The Structure of a Glycopeptide from Human Orosomucoid (α₁-Acid Glycoprotein)*

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SUMMARY

Four glycopeptides were isolated from desialized orosomucoid following digestion with Pronase and chromatography on sulfoethyl cellulose. The structure of the major glycopeptide was studied with sequential hydrolysis with β-galactosidase, β-acetylglucosaminidase, and α-mannosidase, as well as by oxidation with periodate.

Evidence for a glycosylamine type linkage between aspartic acid and acetylglucosamine was provided by isolation of 2-acetamido-1-fl-(L-p-aspartamido)-1,2-dideoxy-D-glucose (β-aspartyl acetylglucosaminylamine).

The major glycopeptide contained 4 moles of galactose, 3 of mannose, 6 of acetylglucosamine, and 1 each of aspartic acid and threonine. The molecular weight of the glycopeptide was 2540 by vapor pressure osmometry and 2840 as measured by forming the trinitrophenyl derivative.

Four moles of galactose followed by 4 moles of acetylglucosamine were released per mole of glycopeptide when it was treated sequentially with highly purified β-galactosidase from Phaseolus vulgaris and β-acetylglucosaminidase from bovine liver. This treatment resulted in the exposure of an inner core containing 3 moles of mannose and 2 of acetylglucosamine per mole of aspartic acid.

Two moles of mannose and 1 mole of acetylglucosamine were split from the inner core of the oligosaccharide with a crude α-mannosidase preparation from ivory nut, with or without added purified α-acetylglucosaminidase from bovine liver.

The intact glycopeptide and its “inner core” were oxidized with sodium periodate. The results from the oxidation and enzymatic studies revealed that the acetylglucosamine linked to asparagine served as a branch point to 2 mannose residues.

The likely structure of the glycopeptide is presented.

Since the isolation of human plasma orosomucoid (α₁-acid glycoprotein) in 1950 (1, 2), investigations on the sequence of monosaccharides and the nature of the carbohydrate-peptide linkage have been pursued in several laboratories (see Reference 3 for review).

Several reports indicate that the carbohydrate moiety of orosomucoid may consist of several oligosaccharide units of different size, composition, and structure (4-8). Hughes and Jeanloz (8-10) and Eylar and Jeanloz (11, 12), have provided evidence that the oligosaccharides terminate in a trisaccharide (sialyl or fucosyl-galactosyl-N-acetylglucosamine, or both). It has also been shown that the linkage between the oligosaccharide units and the peptide chain involves a bond between the reducing group of acetylglucosamine and the amide nitrogen of asparagine (5, 13-16). However, the structure of the oligosaccharide between the peptide-bound acetylglucosamine and the terminal trisaccharide has remained uncertain. The present studies have, therefore, been concerned with the structure of a major glycopeptide from orosomucoid.

EXPERIMENTAL PROCEDURE

Preparation of Glycopeptides from Orosomucoid—Orosomucoid was isolated from pooled human plasma by ion exchange chromatography (17). It was desialized by heating in 0.1 M HCl at 80° for 1 hour. The released sialic acid was removed either by extensive dialysis against cold water or by gel filtration on Sephadex G-25. The desialized orosomucoid was incubated with Pronase (50 mg of protein-1 mg of Pronase) in 20 ml of 0.1 M borate, pH 7.9, containing 0.01 M calcium acetate for 48 hours at 37°, essentially as described by Izumi, Makino, and Yamashina (18). The enzyme was removed by precipitation with 5% trichloracetic acid, and the supernatant was subjected to gel filtration on Sephadex G-25. The hexose-containing material...
in the void volume was purified by cellulose powder chromatography as described by Kamitani and Schmid (4). Recovery of the glycopeptide mixture thus obtained was 21 to 22% by weight of the parent glycoprotein and contained 95 to 100% of the hexose.

Separation of Glycopeptides—The glycopeptide mixture contained four distinguishable fractions as evidenced by high voltage electrophoresis at pH 3.7 in pyridine-acetic acid-water buffer. The mixture was chromatographed on sulfoethyl cellulose (H⁺ form) and eluted as shown in Fig. 1, resulting in the elution of four hexose-containing fractions designated GP I, GP II, GP III + IV, and GP V. The recovery of hexose from the column was 85 to 90%. The major glycopeptide (GP V) was eluted with 0.025 M citrate buffer at pH 3.0. After desalting on a mixed bed resin (MB-3), the preparation was lyophilized.

Chemical Determinations—Galactose, mannose, and fucose were quantitatively determined by gas-liquid partition chromatography with the alditol acetate method (19). For routine purposes, the neutral sugars were estimated by the orcinol reaction (20), with an equimolar mixture of galactose and mannose as standard. β-Galactose was also determined by oxidation with galactose oxidase (21) and reducing sugars by the Nelson method (22). Sialic acid was determined by the method of Spackman, Stein, and Moore (26) with a Spinco amino acid analyzer following hydrolysis of the glycopeptide or its derivatives with 6 N HCl for 16 hours at 115°C.

Determination of Molecular Weight—Molecular weights were determined by reaction with trinitrobenzenesulfonic acid (27), assuming an extinction coefficient of 1.05 × 10⁻⁴ for the trinitrophenol group. Molecular weights were also determined with the aid of a thermoelectric type vapor pressure osmometer (model 301A Medichab, Mountain View, California), with a sucrose standard.

High Voltage Electrophoresis—A potential of 35 to 43 volts per cm was generally used with Varsol as coolant. Electrophoresis was carried out at pH 3.7 in pyridine-acetic acid-water (1:10:250, v/v/v). Free amino groups were detected with ninhydrin, oligosaccharides with periodate-cuprate-para-rosaniline (28), and monosaccharides with alkaline silver nitrate (29).

Thin Layer Chromatography—Free sugars were separated on cellulose powder plates with n-butyl alcohol-pyridine-water (6:4:3, v/v/v), and detected by aniline-diphenylamine-phosphoric acid reagent (30).

RESULTS

The composition of glycopeptide V is given in Table I, which also gives the molar ratios of the sugars and amino acids with aspartic acid taken as unity. These data indicate a glycopeptide containing 1 mole of aspartic acid, 4 moles of galactose, 3 moles of mannose, 6 of acetylgalactosamine, and 1 of threonine. Fractional amounts of fucose and of other amino acids were present. The calculated molecular weight of such a glycopeptide is 2580. This is in reasonable agreement with a value of 2840 by the trinitrobenzene sulfonic acid method (27) and of 2540 by vapor phase osmometry.

Nature of Linkage between Carbohydrate and Peptide—It is already reasonably well established that the carbohydrate moieties of orosomucoid are linked to the protein through the reducing group of acetylgalactosamine and the amide nitrogen of asparagine (β-asparyl acetylgalactosaminylamine) (5, 13-16). This conclusion is further strengthened by the following observations.

1.Treatment of glycopeptide V with 0.0 N NaOH by the method of Anderson et al. (31) did not significantly change the ratio of aspartic acid to threonine (Table II). This eliminates the possibility that threonine is linked to carbohydrate through its hydroxyl group.

2. β-Asparyl acetylgalactosaminylamine could be isolated in reasonable yield essentially by the method of Bogdanov, Kaverzneva, and Andreyeva (32) as follows:

The glycopeptide was hydrolyzed with 2 N HCl for 20 min at 100°C. Quantitative determination of β-asparyl acetylgalactosaminylamine was accomplished by ion exchange chromatography of the hydrolysates, with the amino acid separation procedure (26). A synthetic preparation of the compound was used as the

<table>
<thead>
<tr>
<th>Component</th>
<th>Composition</th>
<th>Molar ratio to aspartate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose⁵</td>
<td>1.29</td>
<td>23.3</td>
</tr>
<tr>
<td>Mannose⁴</td>
<td>0.94</td>
<td>16.9</td>
</tr>
<tr>
<td>Fucose⁶</td>
<td>0.10</td>
<td>1.7</td>
</tr>
<tr>
<td>N-Acetyl-β-glucosamine⁷</td>
<td>2.01</td>
<td>45.6</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.32</td>
<td>4.2</td>
</tr>
<tr>
<td>Threonine⁸</td>
<td>0.28</td>
<td>3.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.09</td>
<td>0.7</td>
</tr>
</tbody>
</table>

⁵ Neutral sugars were quantitatively determined by gas-liquid chromatography (19).
⁶ Measured as hexosamine by the Good and Bessman method (24) and identified as glucosamine by ion exchange chromatography on a Spinco analyzer.
⁷ Uncorrected for destruction by acid hydrolysis.
Effect of mild alkaline treatment and of periodate oxidation and borohydride reduction on amino acids of glycopeptide V

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NaIO₄ added</th>
<th>Asp:Thr ratio</th>
<th>Threonine recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None (native GP V)</td>
<td>0</td>
<td>1:0.875</td>
<td>100</td>
</tr>
<tr>
<td>2. 0.5 N NaOH, 4°, 10 hrs</td>
<td>0</td>
<td>1:0.835</td>
<td>95</td>
</tr>
<tr>
<td>3. 30 °C, 2 min NaBH₄</td>
<td>2.5</td>
<td>1:0.505</td>
<td>58</td>
</tr>
<tr>
<td>4. 4 min NaIO₄, 10 min NaBH₄</td>
<td>6.3</td>
<td>1:0.478</td>
<td>55</td>
</tr>
<tr>
<td>5. 10 min NaIO₄, 20 min NaBH₄</td>
<td>12.5</td>
<td>1:0.496</td>
<td>57</td>
</tr>
<tr>
<td>6. 20 hrs NaIO₄, 20 hrs NaBH₄</td>
<td>40.0</td>
<td>1:0.492</td>
<td>50</td>
</tr>
</tbody>
</table>

a Calculated from the change in ratio of aspartic acid to threonine as compared to native GP V.
b Fifteen milligrams of GP V were treated as indicated with 10 ml of periodate as shown. Excess ethylene glycol was added, followed by alkaline NaBH₄. The borohydride was destroyed; the samples were lyophilized and analyzed by the amino acid analyzer.

Recovery from periodate-treated glycopeptide was the same as that from the untreated glycopeptide. The identity of the compound from the glycopeptide hydrolysates was also established by high voltage electrophoresis in pyridine-acetic acid-water at pH 3.7 in which the compound separated as a single band from threonine and other ninhydrin-reactive products.

Structure of Oligosaccharide—Two approaches have been made toward elucidating the structure of the carbohydrate portion of glycopeptide V. One has been the sequential use of glycosidases to remove the sugars. The other has been the selective destruction of the sugar components by periodate oxidation.

A number of purified and partially purified glycosidase preparations have been used for the sequence studies. These are listed in Table IV along with their specific activities and pH optima.

The effect of a crude glycosidase mixture from rat epididymis on glycopeptide V is shown in Fig. 3. At pH 3.3, optimal for β-galactosidase, only galactose was released from the glycopeptide. At pH 5, both galactose and acetylgalactosamine were split off, with eventually 4 moles of each sugar appearing in the medium. Even at pH 4.6, optimal for α-mannosidase activity, no mannose was split off. Only galactose and acetylgalactosamine were found in the retarded fraction on Sephadex G-25 as identified by thin layer chromatography. These data confirmed previous observations (8-12), that the sugars internal to sialic acid are α-galactose and acetyl-α-galactosamine in β linkage. This observation was further supported by sequential use of purified β-galactosidase and β-acetylgalactosaminidase described below.

The digest resulting from the above experiment was treated with 5% trichloroacetic acid to precipitate the protein which was removed by centrifugation at 27,000 × g for 30 min. The TCA supernatant was lyophilized and subjected to gel filtration on Sephadex G-25. The nonretarded hexose-containing peak was designated GP Vw and the retarded GP Vx. The lyophilized, nonretarded peak (GP Vw) was analyzed for carbohydrate and amino acids, and was found to contain 3 moles of mannose and 2 moles of acetylgalactosamine per mole of aspartic acid. Treatment of this residue with ivory nut glycosidases for 92 hours resulted in the further release of 2 of the 3 moles of mannose and 1 mole of free acetylgalactosamine per mole of aspartic acid. This digest was also treated with 5% TCA and centrifuged as described above. The TCA supernatant was lyophilized and the components were separated on Sephadex G-15. The nonretarded hexose-containing peak was designated GP Vw and the retarded peak GP Vx. The lyophilized, nonretarded peak (GP Vw) contained 1 mole of mannose and 1 mole of acetylgalactosamine per mole of aspartic acid. Since the acetylgalactosamine is linked to asparagine, it must be assumed that the man-
TABLE IV

Specific activities of glycosidases used in enzymatic hydrolysis of glycopeptide V and its derivatives

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>β-Galactosidase</th>
<th>Mannosidase</th>
<th>β-Acetylglucosaminidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Prepar-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>aration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat epididymis</td>
<td>1^a</td>
<td>1.36 (3.3)^b</td>
<td>1.13 (4.6)^b</td>
</tr>
<tr>
<td>Ivory nut</td>
<td>2^a</td>
<td>0.005</td>
<td>0.15 (4.6)^b</td>
</tr>
<tr>
<td>Pinto bean</td>
<td>3^a</td>
<td>3.98 (4.0)^b</td>
<td>0</td>
</tr>
<tr>
<td>Bovine liver</td>
<td>4^a</td>
<td>0</td>
<td>15.08 (4.5)^b</td>
</tr>
<tr>
<td>Jack bean</td>
<td>5^a</td>
<td>0</td>
<td>7.20 (4.5)^b</td>
</tr>
</tbody>
</table>

^a Preparations were made from adult rat epididymis by the method of Coneche and Hay (33). The fractions precipitating between 20 and 60% ammonium sulfate saturation were dialyzed against cold water and then against 0.05 M sodium citrate, pH 6.0.

^b pH optimum.

^c Crude enzyme preparation was made by extracting finely ground ivory nut fragments with water containing 33% ammonium sulfate saturation. The protein precipitated between 33 and 70% ammonium sulfate saturation was dialyzed against water and then against 0.05 M sodium citrate, pH 6.0, and subjected to gel filtration on Sephadex G-100. The activities occurred near the void volume.

^d Gift of Dr. O. P. Bahl (34).

^e Gift of Dr. B. Weissmann (35).

^n Gift of Dr. Y. T. Li (36).

TABLE V

Enzymatic release of monosaccharides from glycopeptide V

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hexose^a</th>
<th>Hexosamine^a</th>
<th>Galactose^b</th>
<th>Mannose^b</th>
<th>Acetylglucosamine^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP V (native)</td>
<td>2.06</td>
<td>1.80</td>
<td>4</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>GP Vw</td>
<td>1.85</td>
<td>1.28</td>
<td>0</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>GP Vx</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>GP Vy</td>
<td>2.03</td>
<td>1.79</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>GP Vz</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

^a Galactose and mannose were determined by gas-liquid chromatography. Hexosamine was analyzed by the method of Good and Bessman (24).

^b Nearest integral molar ratio.
Fig. 4. Release of galactose from glycopeptide V by β-galactosidase. Glycopeptide (100 mg in 20 ml) was hydrolyzed with 1 unit of β-galactosidase (pinto beans) in 0.05 M sodium citrate buffer at pH 4.0. At 11, 23, 37, and 56 hours, 0.5, 19.9, and 1.5 enzyme units were added. The reducing sugar in the reaction mixture at each interval was determined (22) with a galactose standard. Identity of released sugar as galactose was established with gas-liquid chromatography. Colorimetric reaction for acetylg glucosamine was negative.

TABLE VI
Composition of glycopeptide V and its fragments after two sequential enzymatic degradations

| Componenta | Composition | Molar ratiob | GP V | | GP Va | | GP Vb | | Molar ratiob |
|------------|-------------|--------------|------|------|---|---|-----|---|
| Galactose  | 1.134 mg | 0.013 | 1.142 mg | 0.014 | 0.150 mg | 0.015 |
| Mannose    | 0.825 mg | 0.082 | 0.799 mg | 0.080 | 0.039 mg | 0.004 |
| Fucose     | 0.082 mg | 0.008 | 0.057 mg | 0.006 | 0.042 mg | 0.004 |
| N-acetyl-D-glucosamine | 1.797 | 0.179 | 2.165 | 0.216 | 0.976 | 0.098 |
| Aspartate  | 0.280 mg | 0.028 | 0.348 mg | 0.035 | 0.532 mg | 0.053 |
| Threonine  | 0.245 mg | 0.025 | 0.267 mg | 0.027 | 0.460 mg | 0.046 |

* Analyses were done as given in footnotes of Table I.

The residue (GP Vb) containing 3 moles of mannose and 2 moles of acetylg glucosamine per mole of aspartic acid did not release galactose on treatment with β-galactosidase (pinto bean). When it was subjected to enzymatic digestion with ivory nut glycosidases it released 2 moles of mannose. This enzyme preparation had no effect on GP Vb. This indicates that 2 of the 3 mannose residues in GP Vb were protected by acetylg glucosamine and became vulnerable to mannosidase after treatment with acetylg glucosaminidase. Further digestion of GP Vb with purified β-acetylg glucosaminidase from bovine liver cleaved off 1 mole of acetylg glucosamine. The course of sequential hydrolysis of GP Vb with mannosidase and acetylg glucosaminidase is illustrated in Fig. 6. Because of exhaustion of the incubation mixture at the end of the reaction, we were unable to determine directly the composition of residual fragment from GP Vb, but we presume it to be mannosyl acetylg glucosaminy1-asparagine.

The results of these experiments with exoglycosidases are consonant with several possible structures for GP V, the most likely of which are those shown in Fig. 7. Since the glycosidases used in these studies failed to distinguish between these three possible structures, a study of periodate oxidation on the intact GP V and its derivative (GP Vb) was undertaken. The consumption of periodate, measured spectrophotometrically (37,38), shown in Fig. 8, indicates that GP V and GP Vb consumed 8.9 and 4.7 moles of periodate per mole of glycopeptide, respectively.
Fig. 7. Three possible structures of glycopeptide consonant with enzymatic degradations.

Fig. 8. Consumption of periodate by glycopeptide V and GP Vb. Glycopeptide V (20 mg in 0.08 M NaI04, 10 ml) was oxidized for 20 hours in the dark at 4°. GP Vb (1.37 mg containing 1.86 μmoles of mannose) was oxidized for 94 hours at 4° in the dark with 10 μmoles of NaI04 in 1.0 ml. The consumption of periodate was measured spectrophotometrically (37, 38). Molar ratios were calculated with the molecular weight 2580 for glycopeptide V and 1110 for GP Vb. Spectrophotometric measurements were done at 220 μm (38) for glycopeptide V and at 222.5 μm (37) for GP Vb. \( \Delta \), GP V; \( \bullet \), GP Vb.

### Table VII

Carbohydrate composition of glycopeptide V and its derivatives after periodate oxidation and borohydride reduction

<table>
<thead>
<tr>
<th>Compound</th>
<th>GP V</th>
<th>GP V ox-red(^a)</th>
<th>GP V ox-red-hyd(^b)</th>
<th>GP Vb(^c)</th>
<th>GP Vb-ox(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles/mg</td>
<td>Molar ratio(^a)</td>
<td>μmoles/mg</td>
<td>Molar ratio(^a)</td>
<td>μmoles/ml</td>
</tr>
<tr>
<td>Galactose</td>
<td>1.134</td>
<td>4.1 (4)</td>
<td>0.047</td>
<td>0.16 (0)</td>
<td>0.101</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.825</td>
<td>2.9 (3)</td>
<td>0.577</td>
<td>2.0 (2)</td>
<td>0.934</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.092</td>
<td>0.33 (0)</td>
<td>0.026</td>
<td>0.1 (0)</td>
<td>0</td>
</tr>
<tr>
<td>N-Acetyl-d-glucosamine</td>
<td>1.797</td>
<td>6.4 (6)</td>
<td>1.805</td>
<td>6.2 (6)</td>
<td>2.800</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.280</td>
<td>1.0 (1)</td>
<td>0.291</td>
<td>1.0 (1)</td>
<td>0.985</td>
</tr>
</tbody>
</table>

\(^a\) Periodate-oxidized and borohydride-reduced product of GP V (Preparation 6, Table II).

\(^b\) Ninhydrin-positive compound isolated by high voltage electrophoresis in pyridine-acetic acid-water at pH 4.7 from acid hydrolysate of GP V ox-red. Values reported represent composition of the compound eluted from the paper in a final volume of 2 ml.

\(^c\) Enzymatically degraded glycopeptide V. Same as in Table VI. Values reported are for 1.37-mg sample used for oxidation study.

\(^d\) Oxidized and reduced product of GP Vb. Values represent recoveries from the starting material (1.37 mg of GP Vb).

\(^e\) With respect to aspartic acid.

\(^f\) Not determined.

The composition of sugars in the native and oxidized GP V is reported in Table VII. The oxidation of the intact GP V resulted in virtually complete destruction of 1 mole of galactose. However, 2 out of the 3 mannose and all of the acetylglucosamine residues were resistant to periodate oxidation. These results are consistent with the amount of periodate consumed assuming that the mannose oxidized consumed 1 mole of periodate with the 4 galactose molecules consuming 2 moles of periodate each. Mild acid hydrolysis (0.1 N HCl, 4°, 18 hours) of the periodate oxidized and borohydride-reduced GP V followed by preparative high voltage electrophoresis of the hydrolysate in pyridine-acetic acid-water at pH 4.7 resulted in the appearance of a ninhydrin-
reacting compound with the mobility of synthetic $\beta$-aspartyl acetylglucosaminylamine. However, the compound was distinguishable by its color from the synthetic standard. This compound (GP V ox-red-hyd) was eluted from the paper and found to contain mannose, acetylglucosamine, and aspartic acid in the molar proportions 1:3:1 (Table VII).

Periodate oxidation of GP Vb containing 3 mannose and 2 acetylglucosamine residues per mole of aspartic acid resulted in the destruction of all of the mannose and none of the acetylglucosamine. This is shown in Table VII (GP Vb and GP Vb-ox). These results are consonant with those obtained by oxidation of the intact glycopeptide, and indicate that the periodate-resistant mannose residues in GP V are exposed as terminal sugars following digestion with $\beta$-galactosidase and $\beta$-acetylglucosaminidase. These would then be susceptible to oxidation, each consuming 2 moles of periodate per mole of mannose. The internally located mannose would consume 1 mole of periodate for a total of 5 moles per mole of glycopeptide. This is in reasonable agreement with the observed value of 4.7 moles per mole.

On the basis of the periodate oxidation studies, it was concluded that Structure C of Fig. 7 represents the basic structure of glycopeptide V. This conclusion rests primarily on the fact that the innermost mannose is susceptible to periodate oxidation. If this oxidation is followed by reduction and mild acid hydrolysis to cleave the acetal linkages, only Structure C can give a derivative containing aspartic acid, acetylglucosamine, and mannose in a ratio of 1:3:1. Structure A and B should give fragments containing aspartic acid and acetylglucosamine in a ratio of 1:1, with no mannose.

**DISCUSSION**

The structure shown in Fig. 9 is proposed for glycopeptide V from the data presented in this paper and from other information in the relevant literature. Each sugar residue is numbered for ease of identification.

The occurrence of $\beta$-aspartyl acetylglucosaminylamine confirms the results of several investigators (5, 13-16) that the linkage between the carbohydrate and the peptide in orosomucoid is of the glycosaminyl type. That the threonine is fully recovered upon mild alkaline treatment is evidence that threonine is not linked to the oligosaccharide by an $\alpha$-glycosidic linkage. About one-half of threonine is destroyed by oxidation with periodate (Table II), suggesting that the peptide portion of the glycopeptide is a mixture of threonylasparagine and asparaginyl-threonine.

The location of threonine with an unsubstituted hydroxyl group adjacent to or within 1 residue of the asparagine linked to carbohydrate has been observed in other glycoproteins (39-41). Also, isolation from orosomucoid of four separate glycopeptides, all of which contain a threonine residue adjacent to or near the asparagine, has been reported from two different laboratories (5, 15). It is, therefore, tempting to suggest that vicinal location of threonine to asparagine may be required for the action of enzymes attaching the carbohydrate to asparagine during the biosynthesis of orosomucoid. Such a hypothesis has already been proposed (42).

The data presented clearly show that 4 moles of galactose are split from GP V by $\beta$-galactosidase, thus exposing 4 moles of acetylglucosamine to the action of $\beta$-acetylglucosaminidase. Since the disaccharide (galactosyl $1 \rightarrow 4$ acetylglucosamine) has been isolated from orosomucoid, and has been well characterized (11) it is likely that this is the major linkage between galactose and acetylglucosamine. However, a $1 \rightarrow 3$ linkage would also fit the data, since it would also render acetylglucosamine resistant to periodate oxidation.

The inner core of GP V thus consists of 3 mannose (Nos. 2, 4 and 9) and 2 acetylglucosamine residues (Nos. 1 and 3) linked to asparagine through acetylglucosamine 1. Two of the three mannoses (Nos. 4 and 9) in the isolated inner core terminal since they can be cleaved off by $\alpha$-mannosidase from ivory nut. They are susceptible to periodate oxidation, although they are resistant to such oxidation in the intact glycopeptide. This indicates that an acetylglucosamine is linked to position 3 of these 2 mannose residues. Mannose 2 is susceptible to periodate oxidation in the intact glycopeptide as well as in the isolated inner core, indicating a linkage at a position 2 or 4 since only 1 mole of periodate is consumed per mole. After oxidation of the intact GP V and subsequent reduction and mild acid hydrolysis, a fragment containing aspartic acid, 3 acetylglucosamines (Nos. 1, 10, 12) and 1 mannose (No. 9) are released.

The interpretation here is that a periodate-sensitive mannose (No. 2) is linked to the acetylglucosamine (No. 1) which is linked to asparagine. The proposed structure is fully consonant with the data of

\[
\text{Asparagine}
\]

FIG. 9. Proposed structure of glycopeptide V. The sugars are numbered parenthetically for identification purposes.

\[
\text{Asparagine}
\]
Eylar and Jeanloz (11) on the structure of a disaccharide, a tetrasaccharide, and an octasaccharide isolated from mild acid hydrolysates of orosomucoid. The octasaccharide consisting of residues 1 to 8 of Fig. 9 would have all of the properties described by Eylar and Jeanloz (11). The tetrasaccharide studied by these investigators had a periodate-susceptible mannose. Therefore, the tetrasaccharides would have been derived from residues 3 to 6 or from 1, 9, 10, and 11. Mannose residues 4 and 9 would have one of the substitutions in position 3 to account for their periodate resistance. Mannose 2 would be substituted at positions 2 or 4 in order to account for its periodate sensitivity with the consumption of 1 mole of periodate.

The proposed structure for GP V cannot be reconciled with the "average structure of oligosaccharide" studied by Sato et al. (43). This may be due to the fact that these authors studied a mixture of oligosaccharides released from orosomucoid by hydrazinolysis.

The structure of the other glycopeptides—particularly of glycopeptide II (Fig. 1) currently under study—suggests that there may be incompletely oligosaccharides of the basic pattern shown in Fig. 9.

The inner core of orosomucoid (containing 3 residues of man-

References

The Structure of a Glycopeptide from Human Orosomucoid (α₁-Acid Glycoprotein)
P. V. Wagh, I. Bornstein and Richard J. Winzler


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