Carbohydrate structures of a human tissue plasminogen activator variant expressed in recombinant Chinese hamster ovary cells

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The carbohydrate structures of a genetically engineered human tissue plasminogen activator variant bearing a single N-glycosylation site at Asn 448 are reported. After isolation of the tryptic glycopeptide and liberation of the N-linked carbohydrates by polypeptide:*N*-glycosidase F, 6 major oligosaccharide fractions were separated by HPLC on NH_2-bonded phase. Their structures were determined by compositional and methylation analyses combined with fast atom bombardment mass spectrometry. Seventy percent of the carbohydrates were of the biantennary complex type with fucose at the proximal GlcNAc and zero, one or two α2-3 linked NeuAc. The remainder were triantennary structures with one, two or three NeuAc.

Recombinant glycoprotein; Structure of N-linked carbohydrates; Fast atom bombardment mass spectrometry; Human tissue plasminogen activator

1. INTRODUCTION

Human tissue plasminogen activator (t-PA) is an important regulator of the fibrinolytic activity in blood and is produced by endothelial cells at levels of 1–5 ng/ml plasma. In the presence of fibrin it converts plasminogen to plasmin by a specific proteolytic cleavage, which in turn efficiently degrades the fibrin network of thrombin found in the vascular system [1–3]. Recombinant human t-PA produced from Chinese hamster ovary cells (CHO cells) is in clinical use for the treatment of myocardial infarction. Human t-PA is a glycoprotein with 4 potential N-type glycosylation sites of which one (Asn-218) is never occupied. When isolated from human or heterologous mammalian cell lines, the protein can be separated into type I t-PA bearing 3 N-linked glycans at Asn 117, 184 and 448 respectively and type II where only Asn 117 and 448 are glycosylated. Site-specific carbohydrate structure analysis showed Asn 117 to carry high mannose type oligosaccharides, whereas Asn 184 and 448 bear complex type structures displaying cell type specific differences in their terminal substitution [4–7]. Interestingly, type I and II t-PA exhibit different enzymatic activities when tested in vitro [8]. In the present work we describe the detailed structural analysis of a genetically engineered human t-PA variant (containing Gln in place of Asn at positions 117 and 184) which has been expressed and purified from CHO-cells [9].

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2. MATERIALS AND METHODS

2.1. *Materials*

Recombinant human t-PA BC 4′ is a product of Behringwerke AG (Marburg, FRG).

2.2. *Purification of t-PA*

The culture supernatant was concentrated 10-fold by using a Filtron Omega Cassette, and was adsorbed onto heparin-Sepharose as described [10] and eluted with a buffer containing 0.05 M glycine, 0.2 M arginine and 0.2 M lysine adjusted to pH 7.0 (buffer A). The eluate was concentrated 10-fold using the same cassette. The material obtained was adsorbed on a Sepharose-Caproyl-D-Leu-Pro-Arg-CHO. The column was washed with a buffer containing 0.05 M glycine and 0.1 M NaCl (pH 7.0). Bound t-PA protein was eluted with 0.5 M arginine at pH 4.0. In order to separate the t-PA multimers the eluate was adjusted to pH 7.0 and passed through a TSK 3000 column equilibrated with buffer A. The purified protein was lyophilized and stored at −20°C until use.

2.3. *Isolation of the tryptic glycopeptide*

Purified t-PA was reduced, carboxamidomethylated and after dialysis was digested with trypsin at an t-PA:enzyme ratio of 100:3 as described [11]. Peptides were separated by HPLC on a RP-C_{18} column. The glycopeptide was identified by carbohydrate compositional analysis of the HPLC-fractions.

2.4. *Liberation of the oligosaccharides*

The glycopeptide was incubated with polypeptide:*N*-glycosidase F (PNGase) for 24 h in 100 mM Na-phosphate buffer pH 7.0 containing 0.02% NaN_3 as described [11]. Oligosaccharide material was recovered after HPLC on RP-material collecting the first 25 ml. Complete removal of oligosaccharides by PNGase was demonstrated by the failure to detect any carbohydrate in the residual peptide material.

2.5. *Isolation of oligosaccharides*

Oligosaccharides were separated by HPLC on a NH_2-bonded phase (Lichrosorb-NH_2, Merck, Darmstadt, FRG) as described [12].

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2.6. *Carbohydrate composition and methylation analysis*

Monosaccharides were analyzed by GLC on a Carlo Erba Mega Series instrument after methanolysis, reacetylation and trimethylsilylation as the corresponding methyl glycosides on a 30 m DB1 capillary column according to Chaplin [13].

The glycopeptide and the reduced oligosaccharides were methylated according to Hakomori [14]. The permethylated carbohydrate material was purified by chromatography on a Sephadex LH20 column, hydrolyzed, reduced and peracetylated as described [11]. Analyses were performed on a Kratos MS 50 fast scan mass spectrometer connected to a Carlo Erba Mega Series gas chromatograph equipped with a 30 m DB1 column. Ionisation current was 300 μA, ionisation potential 70 eV and a cycle time of 1.5 s was used. Analysis of partially methylated alditol acetates was performed in the SIM-mode monitoring the characteristic fragment ions at m/z = 116, 117, 129, 130, 145, 158, 161, 189, 205, 233, 261, 277, 290, 305, 318, and 333. Quantitation was achieved by peak integration. Values were corrected using response factors obtained from standard oligosaccharides.

2.7. *Fast atom bombardment mass spectrometry*

The reduced and permethylated oligosaccharides were subjected to positive mode FAB mass spectrometry on a Kratos MS50 RF TC equipped with a high field magnet, using a 8 kV xenon beam (source pressure 10^{-5} Torr). Spectra of samples (2–5 μg, dissolved in a matrix of thioglycerol) were recorded at an accelerating potential of 8 kV with a magnet scan rate of 30 s/dec at a resolution sufficient to separate the quasimolecular cluster. 2–5 scans were accumulated using the DS90 datasystem in the raw data mode and then converted to low resolution spectra.

2.8. *High-pH anion-exchange chromatography*

High-pH anion-exchange chromatography was performed using a non-metallic HPLC-system, a pelicular anion exchange column (Carbopac PA1) and a pulsed amperometric detector (HPAE-PAD; Dionex BioLC, Sunnyvale, CA). Elution of oligosaccharides was performed by applying a linear 30 min gradient from 0–10% B, then a 10 min gradient to 20% B followed by a 10 min isocratic run (flow rate was 1 ml/min; solvent A: 0.1 M NaOH, solvent B: 0.1 M NaOH containing 0.5 M NaAc).

3. RESULTS

Methylation data for the glycopeptide (GP) suggest the presence of mainly biantennary (70%) and triantennary structures, the third branch being predominantly bound to position 6 of the common core mannose as shown by the preponderance of the 2,6- compared to the 2,4-disubstituted mannose derivative. The oligosaccharide structures are almost completely fucosylated at the proximal GlcNAc. The degree of sialylation is approximately 70%, NeuAc being exclusively α2-3 linked to Galβ1-4GlcNAc-R. Polylactosamine repeats can be present only in very small amounts (<5%), since the

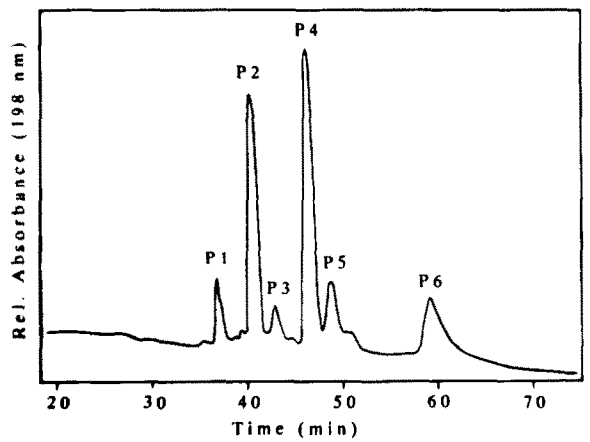

Fig. 1. NH_2-bonded phase HPLC of N-linked oligosaccharides of t-PA. P1–P6 indicate fractions subjected to compositional and methylation analysis as well as FAB mass spectrometry.

Table I

Compositional analysis of the glycopeptide GP and oligosaccharide fractions P1–P6

	Fraction						
	GP	P1	P2	P3	P4	P5	P6
Fuc	0.9	1.0	0.9	0.9	1.0	0.8	0.9
Man[a]	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Gal	2.2	1.9	2.0	2.8	2.0	2.9	3.2
GlcNAc	3.8	3.5	3.7	4.6	3.7	4.5	4.9
NeuAc	1.8	<0.1	1.0	0.9	1.8	1.9	2.9

[a] Values are based on Man = 3.0

Table II

Methylation analyses of the glycopeptide before (GP) and after (GP_h) acid hydrolysis and the oligosaccharide fractions P1–P6

Peracetylated derivative of	GP	GP_h	P1	P2	P3	P4	P5	P6
Fucitol								
2,3,4-Tri-*O*-methyl-	0.9	0.8	1.0	1.0	0.9	0.9	0.9	1.0
Galacitol								
2,3,4,6-Tetra-*O*-methyl-	0.7	2.4	2.1	1.0	1.8	0.1	0.8	0.2
2,4,6-Tri-*O*-methyl-	1.7	<0.1	<0.1	1.1	1.1	2.0	1.8	3.1
Mannitol								
3,4,6-Tri-*O*-methyl-	1.7	1.8	2.1	2.1	1.2	2.2	1.2	1.0
3,6-Di-*O*-methyl-	<0.1	<0.1	–	–	0.2	<0.1	0.2	0.3
3,4-Di-*O*-methyl-	0.2	0.2	<0.1	<0.1	0.7	<0.1	0.8	0.8
2,4-Di-*O*-methyl-[a]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
2-*N*-methylacetamido-2-deoxyglucitol								
1,3,5,6-Tetra-*O*-methyl-	–	0.05	<0.05	<0.05	–	–	–	–
1,3,5-Tri-*O*-methyl-	–	–	0.9	0.8	0.9	0.9	0.8	0.9
3,6-Di-*O*-methyl-	3.2	3.3	2.8	2.6	3.4	2.7	3.5	3.9
3-*O*-methyl-	0.3[b]	0.2[b]	–	–	–	–	–	–

[a] Values are based on 2,4-di-*O*-methylmannitol = 1.0

[b] Due to incomplete hydrolysis of the Asn-GlcNAc-bond, the recovery of the corresponding partially methylated alditol acetate is significantly reduced

3-substituted galactose derivative almost completely disappears after mild acid hydrolysis (0.5 M HAc, 80°C, 1 h) in favour of the 2,3,4,6-tetra-*O*-methyl-galactose derivative.

The HPLC elution profile of the liberated oligosaccharides is shown in Fig. 1. Six major carbohydrate fractions were collected. Compositional and methylation data as well as FAB mass spectrometry of all fractions indicate complete fucosylation at the proximal GlcNAc. Structural analysis of oligosaccharides from fraction P1 indicate a biantennary asialo complex type of structure as demonstrated by the exclusive detection of terminal galactose by methylation analysis, the presence of ions at m/z = 464 (Hex-HexNAc) in the FAB-spectrum, and the absence of NeuAc in the compositional data. Fraction P2 represents a biantennary monosialylated complex type of structure as can be inferred from a 1:1 ratio of terminal and 3-substituted galactose derivatives detected in the methylation analysis and the presence of fragment ions at m/z = 825 (NeuAc-Hex-HexNAc) and m/z = 464 (Hex-HexNAc) shown in the FAB spectrum (Fig. 2). Data for fraction P3 suggest a monosialylated triantennary complex type structure, the third branch being attached mainly to position 6 of the common core mannose (75%), the remainder being bound to position 4 as indicated by the ratio of the corresponding mannose derivatives detected in the methylation analysis. The detection of the molecular ion ($M+H^+$ = 2958) and the presence of only fragment ion m/z = 825 (793) for fraction P4 (as well as detection of exclusively 2,4,6-tri-*O*-methyl galacitol in methylation analysis) indicate a biantennary disialylated complex type of structure. Fraction P5 contains a partially sialylated triantennary oligosac-

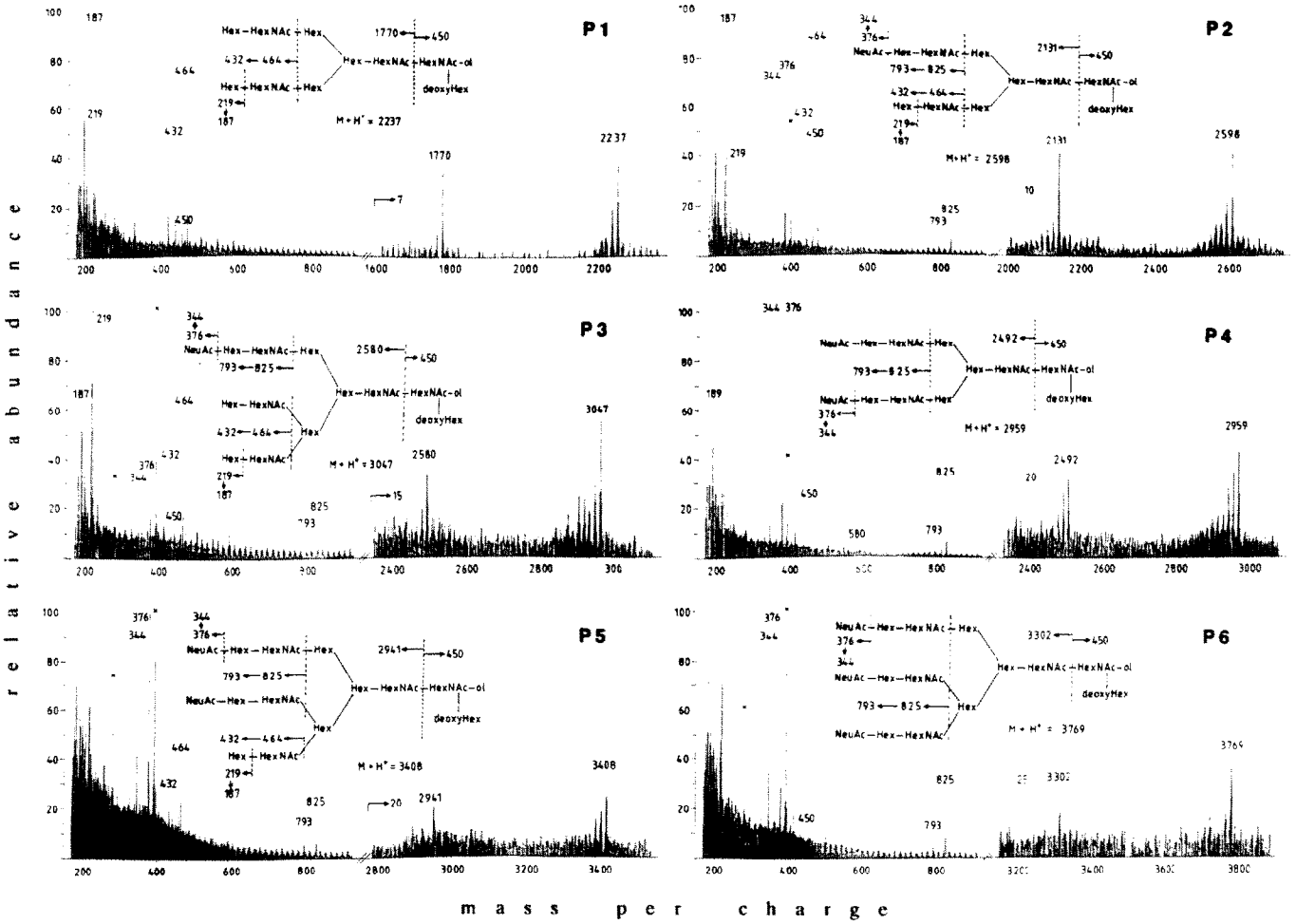

Fig. 2. FAB mass spectra of oligosaccharide fractions P1–P6. In the high mass region the carbohydrates give rise to molecular ions ($M+H^+$) at m/z = 2237 (nominal mass) corresponding to (Hex_5-$HexNAc_3$-deoxyHex-HexNAcol, P1); 2598 (NeuAc-Hex_5-$HexNAc_3$-deoxyHex-HexNAcol, P2); 3047 (NeuAc-Hex_6-$HexNAc_4$-deoxyHex-HexNAcol, P3); 2959 (NeuAc$_2$-Hex_5-$HexNAc_3$-deoxyHex-HexNAcol, P4); 3408 (NeuAc$_2$-Hex_6-$HexNAc_4$-deoxyHex-HexNAcol, P5); and 3769 (NeuAc$_3$-Hex_6-$HexNAc_4$-deoxyHex-HexNAcol, P6). Signals at m/z = $M+H^+-(n\times 14)$ are due to undermethylation. All structures show the characteristic fragment ion at m/z = ($M+H^+$ − 467), which results from loss of deoxyHex-HexNAcol, indicating the presence of a fucose residue attached to the proximal GlcNAc. In the lower mass region the following characteristic fragment ions were observed: at m/z = 825 and 793 (minus CH_3OH): NeuAc-Hex-HexNAc, 464 and 432: Hex-HexNAc, 450: deoxyHex-HexNAcol, 376 and 344: NeuAc, 219 and 187 (Hex) and 189 (deoxyHex). Ions marked by x arise from contaminating dioctylphthalate.

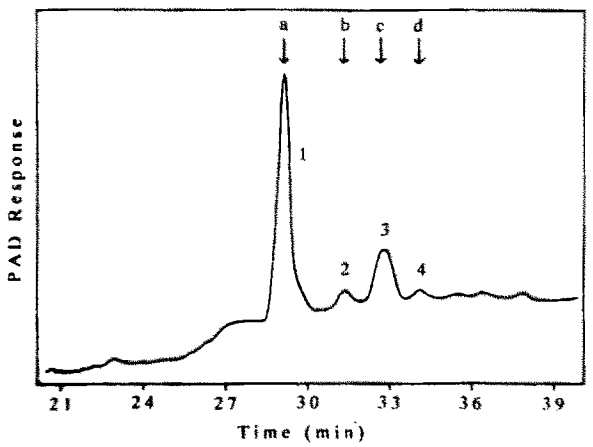

Fig. 3. HPAE-PAD of N-linked desialylated oligosaccharides from t-PA variant BC 4'. Arrows indicate the elution times of fucosylated asialo reference structures (a = biantennary; b = triantennary, 2,4-branched; c = triantennary, 2,6-branched; d = tetraantennary). By integration of peaks 1–4 a relative ratio of 72:4:20:3 for the bi-, tri- (two isomeric forms) and tetraantennary structures was calculated.

charide. Methylation data show the presence of a triantennary complex type structure, the third branch being predominantly bound to position 6 of the common core mannose. The ratio of the 3-substituted galactose derivative to the terminal galactose indicates two sialylated and one asialo branch. This conclusion is supported by the ratios found for fragment ions m/z = 825 (NeuAc-Hex-HexNAc) to m/z = 464 (Hex-HexNAc) and from the molecular ion cluster in the FAB spectrum ($M+H^+$ = 3408). The results obtained for fraction P6 suggest the presence of a triantennary trisialylated oligosaccharide structure, the third branch again being preponderantly attached to position 6 of the common core mannose. From the detection of terminal galactose in the methylation data the presence of some trisialylated tetraantennary structures can be inferred, however, no evidence of partially sialylated structures could be detected in the FAB spectrum.

These results were confirmed by HPAE-PAD. Fig. 3 shows the elution profile of the neuraminidase-treated

Table III

Proposed structures for oligosaccharides from fractions P1–P6

Oligosaccharide	Structure	Rel. Amount (%)
P1	Galβ1-4GlcNAcβ1-2Manα1-6 / Fucα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc / Galβ1-4GlcNAcβ1-2Manα1-3	5
P2	(NeuAcα2-3)$_1$ { Galβ1-4GlcNAcβ1-2Manα1-6 / Galβ1-4GlcNAcβ1-2Manα1-3 } / Fucα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc	29
P3	(NeuAcα2-3)$_1$ { Galβ1-4GlcNAcβ1-6 / Galβ1-4GlcNAcβ1-2Manα1-6 / Galβ1-4GlcNAcβ1-2Manα1-3 } / Fucα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc	3
P4	NeuAcα2-3Galβ1-4GlcNAcβ1-2 Manα1-6 / NeuAcα2-3Galβ1-4GlcNAcβ1-2 Manα1-3 / Fucα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc	36
P5	(NeuAcα2-3)$_2$ { Galβ1-4GlcNAcβ1-6 / Galβ1-4GlcNAcβ1-2Manα1-6 / Galβ1-4GlcNAcβ1-2Manα1-3 } / Fucα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc	11
P6	NeuAcα2-3Galβ1-4GlcNAcβ1-6 / NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6 / NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3 / Fucα1-6 / Manβ1-4GlcNAcβ1-4GlcNAc	14

Fractions P3, P5 and P6 contain approximately 20% of an isomeric triantennary form, the third branch being attached to position 4 of the 3-bound common core mannose.

oligosaccharide mixture. The ratios of the biantennary and the isomeric triantennary structures obtained by peak integration is in good agreement with the results described above. Peak 4 in Fig. 3 (3% of total oligosaccharide material) coelutes with a fucosylated tetraantennary reference sample.

4. DISCUSSION

We report the structures of the N-linked oligosaccharides from a variant of human t-PA expressed in CHO-cells, in which the first two carbohydrate attachment sites have been removed by exchange of Asn with Gln codon. The methodologies applied for structural characterization have made use of several complementary techniques, including compositional and methylation analysis, to obtain information concerning the substitution pattern, antennarity and degree of sialylation. FAB-MS provided a means of determining the size and sequence of the oligosaccharides. Additionally, analytical HPAE-PAD and comparison with reference oligosaccharides was used to confirm the results. These methods provide reliable structural data in a reasonable time for very small sample sizes (less than 5 μg of each oligosaccharide structure).

Seventy percent of the carbohydrate structures were of the biantennary complex type, fucosylated at the proximal GlcNAc, with zero, one or two NeuAc attached α2-3 to galactose (ratio 1:5:6). The remainder were preponderantly 2,6-branched triantennary structures with one, two or three α2-3 linked NeuAc. Tetraantennary structures were detected only in very small amounts. These results are in qualitative agreement with data reported by Spellman et al. [5], who find similar carbohydrate structures at glycosylation site III of wild type human t-PA produced in the same cell line. However, the amount of tri- and tetraantennary structures in their preparation were significantly higher. Interestingly the ratio of 2,6- and 2,4-branched triantennary structures in their t-PA sample was 1.3:1, while in the present work the ratio was 4.4:1. Such a result might be explained by the importance of even minor differences in the protein backbone caused by the different primary structure and the absence of two *N*-glycans for the activity of various glycosyltransferases involved.

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