Locations of Oligosaccharide Chains in Human α_1-Protease Inhibitor and Oligosaccharide Structures at Each Site†

Linda C. Hodges and Shung-Kai Chan*

ABSTRACT: Previous studies in this laboratory had shown that the α_1-protease inhibitor (α_1-PI) molecule contained two types of oligosaccharides, biantennary and triantennary. Cleavage of human α_1-protease inhibitor with CNBr resulted in three carbohydrate-containing fragments, I–III. Composition data revealed that each of these CNBr fragments contained one oligosaccharide chain. The oligosaccharide structure at each glycosylation site on each purified CNBr fragment was determined by compositional analysis, behavior on Con A affinity chromatography, and methylation analysis. From the results of these studies, we propose that fragment I contains one carbohydrate chain that can be of either the biantennary or triantennary type. On the other hand, fragments II and III each contain one carbohydrate chain exclusively by the biantennary type of complex *N*-glycoside.

Human α_1-protease inhibitor (α_1-PI)[1] is a serum glycoprotein of approximately 54 000 molecular weight consisting of a single polypeptide chain and about 12% carbohydrate. An inheritable deficiency of this protein results in familial emphysema and infantile cirrhosis. Much structural work has thus centered on α_1-PI because of its medical and genetic significance [for a review, see Laurell & Jeppsson (1975)].

Previous studies were undertaken in this laboratory to elucidate the detailed structure of the oligosaccharide present in α_1-PI (Hodges et al., 1979). Two types of carbohydrate chains were found. Compositional studies revealed that these oligosaccharide chains were examples of the complex type of *N*-glycoside, each consisting of *N*-acetylneuraminic acid, mannose, galactose, and *N*-acetylglucosamine.

The structure of each type of oligosaccharide was examined in detail by means of sequential exoglycosidase digestion and methylation analysis. The results showed that one type had the biantennary structure, which is known to bind weakly to Con A (Ogata et al., 1975) and is identical with the structure of the oligosaccharide from human transferrin (Spik et al., 1975). A second type had the triantennary structure, which does not bind to Con A and is very similar in structure to the *N*-glycoside of fetuin (Nilsson, 1978; Baenziger & Fiete, 1979). The correctness of these proposed structures were subsequently supported by Mega et al. (1980). The oligosaccharide chain with the biantennary structure was found in approximately 3 times the quantity of the chain with the triantennary structure. On the basis of these data, we originally proposed that four oligosaccharide units are present in α_1-PI, three of which are of the biantennary structure while one is of the triantennary structure.

This problem was further investigated by determining the total number of glycosylation sites along the polypeptide chain and the types of oligosaccharide chain at each glycosylation site. These studies revealed that there are only three glycosylation sites in human α_1-protease inhibitor and that the oligosaccharide unit at two of the sites is exclusively of the biantennary structure, whereas the oligosaccharide unit at the third site can be either of the biantennary or of the triantennary structure. The data supporting this conclusion are the subject of this communication.

Materials and Methods

Chemicals. All chemicals used in these experiments, unless otherwise indicated, are of reagent-grade quality. All organic solvents were redistilled before use. Ultrogel AcA 54 was obtained from LKB, Inc. (Bromma, Sweden). Con A–Sepharose and all other Sephadex products were purchased from Pharmacia (Uppsala, Sweden). Bio-Gels P-4 (200–400 mesh), P-10 (200–400 mesh), and AGA 3 × 4A (200–400 mesh) were from Bio-Rad Laboratories (Richmond, CA). Cyanogen bromide, as well as polyacrylamide (electrophoresis grade), and all associated reagents for gel electrophoresis were obtained from Eastman Kodak (Rochester, NY). Ultrapure guanidine hydrochloride was from Schwarz/Mann (Orangeburg, NY). Ethyl acetate, benzene, and dimethyl sulfoxide were obtained from Burdick-Jackson Laboratories (Muskegon, MI). Constant-boiling HCl, iodoacetamide, fluorescamine, and the sequenal-grade reagents used for both manual and automated Edman degradations were from Pierce Chemical Co. (Rockford, IL). Orcinol and methyl α-D-mannoside were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium borodeuteride was supplied by Stohler Isotope Chemicals (Waltham, MA). Three percent OV-210 on Supelcoport (80–100 mesh) was from Supelco, Inc. (Bellefonte, PA); 2% OV-17 on Gas-Chrom Q (80–100 mesh) was from Applied Science Laboratories (State College, PA).

Proteolytic Enzymes and Exoglycosidases. Pronase was purchased from Calbiochem (San Diego, CA). Trypsin (code TRTPCK-33M945) was obtained from Worthington Biochemical Corp. (Freehold, NJ) and the *Staphylococcus aureus* strain VI protease from Miles Laboratories (Elkhart, IN). Neuraminidase type VI isolated from *Clostridium perfringens* was obtained from Sigma Chemical Co. (St. Louis, MO).

Standards. IgG glycopeptide was kindly provided by Dr. Stuart Kornfeld (Washington University, St. Louis, MO). Fetuin glycopeptide (*N*-glycoside) was prepared by Pronase digestion of fetuin from Sigma Chemical Co. (St. Louis, MO).

Carboxamidomethylation and CNBr Fragmentation of α_1-Protease Inhibitor. Purified α_1-PI was isolated from human plasma or from Cohn fraction IV-1 (a gift from Cutter Lab-

† From the Department of Biochemistry, University of Kentucky, Lexington, Kentucky 40536-0084. *Received September 23, 1981; revised manuscript received January 25, 1982.* This work was supported in part by Research Grant HL-16166 from the National Institutes of Health and by the Graduate School at the University of Kentucky.

[1] Abbreviations: α_1-PI, α_1-protease inhibitor (formerly α_1-antitrypsin); Con A, concanavalin A; NeuNAc, *N*-acetylneuraminic acid; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; AAA, amino acid analysis; Quadrol, *N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine; TFA, trifluoroacetic acid; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/82/0421-2805$01.25/0

oratories, Berkeley, CA) as described previously (Shochat et al., 1978). The amount of protein and peptides used in the experiments herein reported was calculated from values obtained from amino acid analysis of the samples and by knowing the amino acid sequence[2] of the protein and the peptides. Two micromoles of protein was dissolved in 10 mL of 0.4 M Tris-HCl, pH 8.6, containing 0.2% EDTA and 6 M guanidine hydrochloride. The sample was deaerated with a stream of N_2 for 30 min and then reduced with 100 μL of 2-mercaptoethanol for 3 h in the dark at room temperature. Then 268 mg of iodoacetamide dissolved in 1 mL of absolute ethanol was added. After 15 min, the carboxyamidomethylated protein was dialyzed against 0.01 M NH_4HCO_3 and lyophilized. The lyophilized sample was dissolved in 7.5 mL of 70% formic acid, and 350 mg of CNBr was added. The reaction proceeded for 40 h at room temperature. The mixture was then diluted with 2–3 volumes of distilled water, concentrated by rotary evaporation, then diluted with 10 volumes of distilled water, and lyophilized. These conditions resulted in the disappearance of over 96% of the methionine residues in the sample.

Purification of Carbohydrate-Containing CNBr Fragments. The CNBr peptide mixture (500 nmol) was fractionated by gel filtration on Ultrogel AcA 54 in a column (2.5 × 100 cm) equilibrated in 0.05 M NH_4HCO_3 as described previously (Shochat et al., 1978) (see Figure 1). The second and third fractions were further purified by rechromatography on the same column. The first fraction was lyophilized, dissolved in 4 M guanidine hydrochloride in 0.5 M NH_4HCO_3 that contained 0.2 M LiBr, and applied to a column (1.5 × 90 cm) of Ultrogel AcA 54 equilibrated in 0.05 M NH_4HCO_3 containing 0.2 M LiBr (see Figure 2).

Pronase Digestion of Glycopeptides Obtained from CNBr Fragmentation. One micromole of each of purified CNBr fragments I–III (see Figure 2) was extensively digested with Pronase to obtain glycopeptides as described previously (Hodges et al., 1979). Glycopeptides were isolated by gel filtration on a column (1.5 × 90 cm) of Bio-Gel P-10 equilibrated in 4% acetic acid. Aliquots of fractions were assayed for carbohydrate with orcinol and for protein and free amino acids with fluorescamine. Fractions containing carbohydrate were pooled and analyzed for amino acid and carbohydrate content. As it was reported previously (Hodges et al., 1979), the Pronase digestion of the CNBr fragments is quite complete, and each of the resulting glycopeptides contains less than three to four amino acid residues.

Isolation of a Glycopeptide from CNBr Fragment I. A 1.6-μmol sample of CNBr fragment I was digested with *S. aureus* protease (Shochat et al., 1978). The glycopeptide was dissolved in 0.05 M *N*-ethylmorpholine–acetate, pH 8.6, and 0.8 mg of enzyme was added twice over a 30-h period at 37 °C. The peptide mixture was applied to a column (1.5 × 90 cm) of Bio-Gel P-6 in 0.05 M *N*-ethylmorpholine–acetate. Aliquots of the 2-mL fractions were assayed with fluorescamine and orcinol. Orcinol-positive fractions were separated from the enzyme and the other peptides and then were pooled and chromatographed on a column (1.5 × 90 cm) of Sephadex G-50 (40 mesh) superfine in 0.05 M *N*-ethylmorpholine–acetate, pH 8.6. The glycopeptide fraction was pooled, lyophilized, and analyzed for amino acid and carbohydrate content.

Con A–Sepharose Chromatography. Glycopeptides obtained from CNBr fragments I–III by either Pronase or *S. aureus* protease digestions were further resolved on a column (1 × 30 cm) of Con A–Sepharose equilibrated in 10 mM sodium acetate, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.9% NaCl, pH 6.0. The samples were dissolved in the same buffer and applied to the column. The column was first washed with 40 mL of the equilibrating buffer and then with 100 mL of this buffer containing 20 mM methyl α-D-mannoside. Aliquots of each fraction were assayed with fluorescamine and pooled accordingly. Each glycopeptide fraction was then desalted on Bio-Gel P-10 in 4% acetic acid prior to amino acid and carbohydrate analyses.

Neuraminidase Digestions. About 100 nmol of each glycopeptide was dried, dissolved in 2 mL of 0.1 M sodium citrate, pH 5.0, and digested at 37 °C with three additions of 0.1 mg of enzyme over a 48-h period. The asialoglycopeptide was reisolated on Bio-Gel P-10 in 4% acetic acid.

Methylation Analyses. Samples of each Con A fraction from Pronase-digested, desialylated CNBr fragments I–III were methylated according to the procedure of Stellner et al. (1973) with the modifications outlined previously (Hodges et al., 1979). The partially methylated alditol acetates were resolved on a Hewlett-Packard 5830A gas chromatograph with a 2 mm × 6 ft glass column of 2% OV-17 at 180 °C. Each peak was identified by comparison of retention times to those of known standards (IgG and fetuin). Samples were also resolved on a Finnigan 3300-6110 automated gas chromatograph–mass spectrometer–computer system with a 2 mm × 5 ft glass column of 3% OV-210 at 180 °C. Identification of peaks was based on comparison of retention times and mass spectra to those of known standards.

Manual Edman Degradation Procedure. Manual Edman degradations were performed according to the methods of Peterson et al. (1972) and Tarr (1975) with the modifications previously described (Shochat et al., 1978).

NH_2-Terminal Amino Acid Analysis. Samples (0.5 mol) of fragments I–III were each dissolved in 0.5 mL of TFA and applied to a JEOL sequencer, Model JAS-47K (Tokyo, Japan). The method of Edman & Begg (1967) for automated phenyl isothiocyanate degradation was employed, and 0.25 M Quadrol was used as the buffer for the coupling reaction. Fractions, 5 mL each, containing the 2-anilino-5-thiazolinones were obtained after the first step of degradation and were evaporated to dryness under a stream of N_2. Conversion to the corresponding PTH derivatives was performed according to a published method (Edman & Begg, 1967), and the PTH derivatives were identified by thin-layer chromatography, gas–liquid chromatography, and amino acid analysis as described previously (Shochat et al., 1978).

$NaDodSO_4$ Gel Electrophoresis. Polyacrylamide gel electrophoresis (10% acrylamide) in $NaDodSO_4$ was performed according to the procedure of Weber & Osborn (1969).

Amino Acid and Carbohydrate Analysis. Glycopeptides were hydrolyzed as follows: for amino acids, constant-boiling HCl at 110 °C in vacuo for 24 h; for *N*-acetylglucosamine, 4 N HCl at 100 °C in vacuo for 6 h; for neutral sugars (D-galactose and D-mannose), 1 N H_2SO_4 in sealed tubes for 8 h at 100 °C. Amino acids and *N*-acetylglucosamine were analyzed on a modified (Technicon) automated amino acid analyzer with a linear gradient of buffers (Robinson, 1975). Norleucine was used as the internal standard in all analyses. Neutral sugars were determined on an automated sugar analyzer according to Lee et al. (1969). D-Xylose was used as an internal standard.

Results

Purification of CNBr Glycopeptides. Purified α_1-PI was

[2] Subsequent to the work herein reported, the complete amino acid sequence of these fragments has been established in our laboratory.

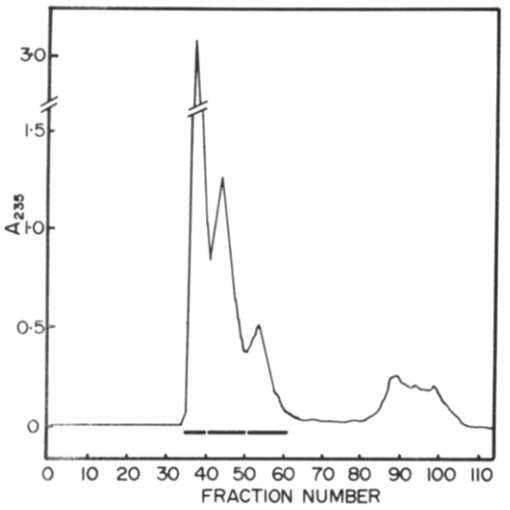


FIGURE 1: Fractionation of the CNBr peptide mixture of α_1-protease inhibitor: gel filtration on Ultrogel AcA 54. The flow rate was 30 mL/h; fractions of 4.5 mL were collected. The effluent was monitored by its absorbance at 235 nm. For experimental conditions, see Materials and Methods. The three fractions containing carbohydrates are indicated by solid bars.

Table I: Amino Acid and Sugar Composition[a] of CNBr Fragments I–III

	no. of residues/molecule of peptide		
	I	II	III
aspartic acid	17.4	10.1	7.1
threonine	11.1	8.9	4.4
serine	4.9	7.2	4.5
glutamic acid	22.0	11.2	5.9
proline	2.8	3.3	3.0
glycine	8.8	8.9	1.6
alanine	6.4	9.4	5.5
valine	10.3	5.2	1.4
isoleucine	4.9	5.7	2.4
leucine	17.0	15.9	3.8
tyrosine	2.6	1.8	0.6
phenylalanine	12.0	4.7	5.2
histidine	4.0	3.8	3.4
lysine	14.1	9.6	3.2
arginine	2.5	2.3	1.1
D-glucosamine	3.4	3.1	2.9
D-mannose	3.3	3.0	3.3
D-galactose	2.8	1.9	2.0

[a] For experimental conditions, see Materials and Methods.

carboxamidomethylated and treated with CNBr, and the resulting peptides were isolated on Ultrogel AcA 54 as described above. The elution profile was the same as that obtained previously (Shochat et al., 1978). Only three carbohydrate-containing peaks were detected (Figure 1). The second and the third fractions were further purified by rechromatography on the same column; each fraction yielded a single glycopeptide, fragments I and II, respectively. The first fraction contained a mixture of fragments I–III. Therefore, this fraction was denatured in 6 M guanidine hydrochloride and gel filtered on Ultrogel AcA 54 in the presence of LiBr (Figure 2). The first fraction eluted on this column contained fragment III, while the subsequent two fractions yielded more of fragments I and II, respectively.

Each of the CNBr fragments I–III showed unique amino acid and sugar compositions (Table I). Fragments II and III each showed a single band upon electrophoresis in the presence of $NaDodSO_4$, while fragment I showed two bands (Figure 3), one major and one minor. The minor band was considered to be an aggregate form of the major band (see Discussion),

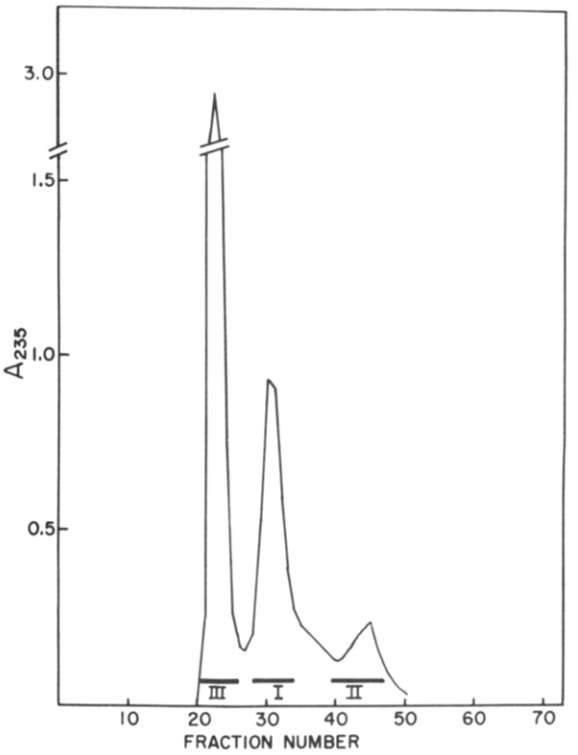


FIGURE 2: Fractionation of the first carbohydrate-containing fraction from Figure 1: gel filtration on Ultrogel AcA 54. The flow rate was 8 mL/h; fractions of 2 mL were collected. The effluent was monitored at 235 nm. For experimental conditions, see Materials and Methods.

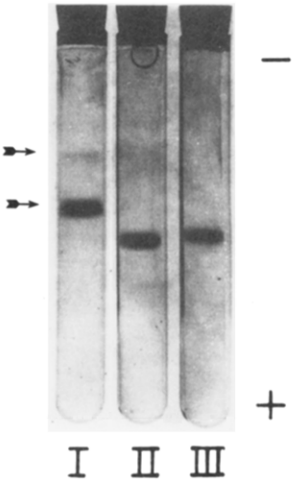


FIGURE 3: Homogeneity of purified CNBr fragments: CNBr fragments I–III were electrophoresed in the presence of $NaDodSO_4$. For experimental conditions, see Materials and Methods. (+) denotes anode; (–) denotes cathode.

and each has the same primary structure. A single NH_2-terminal amino acid residue was identified for each fragment: leucine for fragment I, lysine for fragment II, and glutamic acid for fragment III. These data established that each of the three fragments is a unique single glycopeptide derived from a different region of the intact α_1-PI molecule.[2] Assuming that intact α_1-PI has a molecular weight of 54000 and contains 43 aspartic–asparagine residues, we calculated its carbohydrate composition to be 9 mannose, 7 galactose, 6 *N*-acetylneuraminic acid, and 12 *N*-acetylglucosamine residues per molecule (Hodges et al., 1979). Thus, the carbohydrate composition of fragments I–III together account for the total content of sugars in the intact molecule (Table I).

Identification of Number and Type of Oligosaccharide Unit in CNBr Fragments I–III. Our earlier studies showed that

Table II: Amino Acid and Sugar Composition[a] of Glycopeptides Resulting from Pronase Digest of CNBr Fragment I

	glycopeptide A	glycopeptide B
aspartic acid	1	1
D-mannose	2.9	3.0
D-galactose	3.0	2.4
D-glucosamine	3.3	2.9

[a] For experimental conditions, see Materials and Methods. The molar ratio was calculated on the basis of the assumption that each glycopeptide contains one residue of Asp per molecule of peptide.

each type of oligosaccharide chain in α_1-PI contains three mannose residues (Hodges et al., 1979). On the basis of the carbohydrate composition data (Table I), fragments I–III each contain one of the oligosaccharide chains found in α_1-PI.

To determine the type of oligosaccharide found in each carbohydrate-containing CNBr peptide, we extensively digested purified fragments I–III with Pronase. The glycopeptides were isolated on Bio-Gel P-10 and then applied to Con A–Sepharose. The oligosaccharides in fragment I were resolved into two components on the Con A column, one that did not bind to Con A and one that did bind and could be eluted with 20 mM methyl α-D-mannoside. The carbohydrate composition of each is given in Table II. These data imply that fragment I contains both triantennary and biantennary oligosaccharide structures.

When fragments II and III were each similarly digested with Pronase and the resulting glycopeptides were applied to a Con A–Sepharose column, all of the oligosaccharides were bound to the column and were eluted with 20 mM methyl α-D-mannoside. No detectable material was found in the initial elution with buffer. These data imply that fragments II and III contain only the biantennary oligosaccharide structure. These conclusions are further substantiated by the chemical evidence shown below.

Aliquots of each fraction obtained from Con A chromatography of the Pronase digest of fragment I were desialylated and methylated. The resulting partially methylated alditol acetates were resolved and quantitated by gas chromatography and were identified by comparison of retention times and mass spectra to those of known standards. The glycopeptide from fragment I that did not bind to Con A contained about three terminal galactose residues, one 2-linked mannose, one 3,6-disubstituted mannose, one 2,4-disubstituted mannose, and five 4-linked *N*-acetylglucosamine residues per molecule of peptide (Table III). These data are identical with those obtained for the triantennary oligosaccharide structure of α_1-PI (Hodges et al., 1979). The glycopeptide from fragment I that bound to Con A contained about two residues of terminal galactose, two residues of 2-linked mannose, one 3,6-disubstituted

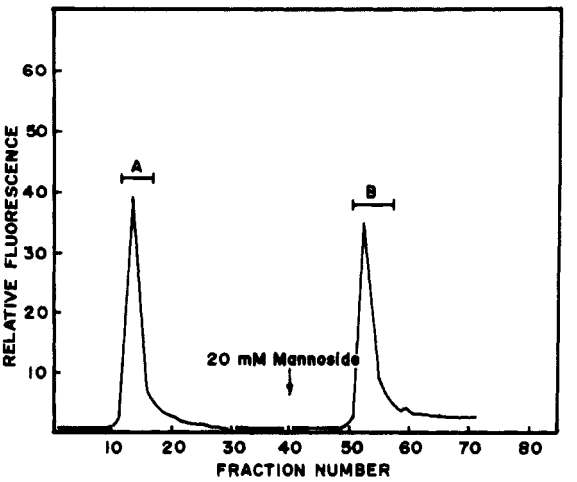


FIGURE 4: Affinity chromatography on Con A–Sepharose of glycopeptides from an *S. aureus* protease digest of CNBr fragment I. The flow rate was 30 mL/h; fractions of 1 mL were collected. (↓) denotes the beginning of elution with 20 mM methyl α-D-mannoside.

mannose, and four residues of 4-linked *N*-acetylglucosamine per molecule of peptide (Table III). These data confirm that fragment I contained both the biantennary and the triantennary oligosaccharide structures found in α_1-PI (Hodges et al., 1979).

Aliquots of fragments II and III after Pronase digestion were desialylated and methylated without prior Con A chromatography. The resulting partially methylated alditol acetates were those characteristic of the biantennary oligosaccharide structure of α_1-PI (see Table III). No significant amount of the methylated products indicative of the triantennary structure was detected. Since methylation analysis is a very sensitive technique, these results indicate that the glycosylation site in each fragment II and III contains exclusively the biantennary oligosaccharide structure.

Amino Acid Sequence around the Glycosylation Site of CNBr Fragment I. Fragment I was digested with *S. aureus* protease, and the peptide mixture was fractionated on Bio-Gel P-6 and Sephadex G-50 (40 mesh) superfine. Only one carbohydrate-containing fraction was obtained, this fraction was applied to a Con A–Sepharose column, and two fractions were obtained as shown in Figure 4: one (B) that bound to Con A and one (A) that did not. The two fractions had identical amino acid but different carbohydrate compositions as shown in Table IV. Fraction A showed three mannose and three galactose residues, which is indicative of the triantennary oligosaccharide structure of α_1-PI. Fraction B showed three mannose and two galactose residues, which is indicative of the biantennary oligosaccharide structure. Samples from both were also taken for amino acid sequence analysis. Six cycles of manual Edman degradation were performed (Table V), and

Table III: Relative Amounts[a] of Partially Methylated Alditol and Aminodeoxyalditol Acetates in Glycopeptides after Desialylation and Methylation

	galactose, 2,3,4,6-Me$_4$	mannose			GlcNAc, 3,6-Me$_2$
		3,4,6-Me$_3$	3,6-Me$_2$	2,4-Me$_2$	
CNBr fragment I					
A	2.77	0.93	1.04	1.00[b]	5.40
B	1.44	2.00[b]		0.87	3.60
CNBr fragment II	1.40	2.00[b]		1.06	3.96
CNBr fragment III	1.20	1.50		1.00[b]	3.97

[a] For experimental conditions, see Materials and Methods. Quantitatively determined on a 5830 A Hewlett-Packard gas chromatograph with a column of 2% OV-17 by using response factors obtained from the partially methylated alditol acetates of IgG glycopeptide and fetuin glycopeptide. [b] Ratios based on this derivative.

Table IV: Amino Acid and Sugar Composition[a] of Glycopeptides Resulting from *S. aureus* Protease Digestion of CNBr Fragment I

	glycopeptide A	glycopeptide B
aspartic acid	1.7	2.0
threonine	1.0	0.9
glutamic acid	1.0	1.3
glycine	1.0	1.0
leucine	1.5	1.8
phenylalanine	0.9	0.9
D-mannose	3.2	3.6
D-galactose	2.9	2.3
D-glucosamine	3.8	3.1

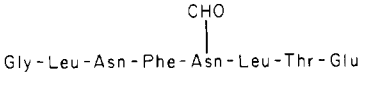
[a] For experimental conditions, see Materials and Methods. The molar ratio was calculated on the basis of the assumption that each glycopeptide contains one residue of glycine.

Table V: Edman Degradation of Glycopeptides from CNBr Fragment I after Digestion with *S. aureus* Protease

step	glycopeptide A PTH-amino acid	glycopeptide B PTH-amino acid
1	Gly (TLC, GC, AAA)[a]	Gly (TLC, GC, AAA)
2	Leu (TLC, GC, AAA)	Leu (TLC, GC, AAA)
3	Asn (TLC, AAA)	Asn (TLC, AAA)
4	Phe (TLC, GC, AAA)	Phe (TLC, GC, AAA)
5		
6	Leu (TLC, GC, AAA)	Leu (TLC, GC, AAA)
7	Thr (TLC)	Thr (TLC)
8	Glu (TLC, AAA)	Glu (TLC, AAA)

[a] Methods used to identify the amino acid residue. For experimental conditions, see Materials and Methods.

the following sequence was obtained for both the glycopeptide fractions.



Since no PTH-amino acid was postively identified on the fifth cycle and since Asx disappeared from the amino acid analysis of the residual peptide after the fifth cycle, we concluded that this was the site of attachment of the oligosaccharide.

Discussion

Much progress has been made in recent years in elucidating the detailed structures of oligosaccharide units of glycoproteins. While many reports concerning the structures of the oligosaccharide units have now appeared in the literature, relatively little attention has focused on determining the uniformity at each of the glycosylation sites in glycoproteins (Mizuochi & Kobata, 1980). We feel that such information is important if a complete knowledge of structure–function relationships and biosynthetic and degradative pathways for oligosaccharide units are to be ascertained. It should be emphasized that vigorous proof on the homogeneity and/or heterogeneity of oligosaccharide structures at a given glycosylation site is difficult to obtain. To establish that a single glycosylation site could accommodate more than one type of oligosaccharide structure, one must extensively purify the glycopeptide and clearly establish its purity. The purified peptide should contain only one glycosylation site. The next step is to establish the primary structure of the glycopeptide and show that only a single set of amino acid sequence is present in that peptide. Then with the elucidated oligosaccharide structures on hand, one can conclude that indeed one or more different oligosaccharide structural units are attached to the same unique site of the polypeptide chain.

In this communication, we have established that in human α_1-PI there are three glycosylation sites. On the basis of our amino acid sequence studies on the human protein[2] and the nucleotide sequence recently elucidated for the corresponding baboon gene by Kurachi et al. (1981), we can now identify the three glycosylation sites as amino acid residues 46, 83, and 247, numbering from the NH_2 terminus of the polypeptide. Only the biantennary oligosaccharide chain is found in the first and the third glycosylation sites, but either the triantennary or the biantennary oligosaccharide chain may be found in the second site.

To reach this conclusion, we fragmented α_1-PI using CNBr and obtained three carbohydrate-containing peptides, fragments I–III. The purity of each fragment was demonstrated by $NaDodSO_4$ gel electrophoresis and by the presence of a single NH_2-terminal residue in each fragment. Even though two bands were repeatedly observed (Figure 3) when fragment I was subjected to electrophoresis in the presence of $NaDodSO_4$, we believe that the upper band is a dimer and that the two bands have the same primary structure. This conclusion was reached on the basis of the fact that only one set of amino acid sequence was obtained from this sample.[2]

Sugar analysis on the three CNBr fragments shows that together they account for the total carbohydrate content in α_1-PI and that each fragment contains sufficient sugars for approximately one oligosaccharide unit (Table I). Previously we established that there are two types of oligosaccharide unit in α_1-PI, triantennary and biantennary (Hodges et al., 1979). On the basis of their affinity to Con A and on chemical evidence derived from the analysis of the sugar derivatives after methylation, only the biantennary oligosaccharide chain is found in fragments II and III, while both triantennary and biantennary are found in fragment I. Since composition data supported the presence of only one carbohydrate chain in fragment I, we concluded that the glycosylation site in fragment I is heterogeneous. This observation is further substantiated by the amino acid sequence analysis of the glycopeptides derived from fragment I. After *S. aureus* protease digestion of fragment I, only one carbohydrate-containing fraction was obtained on gel filtration. However, this fraction could be resolved into two glycopeptide-containing fractions on Con A–Sepharose (Figure 4). The peptides in the two resolved fractions had identical amino acid sequences, but one contained the triantennary oligosaccharide structure and the other the biantennary oligosaccharide structure (Table IV).

As pointed out in our earlier paper (Hodges et al., 1979), a discrepancy exists between the amounts of *N*-acetylglucosamine in the glycopeptides as determined by anion-exchange chromatography after hydrolysis in 4 N HCl (Tables I, II, and IV) and amounts obtained by integration of the peak areas of the products of methylation (Table III). This difference may arise from incomplete hydrolysis using 4 N HCl in vacuo at 100 °C for 4–6 h. These conditions can result in a rapid loss of the acetyl group from *N*-acetylglucosamine and can generate a positively charged amino species near the glycosidic bond, making that bond highly resistant to hydrolysis.[3] Other workers using 4 N HCl for hydrolysis as opposed to techniques such as methanolysis also show low *N*-acetylglucosamine content compared to their proposed glycopeptide structures (Zinn et al., 1978; Kawasaki & Ashwell, 1976). The aceto-

[3] S. Svensson (personal communication).

lysis–hydrolysis (Stellner et al., 1973) appears to circumvent this problem.

Human α_1-PI is heterogeneous, showing two major and several minor bands upon isoelectric focusing. This multiplicity presumably arises from a postribosomal event and can be partially explained by variations in the content of NeuNAc in the protein (Jeppsson et al., 1978). On the basis of the data herein reported, we now propose that one of the major contributions to the heterogeneity in α_1-PI is the content of the triantennary oligosaccharide chain. Because the triantennary chain has three NeuNAc residues and the biantennary chain has only two, the variation in the type of oligosaccharide chain present in the protein will result in electrophoretic heterogeneity.

References

Baenziger, J. U., & Fiete, D. (1979) *J. Biol. Chem. 254*, 789.

Edman, P., & Begg, G. (1967) *Eur. J. Biochem. 1*, 80.

Hodges, L. C., Laine, R., & Chan, S. K. (1979) *J. Biol. Chem. 254*, 8208.

Jeppsson, J.-O., Laurell, C.-B., & Fagerhol, M. (1978) *Eur. J. Biochem. 83*, 143.

Kawasaki, T., & Ashwell, G. (1976) *J. Biol. Chem. 252*, 5292.

Kurachi, K., Chandra, T., Degen, S. J. F., White, T. T., Marchioro, T. L., Woo, S. L., & Davie, E. W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6826.

Laurell, C. B., & Jeppsson, J.-O. (1975) in *The Plasma Proteins* (Putnam, F. W., Ed.) 2nd ed., Vol. 1, pp 229–245, Academic Press, New York.

Lee, Y. C., McKelvy, J. F., & Lang, D. (1969) *Anal. Biochem. 27*, 567.

Mega, T., Lujan, E., & Yoshida, A. (1980) *J. Biol. Chem. 255*, 4053.

Mizuochi, T., & Kobata, A. (1980) *Biochem. Biophys. Res. Commun. 97*, 772.

Nilsson, B. (1978) Ph.D. Thesis, University of Lund, Lund, Sweden.

Ogata, S.-I., Muramatsu, T., & Kobata, A. (1975) *J. Biochem. (Tokyo) 78*, 687.

Peterson, J. D., Nehrlich, S., Oyer, P. E., & Steiner, D. F. (1972) *J. Biol. Chem. 247*, 4866.

Robinson, G. W. (1975) *J. Chromatogr. 111*, 416.

Shochat, D., Staples, S., Hargrove, K., Kozel, J. S., & Chan, S. K. (1978) *J. Biol. Chem. 253*, 5630.

Spik, G., Bayard, B., Fournet, B., Strecker, G., Bouquelet, S., & Montreuil, J. (1975) *FEBS Lett. 50*, 296.

Stellner, K., Saito, H., & Hakomori, S.-I. (1973) *Arch. Biochem. Biophys. 155*, 464.

Tarr, G. E. (1975) *Anal. Biochem. 63*, 361.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Zinn, A. B., Marshall, J. S., & Carlson, D. M. (1978) *J. Biol. Chem. 253*, 6768.