideration of the abnormal depression of freezing temperature in relation to the melting temperature in certain cross-linked gel solutions, it has been proposed that the gel greatly retards or prevents the annealing of ice nuclei (or microcrystals) into ice crystals (19). A surface mechanism was postulated for prevention of this annealing process. However, any mechanism postulating function of the AFGP at surfaces is also speculative at this time. Models involving interaction with only the water phase appear less probable but they must still be considered (7).

Requirements for inhibition of the lectin include the poly-peptide structure (6) as well as the integrity of the polyhydroxy side chain groups of the carbohydrate and the absence of any charge on these side chains (e.g. the oxidation of the C-6 alcohol groups to carboxy groups or the formation of the bisulfite adducts). The structural requirements for the strong antilectin activities of AFGP therefore include multiplicity of combining sites on the polymer backbone. However, the requirement or lack of requirement of macromolecular structure for the function of other lectin inhibitors does not appear to be clearly shown at this time (21, 22). The small hydrophobic methyl groups would not interfere with these reactions, and the random coil characteristics (8) of the molecule might be suitable for approximation of the hydrophilic surfaces of the carbohydrate side chains to the surfaces of lectin molecules. Physical studies of the complexes with lectins obviously need to be made. Similarly, physical studies of the structure of the AFGP and its relationship to ice formation are underway and, of course, are essential in obtaining the information necessary to support this or other possible hypotheses.

The similarities in the structural requirements for activities also indicate that the antilectin assay can be a useful micromethod for studying the presence and concentrations of AFGP in polar fishes as well as in experimental systems (6).

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REFERENCES
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