Structure of the Tryptic Glycopeptide Isolated from Rabbit Transferrin†

Dudley K. Strickland,‡ James W. Hamilton, and Billy G. Hudson*

ABSTRACT: The structure of the tryptic glycopeptide isolated from rabbit transferrin was elucidated by use of sequential Edman degradations, specific exoglycosidases, endo-β-*N*-acetylglucosaminidases, methylation analyses, and periodate oxidation studies. The glycopeptide consists of a heteropolysaccharide, AcNeuα2→6Galβ1→4GlcNAcβ1→2Manα1→3[AcNeuα2→6Galβ1→4GlcNAcβ1→2Manα1→6]-Manβ1→4GlcNAcβ1→4GlcNAc, attached to a peptide, Asn-Ser-Ser-Leu-Cys, via a linkage involving *N*-acetylglucosamine and asparagine. The stoichiometry of this glycopeptide is 2 mol/mol of protein, indicating that rabbit transferrin contains two structurally identical glycopeptide segments.

Transferrin is a glycoprotein that plays a vital role in iron metabolism, including binding, transport, and transfer of iron to a variety of cells, such as the reticulocyte where the iron is utilized in hemoglobin biosynthesis (Aisen & Brown, 1977). The homologous system of rabbit transferrin and rabbit reticulocytes has been used extensively as a model system for investigating the mechansim of iron transfer. Initially, transferrin binds to receptors located on the recticulocyte surface (Jandl et al., 1959; Jandl & Katz, 1963; Leibamn & Aisen, 1977), and then the iron is released to the reticulocyte via a mechanism not fully understood. Ultimately, the elucidation of the detailed mechanism of iron transfer will require a knowledge of the chemical structure of rabbit transferrin.

Rabbit transferrin is a single-chain glycoprotein (Hudson et al., 1973) that contains two identical heteropolysaccharide units which are both attached to the chain within the same region, representing approximately one-third of the molecule (Strickland & Hudson, 1978). These units can be isolated in the form of a single tryptic glycopeptide with a stoichiometry of 2 mol of glycopeptide/mol of transferrin, indicating that both units have identical amino acid residues in the immediate vicinity of the carbohydrate–peptide attachment sites. The heteropolysaccharide units are of the complex type (Kornfeld & Kornfeld, 1976), each composed of two sialic acid, two galactose, three mannose, and four *N*-acetylglucosamine residues.

The purpose of the present study was to determine the detailed structure of the tryptic glycopeptide from rabbit transferrin with regard to amino acid and monosaccharide sequence, anomeric configurations, monosaccharide linkage positions, and the chemical nature of the carbohydrate–peptide linkage.

Experimental Procedures

Materials. Jack bean meal was obtained from ICN Pharmaceuticals. *p*-Nitrophenyl *N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl α-D-galactopyranoside, *p*-nitrophenyl β-D-galactopyranoside, and *p*-nitrophenyl α-D-mannopyranoside were obtained from Sigma. *p*-Nitrophenyl β-D-mannopyranoside was purchased from Calbiochem. Sodium metaperiodate, stachyose, β-D-methylmannopyranoside and D-glucosamine hydrochloride were purchased from Sigma, while methyl α-D-galactopyranoside was obtained from Pfanstiehl Laboratories. 2,3,4,6-Tetra-*O*-methylglucose and phenylthiohydantoin amino acid derivatives were obtained from Pierce. All other reagents were the best available commerical grade and used without further purification.

Isolation of the Tryptic Glycopeptides from Rabbit Transferrin. The tryptic glycopeptides from S-aminoethylated rabbit transferrin were isolated as previously described (Strickland & Hudson, 1978).

Amino Acid Sequence of the Tryptic Glycopeptides. Sequential amino-terminal analysis of the glycopeptide was determined by the automated method of Edman & Begg (1967) by using a Beckman 890C automated sequencer. A DMAA[1] program (Beckman Sequencer Program 102974) was used for amino-terminal degradation. For sequencing purposes 100 nmol of the tryptic glycopeptide was used. Phenylthiazolinone derivatives were converted to the phenylthiohydantoin derivatives by adding 1 M HCl and heating at 80 °C for 10 min. For the phenylthiazolinone derivatives of serine, the time was reduced to 6 min.

The phenylthiohydantoin amino acids were identified by high-pressure liquid chromatography essentially as described by Downing & Mann (1976) by using a Waters Associates high-pressure liquid chromatograph.

Proteolytic Digestion of the Tryptic Glycopeptide and Subsequent Alkaline-Sulfite Treatment. The tryptic glycopeptide (200 nmol) was digested with carboxypeptidase B (1.0 unit) for 12 h, followed by extensive digestion with Pronase. Subsequent alkaline-sulfite treatment on this fraction was carried out as described by Spiro (1972).

Glycosidases. α-Mannosidase, β-*N*-acetylhexosaminidase, and β-galactosidase were purified from jack bean meal by the procedures described by Li & Li (1972). The activity of the various glycosidases was measured by using the appropriate *p*-nitrophenyl glycoside. Each purified enzyme was checked for contaminating exoglycosidase activity. The specific activities of these enzyme preparations were comparable to the literature values. Neuraminidase (Type IX) from *Clostridium perfringens* was purchased from Sigma. β-Mannosidase from *Polysporous sulfureus* was a generous gift from Dr. Yu-Teh Li. Endo-β-*N*-acetylglucosaminidase D from *Diplococcus pneumoiae* and endo-β-*N*-acetylglucosaminidase H from

† From the Department of Biochemistry, University of Kansas Medical Center, Kansas City, Kansas 66103, and the Calcium Research Laboratory, Veterans Administration Hospital, Kansas City, Missouri 64128. *Received October 5, 1978; revised manuscript received January 29, 1979.* This work was supported by the Herman Frasch Foundation and by a grant from the National Institutes of Health (AM 18381).

* Address all correspondence to this author at the Department of Biochemistry, University of Kansas Medical Center, Kansas City, KS 66103.

‡ Submitted by D.K.S. to the Graduate School of Kansas University in partial fulfillment of the requirements for the Ph.D. degree. Present address: Department of Chemistry, University of Notre Dame, Notre Dame, IN.

[1] Abbreviations used: DMAA, dimethylallylamine; PTH, phenylthiohydantoin.

0006-2960/79/0418-2549$01.00/0

Streptomyces plicatus were generous gifts from Dr. Akira Kobata.

Glycosidase Digestion. The monosaccharide sequence was determined as described by Kornfeld et al. (1971). Glycopeptides (50–200 nmol) were digested with 5% (w/w) neuraminidase in 5 mM sodium citrate, pH 5.6, at 37 °C for 12 h under a toluene atmosphere. The amount of sialic acid released was determined by measuring the free sialic acid content and comparing this value with that obtained from an acid hydrolysis of the digest. Glycopeptides were then incubated with either β-*N*-acetylhexosaminidase (0.5 unit) or α-mannosidase (0.5 unit) by dissolving the sample in 200–300 μL of 0.02 M sodium citrate, pH 4.6, followed by addition of the appropriate enzyme. Incubation was carried out for 12 h at 37 °C under a toluene atmosphere. After incubation, an aliquot containing 50 nmol each of arabinose and inositol was added to the digest. The enzyme activity was destroyed by heating at 100 °C for 2 min, and the sample was centrifuged prior to applying to a Bio-Gel P-2 column (0.6 × 50 cm). The residual glycopeptide was pooled, concentrated, and digested with the next glycosidase, while the salt fraction was analyzed by gas–liquid chromatography for neutral sugar content and by use of the amino acid analyzer or the modified Elson–Morgan reaction (Levvy & McAllen, 1959) for amino sugar content.

Digestion of the tryptic glycopeptide with β-galactosidase from *Streptococcus pneumoniae* (Paulson et al., 1978) was carried out in Dr. Robert L. Hill's laboratory. Tryptic asialoglycopeptide (20 nmol) was dissolved in 50 μL of 50 mM sodium cacodylate, pH 6.0, and incubated with 2 milliunits of enzyme for 6 h at 37 °C.

Preparation of $(Man)_3(GlcNAc)_2$-peptide was accomplished by simultaneous digestion of the asialoglycopeptide with β-galactosidase (0.5 unit) and β-*N*-acetylhexosmainidase (0.5 unit), followed by gel filtration on a Bio-Gel P-2 column. Digestion with β-mannosidase (0.4 units) was carried out in 0.1 M glycine hydrochloride, pH 3.5, for 12 h at 37 °C. The determination of the sequence of the core region was accomplished by simultaneous digestion of $Man(GlcNAc)_2$-peptide with β-mannosidase and β-*N*-acetylhexosaminidase. Following incubation, an internal standard consisting of arabinose and inositol was added, and the digest was applied to a Dowex 50 X-2 column (0.5 × 2 cm) and washed with H_2O. The effluent was analyzed for mannose content by gas–liquid chromatography and for amino sugar content by the modified Elson–Morgan reaction.

Endo-β-*N*-acetylglucosaminidase D digestion was carried out in 0.15 M sodium phosphate, pH 6.5 (Tai et al., 1975a,b), while endo-β-*N*-acetylglucosaminidase H digestion was carried out in 0.1 M sodium citrate and 0.1 M sodium phosphate, pH 5.0, containing 0.1 M NaCl (Tai et al., 1975a,b). Following incubation for 6 h at 37 °C, the digests were applied to Bio-Gel P-2 columns (0.6 × 50 cm).

The product of the endo-β-*N*-acetylglucosaminidase D digestion from the Bio-Gel P-2 column contained both carbohydrate and peptide in a single fraction. These were separated from each other by use of a Dowex 50 X-2 column (0.5 × 2.0 cm) equilibrated with 0.10 M pyridine–acetate, pH 3.5. Under these conditions, the hexose-containing material passed directly through the column, while the peptide portion was eluted with 0.1 N NH_4OH. Each of these fractions was pooled for further analyses.

Periodate Oxidation. Periodate oxidation of the tryptic glycopeptides was accomplished essentially as described by Baenziger et al. (1974). Tryptic glycopeptide (20 nmol) was dissolved in 100 μL of 0.04 M sodium metaperiodate in 0.05 M sodium acetate buffer, pH 4.6. The oxidation was carried out at 4 °C in the dark. Aliquots of 25 μL were removed at various times and assayed for their periodate content, by using the procedure described by Avigad (1969). Control samples included a blank containing only periodate, a sample containing β-methylmannopyranoside (50 nmol), and a sample containing α-methylgalactopyranoside (50 nmol).

In order to assess which monosaccharide residues were destroyed by periodate treatment, 40 nmol of tryptic glycopeptide was oxidized at 4 °C for 24 h in the dark. Excess periodate was then destroyed by addition of ethylene glycol (15 μL at room temperature for 1.5 h). The oxidized glycopeptide was placed on a Bio-Gel P-2 column (0.6 × 50 cm) equilibrated with H_2O, and the glycopeptide material was analyzed for neutral sugar and amino sugar content by using the methods described under Analytical Procedures. The oxidation of sialic acid by periodate was monitored as described by Sprio (1964).

Methylation Analysis. The tryptic glycopeptide (100–500 nmol) was methylated according to the procedure developed by Hakomori (1964), and the methylated glycopeptide was separated from the reaction products by chromatography over a Sephadex LH-20 column (1.0 × 15 cm) equilibrated with chloroform–methanol (1:1 v/v). Acetolysis, hydrolysis, reduction, and acetylation of partially methylated derivatives were carried out as described by Stellner et al. (1973). Hydrolysis was carried out at 80 °C for 7 h, and the acetylation time was extended to 4 h (Yamashita et al., 1978). The partially methylated alditol acetate derivatives of mannose and galactose were identified by their relative retention times (Lindberg, 1972), by using a glass column of 3% OV-225 (on Supelcoport, 100–120 mesh) on a Hewlett-Packed 5830A gas chromatograph equipped with a flame ionization detector. For identification, an isothermal (170 °C) program was used. In order to quantitate the various derivatives, a temperature program was used in which the temperature was varied between 150 and 200 °C at the rate of 2 °C/min. A flow rate of N_2 of 20 mL/min was used. The partially methylated derivatives of *N*-acetylglucosamine were identified by their retention times (Yamashita et al., 1978) by using a glass column of 3% OV-17 (on Supelcoport, 100–120 mesh). The temperature was varied between 150 and 240 °C at the rate of 4 °C/min. A flow rate of 30 mL/min was used. Since all of the methylated sugars were resolved under these conditions, quantitation of the *N*-acetylglucosamine derivatives was possible. It was assumed that response factors for the various partially methylated alditol acetates were identical.

The following standards were used for identification of methylated derivatives of mannose. 2,3,4,6-Tetra-*O*-methylmannose and 3,4,6-tri-*O*-methylmannose were obtained from methylation and hydrolysis of a mannose trisaccharide obtained from Dr. Gary Gray. The remainder of the mannose derivatives were identified by partial methylation of β-methylmannopyranoside by using Kuhn's procedure (Kuhn & Egge, 1963) as described by Handa & Montgomery (1969).

2,3,4,6-Tetra-*O*-methylgalactose was identified by exhaustive methylation of α-methylgalactopyranoside. 2,3,4-Tri-O-methylgalactose was identified by the exhaustive methylation of stachyose.

Pentacetyl-*N*-methyl-α-D-glucosamine (I) was synthesized from D-glucosamine hydrochloride as described by Kuehl et al. (1947). Methyl 2-(*N*-methylacetamido)-2-deoxyglucopyranoside (II) was synthesized from I by utilizing the procedure described by Tai et al. (1975a,b). The 3,4,6-tri-

Table I: Composition and Sequence Analysis of the Tryptic Glycopeptide from Rabbit Transferrin

composition		sequence analysis		
components	residues/mol[a]	cycle no.	PTH amino acid[c]	yield (nm)[d]
amino acid		1		
aspartic acid	1.02	2	serine	15.2
serine	1.78 (2)[b]	3	serine	10.4
leucine	1.05 (1)	4	leucine	41.9
S-(aminoethyl)-cysteine	1.00 (1)	5	*S*-(aminoethyl)-Cys	41.0
monosaccharide		6		
mannose	2.95 (3)			
galactose	2.20 (2)			
glucosamine	4.06 (4)			
N-acetylneuraminic acid	2.05 (2)			

[a] Based on *S*-(aminoethyl)cysteine. [b] Values in parentheses are residues per mole derived from amino acid and carbohydrate sequence studies. [c] PTH derivatives were identified and quantitated by high-pressure liquid chromatography. [d] Glycopeptide (100 nmol) was used for sequencing.

O-methyl derivative of 2-*N*-methyl-*N*-acetylglucosamine was prepared by exhaustive methylation of II, followed by acetolysis, hydrolysis, reduction, and acetylation as described by Stellner et al. (1973). The remainder of the *N*-acetylglucosamine derivatives was prepared by partial methylation of II according to Kuhn's procedure (Kuhn & Egge, 1963) as described by Tai et al. (1975a,b).

Analytical Procedures. Neutral sugars, amino sugars, amino acids, and sialic acid were quantitated as previously described (Strickland & Hudson, 1978). *N*-Acetylglucosamine released as the result of β-*N*-acetylhexosaminidase hydrolysis was also quantitated by the Elson–Morgan reaction (Levvy & McAllen, 1959). Protein concentration of the purified glycosidases was determined by the method described by Hartree (1972).

Results

Amino Acid Sequence of the Tryptic Glycopeptide and Nature of the Peptide–Carbohydrate Linkage. The composition of the tryptic glycopeptide isolated from rabbit transferrin is summarized in Table I. This fraction was subjected to sequential Edman degradations, and the results of these studies are summarized in Table I. A PTH amino acid derivative resulting from the first cycle of the Edman degradation was not observed. The second and third cycles of the Edman degradation were both identified as the PTH derivatives of serine, while PTH-leucine and PTH-*S*-(aminoethyl)cysteine were identified in the fourth and fifth cycle, respectively. Since the composition data indicate the presence of one aspartic acid residue (Table I), this residue was placed at the amino-terminal position.

After complete degradation of the peptide, an aliquot of the sample remaining in the reaction cup was removed and analyzed for amino acid and amino sugar content. These results indicated that this fraction contained 1 mol of aspartate and 4 mol of glucosamine in addition to traces of other amino acids and, thus, suggest that the carbohydrate unit is attached to an asparagine residue via an N-glycosidic linkage. The presence of carbohydrate would explain why a PTH-asparagine was not observed during the first cycle of the Edman degradation. Thus, the following sequence for the tryptic glycopeptide is proposed: –Asn(CHO)-Ser-Ser-Leu-Cys–.

In order to further substantiate that the carbohydrate unit is attached to asparagine, tryptic glycopeptide was subjected to extensive digestion with carboxypeptidase B and Pronase, and the resultant glycopeptide was isolated. This fraction was found to contain, in addition to the carbohydrate, one aspartic acid and two serine residues upon acid hydrolysis. Treatment of this fraction with alkaline sulfite did not result in the destruction of either serine or glucosamine (data not shown), indicating that the heteropolysaccharide unit is not attached via an O-glycosidic linkage to serine. Thus, these results are consistent with the carbohydrate unit being N-glycosidically linked to asparagine.

Table II: Methylated Sugars Found in the Tryptic Glycopeptide from Rabbit Transferrin

	residues/mol	
	intact glycopeptide[a]	core glycopeptide[b]
galactose		
2,3,4-tri-*O*-methyl	1.8	0.2
mannose		
2,3,4,6-tetra-*O*-methyl		2.0
3,4,6-tri-*O*-methyl	2.0	0.1
2,4-di-*O*-methyl	1.0	1.0
2-*N*-methyl-*N*-acetylglucosamine		
3,6-di-*O*-methyl	3.9	ND[c]

[a] Based on 2.0 residues of 3,4,6-tri-*O*-methylmannose. Average of triplicate analyses. [b] Based on 2.0 residues of 2,3,4,6-tetra-*O*-methylmannose. [c] Not determined.

Table III: Effect of Periodate Oxidation on the Tryptic Glycopeptide from Rabbit Transferrin[a]

	residues/mol			
	AcNeu	Gal	Man	GlcNAc
intact glycopeptide	2.04	2.18	2.80	3.85
glycopeptide + IO_4^-	0.05	0.20	1.12	3.78

[a] Based on duplicate analyses.

Methylation Analysis. In order to determine the linkage positions of the various monosaccharide residues, the tryptic glycopeptide was subjected to methylation analysis. The results are summarized in Table II. All of the galactose residues were present as the 2,3,4-tri-*O*-methyl derivative, indicating that both galactose residues are substituted at C-6. Two of the three mannose residues were present as the 3,4,6-tri-*O*-methyl derivative, indicating substitution at C-2, while the remaining residue was present as the 2,3-di-*O*-methyl derivative, indicating substitution at both C-3 and C-6, providing a single branch point. All of the *N*-acetylglucosamine residues were found as 3,6-di-*O*-methyl derivatives, indicating substitution at C-4.

Periodate Oxidation Studies. In order to confirm the results obtained from methylation analysis, the tryptic glycopeptide was subjected to periodate oxidation studies. Quantitation of periodate consumption indicated an initial, rapid uptake of periodate, followed by a prolonged, more gradual phase. After 28 h the periodate consumption remained constant, and the tryptic glycopeptide had consumed 9.8 mol of periodate/molecule. As a control, the periodate consumption of β-methylmannopyranoside and α-methylgalactopyranoside were monitored. After 28 h, β-methylmannopyranoside consumed 1.8 mol of periodate/mol while α-methylgalactopyranoside consumed 2.0 mol of periodate/mol.

The effect of periodate oxidation on individual monosaccharide residues is summarized in Table III. Periodate treatment destroyed all of the sialic acid galactose residues. These results indicate that galactose must be substituted at either C-2, C-4, or C-6 and thus are in agreement with the methylation data which indicate that galactose is substituted

Table IV: Effects of Glycosidase Treatment on the Tryptic Glycopeptide from Rabbit Transferrin

experiment	residues released by glycosidase treatment per mole of glycopeptide			
	AcNeu	Gal	GlcNAc	Man
(A) intact glycopeptide	2.04	2.18	3.85	2.80
(1) + α-mannosidase	–	–	–	–
(2) + β-galactosidase	–	–	–	–
(3) + β-*N*-acetylhexosaminidase	–	–	–	–
(4) + neuraminidase	2.10	–	–	–
(5) 4 + α-mannosidase	–	–	–	–
(6) 4 + β-*N*-acetylhexosaminidase	–	–	–	–
(7) 4 + β-galactosidase	–	2.10	–	–
(8) 7 + α-mannosidase	–	–	–	–
(9) 7 + β-*N*-acetylhexosaminidase	–	–	2.00	–
(B) core glycopeptide			1.90	2.70
(1) + β-mannosidase			–	–
(2) + α-mannosidase			–	2.00
(3) 2 + β-mannosidase			–	1.10
(4) 3 + β-*N*-acetylhexosaminidase			0.70	–

at C-6. Periodate oxidation also destroyed two of the three mannose residues, indicating that two residues must be substituted at C-2, C-4, or C-6 and one residue is substituted at C-3 and/or disubstituted, which is in agreement with methylation data. All of the *N*-acetylglucosamine residues were completely resistant to periodate treatment, indicating that these residues are substituted at either the C-3 or the C-4 position, again confirming the data obtained by methylation analysis.

Monosaccharide Sequence of the Heteropolysaccharide Units. The monosaccharide sequence of the tryptic glycopeptide was determined by sequential use of purified glycosidases, and the results obtained are summarized in Table IV. Treatment of the tryptic glycopeptide with α-mannosidase, β-galactosidase, or β-*N*-acetylhexosaminidase did not release any monosaccharide residues, indicating that mannose, galactose, and *N*-acetylglucosamine are not terminally linked in the heteropolysaccharide unit. An alternate explanation of these results is that the anomeric linkages of the monosaccharide residues are such that they cannot be hydrolyzed by the enzymes. However, the results of the following sequence studies indicate that this is not the case.

Treatment of the tryptic glycopeptide with neuraminidase resulted in the release of two residues of *N*-acetylneuraminic acid, indicating that these residues occupy the terminal nonreducing position in the carbohydrate unit. Treatment of the asialoglycopeptide with α-mannosidase or β-*N*-acetylhexosaminidase did not release any mannose, *N*-acetylglucosamine, or galactose. Treatment with β-galactosidase from either jack bean meal or *S. pneumoniae* resulted in the release of two galactose residues, indicating that these residues are β-linked and occupy penultimate positions. Subsequent treatment with α-mannosidase had no effect, while treatment with β-*N*-acetylhexosaminidase resulted in the release of two of the four *N*-acetylglucosamine residues.

Since β-galactosidase from both jack bean meal (Li & Li, 1972) and *S. pneumoniae* (Paulson et al., 1978) appears to be specific for β1→4 galactosides, the data obtained from use of these enzymes confirm the methylation data, indicating that the two *N*-acetylglucosamine residues released are substituted at C-4. The results obtained indicate that the nonreducing terminal region consists of two *N*-acetylneuraminyl-α(2→6)-*N*-acetyllactosamine chains.

Analysis of the remaining "core" glycopeptide indicated that this fraction contained three mannose residues and two *N*-acetylglucosamine residues. Treatment of the core glycopeptide with β-mannosidase did not release any mannose residues. Two of the three mannose residues could be released by treatment of the core glycopeptide with α-mannosidase. Subsequent treatment with β-mannosidase released the remaining mannose residue. The glycopeptide now contained two residues of *N*-acetylglucosamine, one of which could be released with β-*N*-acetylhexosaminidase.

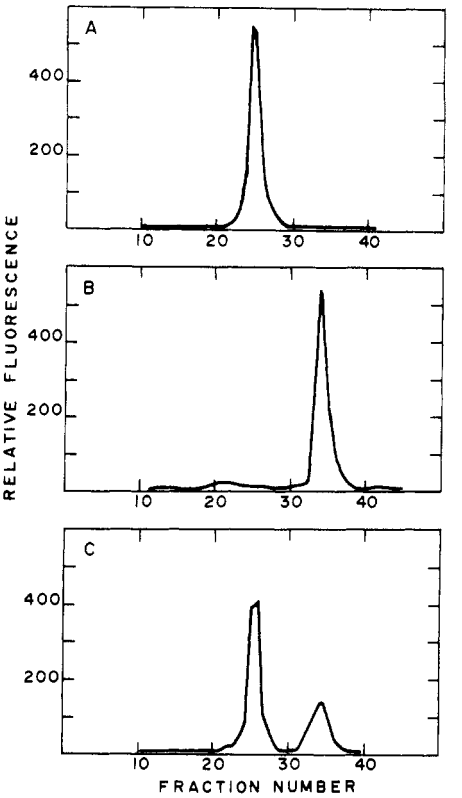


FIGURE 1: Endoglycosidase digestion of $(Man)_3(GlcNAc)_2$-peptide from rabbit transferrin. $(Man)_3(GlcNAc)_2$-peptide (20 nmol) was incubated with endo-β-*N*-acetylglucosaminidase D (2 milliunits) or endo-β-*N*-acetylglucosaminidase H (5 milliunits) at 37 °C for 12 h. Each digest was then applied to a Bio-Gel P-2 column (0.6 × 50 cm) equilibrated with H_2O. The column was maintained at a flow rate of 2.5 mL/h, and 0.38-mL fractions were collected. Peptide material was identified by the fluorescamine method. (A) Elution profile for core glycopeptide. (B) Elution profile for endo-β-*N*-acetylglucosaminidase D digestion of core glycopeptide. (C) Elution profile for endo-β-*N*-acetylglucosaminidase H digestion of core glycopeptide.

Endoglycosidase Digestion of $(Man)_3(GlcNAc)_2$-peptide and Characterization of the Digestion Products. Since the substrate specificities of the endo-β-*N*-acetylglucosaminidases from *S. plicatus* (H) and *D. pneumoniae* (D) have been examined in considerable detail (Tai et al., 1977; Ito et al., 1975; Trimble & Maley, 1977; Tarentino & Maley, 1975), these enzymes have become useful tools for examining the carbohydrate structure of glycopeptides and glycoproteins. In order to further examine the structure of the core region of the glycopeptide from rabbit transferrin, the tryptic glycopeptide was subjected to digestion by using these enzymes. The results of this study are summarized in Figure 1. When the core glycopeptide was digested with endo-β-*N*-acetylglucosaminidase D for 6 h, the elution volume on a Bio-Gel P-2 column was altered, suggesting that cleavage into smaller fragments had occurred. Treatment of the core glycopeptide with endo-β-*N*-acetylglucosaminidase H for 6 h resulted in partial cleavage.

In order to substantiate that cleavage of the core glycopeptide had occurred, the digestion products resulting from endo-β-*N*-acetylglucosaminidase D cleavage were isolated.

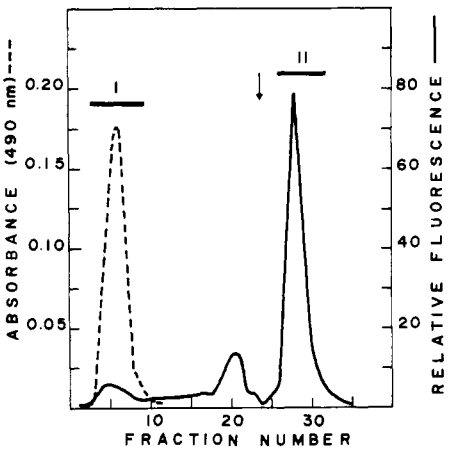


FIGURE 2: Further purification of the products resulting from endo-*N*-acetylglucosaminidase D digestion. The pooled fraction from Figure 1 was lyophilized, redissolved in 0.5 mL of 0.1 M pyridine–acetate, pH 3.5, and applied to a Dowex 50 X-2 column (0.5 × 4 cm) equilibrated with 0.1 M pyridine–acetate, pH 3.5. At the position indicated by the arrow, the column was eluted with 0.1 N NH_4OH. Individual fractions were assayed for hexose by using the phenol–sulfuric acid assay and for peptides by using the fluorescamine assay. The indicated fractions were pooled and used for subsequent compositional analyses.

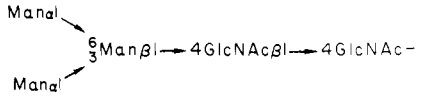
Table V: Analyses of the Products Resulting from Endo-β-*N*-acetylglucosaminidase D Digestion of the Core Glycopeptide from Rabbit Transferrin

component	residues/mol: core glycopeptide[a]	fraction I[b]	fraction II[a]
amino acid			
Asp	1.02		1.31
Ser	1.78		2.09
Leu	1.05		1.14
S-(aminoethyl)-Cys	1.00		1.00
carbohydrate			
mannose	2.70	3.00	
glucosamine	1.90	1.20	0.97

[a] Based on *S*-(aminoethyl)cysteine. [b] Based on three mannose residues.

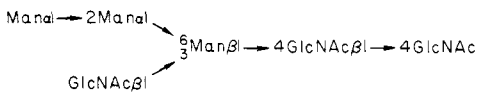
Since both products appeared in the same fraction upon gel filtration on a Bio-Gel P-2 column (Figure 1), further purification was achieved by chromatography on a Dowex 50 X-2 column (Figure 2), and each fraction was analyzed for neutral sugar, amino sugar, and amino acid content. These results are summarized in Table V and show that fraction I (Figure 2) contains three mannose residues and one *N*-acetylglucosamine residue, while fraction II (Figure 2) contains the pentapeptide and one residue of *N*-acetylglucosamine.

These data indicate that the core glycopeptide was hydrolyzed by endo-β-*N*-acetylglucosaminidase D. Based on the known specificity of this enzyme, the following structure for the core region can be proposed.



I

However, the possibility does remain that two alternate structures could exist.



II

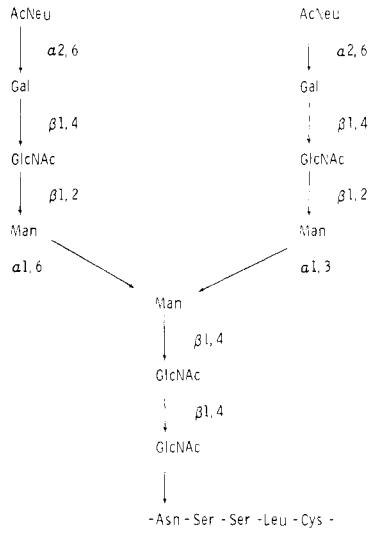
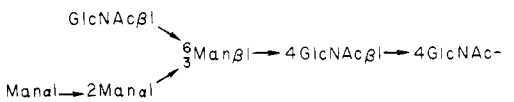


FIGURE 3: Proposed structure for the tryptic glycopeptide isolated from rabbit transferrin.



III

Although structures II and III are not consistent with the known specificity of endo-β-*N*-acetylglucosaminidase D, in order to determine unequivocally the structure of the core region, the $(Man)_3(GlcNAc)_2$-peptide was subjected to methylation analysis. The results of this analysis (Table II) indicate that the core glycopeptide contains two terminal mannose residues and one mannose residue substituted at C-3 and C-6. These results are consistent only with structure I.

Discussion

The structure of the tryptic glycopeptide isolated from rabbit transferrin was investigated with regard to amino acid and monosaccharide sequence, anomeric configurations, monosaccharide linkage positions, and the nature of the carbohydrate-peptide linkage. This was accomplished by use of sequential Edman degradations, specific exoglycosidases, endo-β-*N*-acetylglucosaminidases, methylation analyses, and periodate oxidation studies. The results are consistent with the structure proposed in Figure 3. Since the stoichiometry of this glycopeptide is 2 mol/mol of protein (Strickland & Hudson, 1978), rabbit transferrin contains two structurally identical glycopeptide segments, both of which are located within a polypeptide region representing approximately one-third of the transferrin molecule (Strickland & Hudson, 1978).

The structure of the carbohydrate units of rabbit transferrin is identical with that proposed by Spik et al. (1975) for one of the glycopeptides isolated from human transferrin but differs from that proposed by Jamieson et al. (1971) for human transferrin. A second type of carbohydrate unit apparently exists in human transferrin in which an additional *N*-acetylneuraminyl-α(2→6)-*N*-acetyllactosamine chain is attached to the same mannose core (Montreuil, 1975). This type of unit is absent in rabbit transferrin since only one residue of disubstituted mannose was found by methylation analysis (Table II). The carbohydrate units of rabbit transferrin are also identical with that found in thyroxine-binding globulin (Zinn et al., 1978), lactoferrin (Spik & Mazurier, 1977), and human IgG, IgE, and IgA except for the presence of fucose in the latter (Kornfeld & Kornfeld, 1976).

The function of the carbohydrate moiety of transferrin is unknown. Recent studies by Struck et al. (1978) have shown that the carbohydrate moiety of rat liver transferrin does not appear to be involved in the secretion process of this protein, suggesting an alternate role for the carbohydrate units. In several glycoproteins, the carbohydrate moiety appears to be involved in a variety of recognition systems (Ashwell & Morell, 1974; Kobata et al., 1968; Stahl et al., 1978). Likewise, it is possible that the carbohydrate units of transferrin play a role in the interaction of transferrin with the reticulocyte receptors (Kornfeld, 1968). These and other structural studies should provide a basis for further investigation into the possible role of the carbohydrate of transferrin in the mechanism of iron transfer.

Acknowledgments

The authors greatly appreciate the assistance of Dr. James C. Paulson in the digestion of the asialoglycopeptide with β-galactosidase from *Streptococcus pneumoniae*. The generous gifts of β-mannosidase from Dr. Y. T. Li and endo-β-*N*-acetylglucosaminidases D and H from Dr. A. Kobata are appreciated.

References

Aisen, P., & Brown, E. B. (1977) *Semin. Hematol. 14*, 31–53.
Ashwell, G., & Morell, A. G. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol. 41*, 99–128.
Avigad, G. (1969) *Carbohydr. Res. 11*, 119–123.
Baenziger, J., Kornfeld, S., & Kochwa, S. (1974) *J. Biol. Chem. 249*, 1889–1896.
Downing, M. R., & Mann, K. G. (1976) *Anal. Biochem. 74*, 298–319.
Edmann, P., & Begg, G. (1967) *Eur. J. Biochem. 1*, 80–91.
Hakomori, S. (1964) *J. Biochem. (Tokyo) 55*, 205.
Handa, N., & Montgomery, R. (1969) *Carbohydr. Res. 11*, 467–484.
Hartree, E. F. (1972) *Anal. Biochem. 48*, 422–427.
Hudson, B. G., Ohno, M., Brockway, W. J., & Castellino, F. J. (1973) *Biochemistry 12*, 1047–1053.
Ito, S., Muramatsu, T., & Kobata, A. (1975) *Biochem. Biophys. Res. Commun. 63*, 938–944.
Jamieson, G. A., Jett, M., & DeBernardo, S. L. (1971) *J. Biol. Chem. 246*, 3686–3693.
Jandl, J. H., & Katz, J. H. (1963) *J. Clin. Invest. 42*, 314–325.
Jandl, J. H., Inman, J. K., Simmons, R. L., & Allen, D. W. (1959) *J. Clin. Invest. 38*, 161–184.
Kobata, A., Grollman, E. F., & Ginsberg, V. (1968) *Biochem. Biophys. Res. Commun. 32*, 272–277.
Kornfeld, R., & Kornfeld, S. (1976) *Annu. Rev. Biochem. 45*, 217–237.
Kornfeld, R., Keller, J., Baenzinger, J., & Kornfeld, S. (1971) *J. Biol. Chem. 246*, 3250–3258.
Kornfeld, S. (1968) *Biochemistry 7*, 945–954.
Kuehl, F. A., Flynn, E. H., Holly, F. W., Mozingo, R., & Folkers, K. (1947) *J. Am. Chem. Soc. 69*, 3032–3035.
Kuhn, R., & Egge, H. (1963) *Chem. Ber. 96*, 3338–3348.
Leibman, A., & Aisen, P. (1977) *Biochemistry 16*, 1268–1272.
Levvy, G. A., & McAllen, A. (1959) *Biochem. J. 73*, 127–132.
Li, Y. T., & Li, S. C. (1972) *Methods Enzymol. 28*, 702–713.
Lindberg, B. (1972) *Methods Enzymol. 28*, 178–195.
Montreuil, J. (1975) *Pure Appl. Chem. 42*, 431–477.
Paulson, J. C., Prieels, J. P., Glasgow, L. R., & Hill, R. L. (1978) *J. Biol. Chem. 253*, 5617–5624.
Spik, G., & Mazurier, J. (1977) in *Proteins of Iron Metabolism* (Brown, E. B., Aisen, P., Fielding, F., & Crichton, R. R., Eds.) pp 143–151, Academic Press, London.
Spik, G., Fournet, B., Stecker, G., Bouqelet, S., & Montreuil, J. (1975) *FEBS Lett. 50*, 296–299.
Spiro, R. G. (1964) *J. Biol. Chem. 239*, 567–573.
Spiro, R. G. (1972) *Methods Enzymol. 28*, 36–37.
Stahl, P. D., Rodman, J. S., Miller, M. J., & Schlesinger, P. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1399–1403.
Stellner, K., Saito, H., & Hakomori, S. I. (1973) *Arch. Biochem. Biophys. 155*, 464–472.
Strickland, D. K., & Hudson, B. G. (1978) *Biochemistry 17*, 3411–3418.
Struck, D., Siuta, P. B., Lane, M. D., & Lennarz, W. J. (1978) *J. Biol. Chem. 253*, 5332–5337.
Tai, T., Yamashita, K., & Kobata, A. (1975a) *J. Biochem. (Tokyo) 78*, 679–686.
Tai, T., Yamashita, K., Ogata-Arakawa, M., Koide, N., Muramatsu, T., Iwashita, S., Inove, Y., & Kobata, A. (1975b) *J. Biol. Chem. 250*, 8569–8575.
Tai, T., Yamashita, K., & Kobata, A. (1977) *Biochem. Biophys. Res. Commun. 78*, 343–441.
Tarentino, A. L., & Maley, F. (1975) *Biochem. Biophys. Res. Commun. 67*, 455–462.
Trimble, R. B., & Maley, F. (1977) *Biochem. Biophys. Res. Commun. 78*, 935–944.
Yamashita, K., Tachibana, Y., & Kobata, A. (1978) *J. Biol. Chem. 253*, 3862–3869.
Zinn, A. B., Marshall, J. S., & Carlson, D. M. (1978) *J. Biol. Chem. 253*, 6768–6773.