The Structure of the Complex Type Oligosaccharide from Rabbit Hepatic Binding Protein

A RE-EXAMINATION*

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The structure of the complex oligosaccharides from the rabbit hepatic binding protein has been re-examined. The structural analysis in this study is based on chemical methods using sugar analysis, methylation analysis, two consecutive Smith degradations, and gas-liquid chromatography/mass spectrometry. Based on these additional results, a carbohydrate structure different from that previously reported is proposed:

\[
\text{NeuNAc}2-3\text{Gal}1-4\text{GlcNAc}1-4\text{Man}1
\]
\[
\text{NeuNAc}2-6\text{Gal}1-4\text{GlcNAc}1-2\text{Man}1
\]

Although most N-linked oligosaccharides described thus far have a common sugar sequence\[1\] these previous structures differ in one mannose residue. Although seemingly minor, this difference predicts the presence of glycosidases or glycosyltransferases that have not yet been implicated in the synthesis of N-linked oligosaccharides (3, 4). A reanalysis of the complex type oligosaccharide from the hepatic binding protein by chemical methods strongly suggests that the structure as originally proposed is incorrect.

EXPERIMENTAL PROCEDURES

Materials—Con A-Sepharose and Sephadex G-50 (fine) were from Pharmacia. Bio-Gel P-2 and P-4 (400 mesh) were purchased from Bio-Rad. Silica Gel G was obtained from Merck. Pronase E was a product of Kakenkagaku, Tokyo. All other reagents were obtained from various sources and were of reagent grade or higher quality.

Analytical Methods—Neutral sugars were assayed by an orcinol procedure (5). Sialic acid was determined by the periodate-resorcinol assay (6). Sugar and methylation analyses were accomplished by published procedures (7, 8). Partially methylated alditol acetates were further purified on Silica Gel G before analysis by gas-liquid chromatography/mass spectrometry. Partially methylated alditol acetates and contaminants were dissolved in a small amount of petroleum ether/ethyl acetate 1:1 (v/v) and applied to a silica gel column (2.0-ml bed volume) that was equilibrated in the same solvent. The column was washed with 5.0 ml of this solvent. The wash contained no sugars and was discarded. The hexose derivatives were eluted with 6.0 ml of petroleum ether/ethyl acetate 2:1 (v/v) and applied to a silica gel column (2.0-ml bed volume) that was equilibrated in the same solvent. The column was washed with 5.0 ml of this solvent. The wash contained no sugars and was discarded. The hexose derivatives were eluted with 6.0 ml of petroleum ether/ethyl acetate 2:1 (v/v). The hexosamine derivatives were then eluted with 6.0 ml of methanol. Gas-liquid chromatography/mass spectrometry were performed as previously described (9).

Preparation of Glycopeptide—The pronase glycopeptides of the rabbit hepatic binding protein were prepared as previously described (2).

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Hepatic Binding Protein Oligosaccharides

The isolated glycoprotein was active in binding asialo α-acid glycoprotein and reacted with goat antibody to authentic rabbit binding protein in double immunodiffusion. (The antibody was a gift from Dr. Gilbert Ashwell, National Institutes of Health, Bethesda, MD.) Two glycopeptides were separated on Sephadex G-50 as described by Kawasaki and Ashwell (2). Only the larger one contained sialic acid. The smaller glycopeptide (Glycopeptide II in Ref. 2) was found to be a high mannose oligosaccharide in agreement with the previous report (2). The purified sialic acid-containing glycopeptide (Glycopeptide I in Ref. 2) was then passed over a Con A-Sepharose column (20-m bed volume) equilibrated in 5 mM sodium acetate, pH 5.0, plus 0.1 mM concentration each of MgCl₂, MnCl₂, and CaCl₂ (10). Virtually all of the hexose as measured by orcinol did not bind to the column. The unbound material was desalted on a P-2 column (1.0 × 20.0 cm) equilibrated in water. Approximately 95% of the sialic acid in the starting material was recovered in the final glycopeptide.

Smith Degradation (11)—The glycopeptide (about 800 µg of sugar) was oxidized with 1.0 ml of 8.0 mM NaIO₄ in 0.05 M sodium acetate buffer, pH 4.5, at 4 °C for 48 h in the dark. A drop of ethylene glycol was added to destroy the excess periodate. The reaction mixture was adjusted to pH 8.0 with 1 N NaOH and solid sodium borohydride was added to a final concentration of 0.2 M. After 20 h at 4 °C, several drops of glacial acetic acid were added to destroy the excess borohydride. The sample was concentrated to dryness, evaporated from methanol, and desalted on a Bio-Gel P-2 column equilibrated in water. The glycopeptide was located by an orcinol assay and the pooled fractions were concentrated to dryness. The sample was dissolved in water and portions were removed for sugar and methylation analysis. To the remaining material was added the appropriate volume of 5 N HCl to give a final concentration of 0.5 N HCl. The mixture was incubated at room temperature for 48 h. The hydrolysate was neutralized with sodium bicarbonate and 3.0 mg of sodium borodeuteride were added. The reduction was continued for 2 h at room temperature and stopped by adding glacial acetic acid. The sample was concentrated to dryness and boric acid was removed by evaporation from methanol. The residue was dissolved in water and fractionated on a Bio-Gel P-4 column (1.0 × 40.0 cm) equilibrated in water. The salt fractions were concentrated by dialysis and the pooled fractions were concentrated to dryness. The fractions containing salt were pooled separately. The pool in front of the salt contains the hydrolyzed glycopeptide and the salt-containing pool contains the oligosaccharides released by mild acid hydrolysis. After removing a portion of the desalted pool for sugar and methylation analysis, the remainder was subjected to a second round of Smith degradation as above except that the reduction with sodium borodeuteride after the mild acid hydrolysis was omitted and the final step was desalting on Bio-Gel P-2 instead of Bio-Gel P-4.

The oligosaccharide alditol released by the first Smith degradation and recovered in the salt volume of the Bio-Gel P-4 column was permethylated as previously described (12) and analyzed by gas-liquid chromatography/mass spectrometry.

RESULTS AND DISCUSSION

The sugar composition of the glycopeptide is given in Table I. Sialic acid, galactose, mannose, and N-acetylgalcosamine were found in the molar ratio of 3:3:3:5. These values differ from the reported data, obtained by enzymatic methods, where the ratio of mannose to galactose was 2:3 (2). In agreement with the previous report, no fucose was detected in the glycopeptide. These results and the fact that the glycopeptide failed to bind to Con A-Sepharose (see "Experimental Procedures") suggest that the oligosaccharide on this glycopeptide has a triantennary structure.

The methylation analysis is also consistent with the presence of a triantennary oligosaccharide (Table II). The di-O-substituted mannose derivatives, 3,6-di-O-methyl-Man and 2,4-di-O-methyl-Man, were present in equimolar proportions. These derivatives are expected in a triantennary structure of the fucosyl type (7) but should not be found in a structure such as that reported for the rabbit hepatic binding protein (2). Another mannose derivative, 3,4,6-tri-O-methyl-Man, was observed in an amount equal to the other mannose derivatives.

M. Lowe and B. Nilsson, unpublished results.

#### Table I

| Sample         | Man | Gal | GlcNAc | NeuNAc |
|----------------|-----|-----|--------|--------|
| Intact glycopeptide | 3.0 | 2.9 | 4.8    | 2.9    |
| First Smith | 2.0 | 1.1 | 4.9    | n.d.   |
| After hydrolysis | 2.0 | 1.1 | 4.2    | n.d.   |
| Second Smith | 2.0 | 0.2 | 3.2    | n.d.   |

#### Table II

| Sugar derivative | T value | Relative molar proportions |
|------------------|---------|----------------------------|
| Intact glycopeptide |         | First | Second |         |
| 2,3,4,6-Tetra-O-Me-Gal | 1.03 | 0 | 0 | 0.7 | Trace |
| 2,4,6-Tri-O-Me-Gal | 1.37 | 1.2 | 1.0 | 0 | 0 |
| 2,3,4-Tri-O-Me-Gal | 1.50 | 2.1 | 0 | 0 | 0 |
| 3,4,6-Tri-O-Me-Man | 1.32 | 1.0 | Trace | 0 | 0 |
| 2,4,6-Tri-O-Me-Man | 1.42 | 0 | 0 | 0.7 | 0.7 |
| 2,3,4,6-Tri-O-Me-Man | 1.35 | 0 | 0 | 0 | 1.0 |
| 3,6-Di-O-Man | 1.70 | 1.0 | 1.0 | Trace | 0 |
| 2,4-Di-O-Man | 1.91 | 1.1 | 1.1 | 0.2 | 0.2 |
| 3,4,6-Tri-O-Me-GlcN(Me)Ac | 2.31 | 0 | 0 | 0 | 0 |
| 3,6-Di-O-Me-GlcN(Me)Ac | 2.80 | + | + | + | + |

* Molar proportions of sugars relative to mannose set to 3.0 or 2.0.
+ n.d., not determined.

Both 2,4,6-tri-O-methyl-Gal and 2,3,4-tri-O-methyl-Gal were present in a ratio of 1:2. This result suggests that galactose is substituted in the α or β position by sialic acid, a result found by Kawasaki and Ashwell (2). Only 4-O-substituted N-acetylgalcosamine (3,6-di-O-methyl-GlcN(Me)Ac) was present.

Additional information about the structure of the oligosaccharide was obtained by Smith degradation. After periodate oxidation and reduction with sodium borohydride, the sugar analysis showed the loss of about two galactose residues and one mannose residue (Table I). No N-acetylgalcosamine was lost. The methylation analysis of this material revealed the disappearance of the 6-O-substituted galactose residues and the 2-O-substituted mannose as expected (Table II).

The periodate-oxidized and reduced glycopeptide was treated with mild acid to hydrolyze the noncyclic acetals. Following reduction with sodium borodeuteride, the sample was fractionated on a Bio-Gel P-4 column. Two sugar-containing peaks were found, one in the included volume and one in the salt volume. The sugar analysis of the included fraction gave a ratio of galactose:mannose:N-acetylgalcosamine of about 1:2:4, demonstrating that one N-acetylgalcosamine residue was released after mild acid hydrolysis (Table I). The methylation analysis of this fraction showed nonreducing terminal galactose (2,3,4,6-tetra-O-methyl-Gal) and N-acetylgalcosamine (3,4,6-tri-O-methyl-GlcN(Me)Ac). A new mannose derivative, 3-O-substituted mannose (2,4,6-tri-O-methyl-Mann), was formed after the first Smith degradation. No other new derivatives were present.

The sugar in the salt fraction was methylated and analyzed directly by gas-liquid chromatography/mass spectrometry. A
disaccharide alditol was found and identified as N-acetylglucosamine-1-2-glycerol-1-d (7). The recovery of N-acetylglucosamine in this fraction accounted for the N-acetylglucosamine which was missing in the larger sugar fraction. The glycerol residue was formed after Smith degradation of the 2-O-substituted mannose.

The presence of N-acetylglucosamine-1-2-glycerol and the formation of a 3-O-substituted mannose after the first Smith degradation, combined with the initial sugar and methylation analyses, suggests that the following structures are present in the glycopeptide from the hepatic binding protein:

\[
\begin{align*}
\text{NeuNAc2-3Galβ1-4GlcNAcβ1} & \quad 4 \\
\text{Manβ1-3} & \\
\text{NeuNAc2-6Galβ1-4GlcNAcβ1} & \quad 3 \\
\text{Manβ1-6} & \\
\text{NeuNAc2-6Galβ1-4GlcNAcβ1} & \quad 2 \\
\text{2Manα1-4GlcNAcβ1} & \quad 1 \\
\text{Manα1-} & \quad 1 \\
\text{GlcNAc1-4GlcNAc1-N-Asn} & \quad 6 \\
\end{align*}
\]

A second round of Smith degradation provided more information about the structure of the oligosaccharide. After oxidation, reduction, and mild acid hydrolysis, the sugar analysis showed that virtually all of the galactose was destroyed. A ratio of mannose to N-acetylglucosamine of 2:3 was found (Table I). The methylation analysis (Table II) gave a new mannose derivative, 4-O-substituted (2,3,6-tri-O-methyl-Man). As predicted, nonreducing terminal N-acetylglucosamine (3,4,6-tri-O-methyl-GlcN(Me)Ac), 3,6-di-O-methyl-mannose (2,4,6-tri-O-methyl-Man), and 4-O-substituted N-acetylglucosamine (3,6-di-O-methyl-GlcN(Me)Ac) were still present. These data indicate that the structure after two consecutive Smith degradations is:

\[
\begin{align*}
\text{GlcNAc1-4Manα1} & \quad 3 \\
\text{Manα1-4GlcNAc1-4GlcNAc1-N-Asn} & \quad 6 \\
\end{align*}
\]

The presence of the 4-O-substituted mannose residue after the second Smith degradation localizes the 2-3-linked sialic acid to the sequence:

\[
\begin{align*}
\text{NeuNAc2-3Galβ1-4GlcNAcβ1} & \quad 4 \\
\text{Manβ1-3} & \\
\text{NeuNAc2-6Galβ1-4GlcNAcβ1} & \quad 3 \\
\text{Manβ1-6} & \\
\text{NeuNAc2-6Galβ1-4GlcNAcβ1} & \quad 2 \\
\text{2Manα1-4GlcNAcβ1} & \quad 1 \\
\text{NeuNAc2-6Galβ1-4GlcNAcβ1} & \quad 6 \\
\text{NeuNAc2-6Galβ1-4GlcNAcβ1} & \quad 2 \\
\text{Manα1-4GlcNAcβ1} & \quad 1 \\
\text{GlcNAc1-4GlcNAc1-N-Asn} & \quad 6 \\
\end{align*}
\]

From the data presented, the following structure for the complex-type oligosaccharide of the hepatic binding protein is proposed:

This structure is supported by the results of sequential exoglycosidase digestions as reported by Kawasaki and Ashwell (2). Only the sequence of treatment with neuraminidase, β-galactosidase, β-N-acetylglucosaminidase, and α-mannosidase released sugars from the glycopeptide. Any other permutation was unsuccessful in cleaving monosaccharides from the glycopeptide. The anomeric configurations are inferred from that work.

The proposed structure differs from the previously reported structure, but it is consistent with the triantennary structures from other glycoproteins and with what is understood about the biosynthesis of N-linked oligosaccharides.

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