Structural analysis of the carbohydrate chains of β -N-acetylhexosaminidases from bovine brain

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The oligosaccharide structures of bovine brain β -N-acetylhexosaminidases A and B (EC 3.2.1.30) were studied at the glycopeptide level by employing 500 MHz ^1H -n.m.r. spectroscopy and methylation analysis involving g.l.c.-m.s. More than 90% of the chains were found to be of the oligomannoside type, containing, on average, five to six mannose residues. Biantennary N-acetyl-lactosamine-type chains terminated in N-acetylneuraminic acid were found to comprise the remaining 5–10% of the total carbohydrate. The isoenzyme forms A and B do not differ from each other in the structure of their carbohydrate moiety, but do deviate in carbohydrate content and, in consequence, in the number of carbohydrate chains per molecule.

INTRODUCTION

Carbohydrate chains of glycoconjugates are known to be involved in recognition processes. This has been elegantly shown for lysosomal enzymes (for a review, see Sly & Fischer, 1982). In human fibroblasts, the newly synthesized lysosomal enzymes bear phosphomannose residues on oligomannoside-type chains. Via binding of these residues to specific membrane receptors the enzymes become incorporated into lysosomes (Sly *et al.*, 1981; Gabel *et al.*, 1982; Campbell & Rome, 1983). Because of the ubiquity of the Man6P receptor (Fischer *et al.*, 1980), it seemed reasonable to suppose that the proposed biosynthetic pathway for lysosomal hydrolases is the only, and universal, route.

The unexpected presence of normal activities of a number of lysosomal enzymes in tissues of I-cell patients, who lack the enzyme responsible for the synthesis of Man6P (Hasilik *et al.*, 1981; Owada & Neufeld, 1982; Waheed *et al.*, 1982a), suggests the existence of an alternative route for newly synthesized hydrolases. The latter is supported by studies of Kornfeld and co-workers (Gabel *et al.*, 1983; Kornfeld *et al.*, 1983) with murine cell lines lacking the Man6P receptor. They demonstrated in these cells the presence of high levels of intracellular acid hydrolase activities, which are contained in dense granules characteristic of lysosomes. These intriguing phenomena have raised renewed interest in the carbohydrate structures of lysosomal enzymes.

The present information on the structure of oligosaccharide chains of lysosomal enzymes indicates that a high proportion of the enzymes contain oligomannosidic chains (Alvares & Balasubramanian, 1982; Goldberg & Kornfeld, 1981; Von Figura & Klein, 1979; Varki & Kornfeld, 1980; Byrd & Touster, 1981; Hasilik & Von Figura, 1981; Mizuochi *et al.*, 1981; Howard *et al.*, 1982; Natowicz *et al.*, 1982; Waheed *et al.*, 1982b; Sweeley *et al.*, 1983). Only a small part of the oligosaccharide chains

was of the complex type. β -Glucocerebrosidase from human placenta was found to be an exception to this rule. For this enzyme, 80% of the N-glycosidic chains comprise complex-type chains (Takasaki *et al.*, 1983).

In a preliminary report we presented information, obtained by sugar analysis and 500 MHz ^1H -n.m.r. spectroscopy, on the tissue and species specificity of the oligosaccharide structure of β -N-acetylhexosaminidase (Overdijk *et al.*, 1983). This enzyme exists in two forms, A and B, differing in various properties. Structurally, the A enzyme consists of two different subunits, α and β , whereas in β -N-acetylhexosaminidase B, only β -subunits are present (Mahuran & Lowden, 1980; Overdijk *et al.*, 1982). In the present paper we substantiate our findings on bovine brain β -hexosaminidases; a detailed description of the structures is presented, as established by n.m.r. spectroscopy and methylation analysis with the aid of g.l.c.-m.s. For these analyses, glycopeptides obtained by digestion with Pronase were used.

MATERIALS AND METHODS

Preparation of glycopeptides

The β -N-acetylhexosaminidase isoenzymes, A and B, were isolated from bovine brain as described by Overdijk *et al.* (1982). In a typical experiment, 54 mg of hexosaminidase A and 23 mg of hexosaminidase B were obtained from 47 kg of bovine brain. The purified enzyme samples were dissolved in a solution of 0.01 M- CaCl_2 /0.02% (w/v) NaN_3 , adjusted to pH 8.0 with 1.0 M-NaOH. Pronase was added, dissolved in the same solution. Digestion was performed at 40 °C for 48 h with 5.2 mg and 3.5 mg of Pronase respectively. The digests were layered on a Bio-Gel P-6 column and eluted with 0.1 M-ammonium acetate, adjusted to pH 5.0 with 0.1 M-acetic acid. The void-volume fractions were freeze-dried and treated again with 3.0 mg of Pronase each for 48 h as described above. Glycopeptides were

Abbreviations used: Man6P, mannose 6-phosphate; g.l.c.-m.s., gas-liquid chromatography-mass spectrometry; example of the abbreviations for methylacetyl hexosides: 2,3,4-Gal, 2,3,4-tetramethyl-6-O-acetylmethyl galactoside, etc.

isolated by Bio-Gel P-6 and P-30 chromatography in the same ammonium acetate buffer, followed by desalting on a Bio-Gel P-2 column, run in water. The dimensions of all Bio-Gel columns were 29 cm long $\times$ 2.5 cm diameter.

Determination of the carbohydrate composition

The carbohydrate composition was determined by g.l.c. as described previously (Overdijk *et al.*, 1982).

Methylation analysis

The methylation of the glycopeptides was performed by the method of Hakomori (1964), as modified by Finne *et al.* (1980). The permethyl oligosaccharides

were methanolysed and acetylated (Fournet *et al.*, 1981). Analysis of the partially methylated, partially acetylated methyl glycosides was done by g.l.c.-m.s. with a Hewlett-Packard 5993 apparatus, equipped with a cross-linked methyl-silicone capillary column (15 m $\times$ 0.2 mm int. diam.). Pressure was 50 kPa. Peak identification was performed by means of computerized comparison with reference substances, based on retention times and mass spectra.

500 MHz ^1H -n.m.r. spectroscopy

The 500 MHz ^1H -n.m.r. analyses were performed as described by Vliegthart *et al.* (1983). The spectra were

Fig. 1. 500 MHz ^1H -n.m.r. spectrum of the glycopeptide mixture obtained from bovine hexosaminidase A, recorded in $^2\text{H}_2\text{O}$ at 27 $^\circ\text{C}$

(a) Overall spectrum; (b) expanded structural-reporter-group regions.

Table 1. Carbohydrate composition of β -*N*-acetylhexosaminidase (Hexase) A and B from bovine brain

The carbohydrate analysis was performed as described by Overdijk *et al.* (1982). The values represent mol of carbohydrate residues/mol of enzyme and are calculated on the basis of an M_r value of 150000 for both isoenzymes

Monosaccharide	Composition (mol/mol)	
	Hexase A	Hexase B
Man	18.9	6.6
Gal	2.0	1.1
GlcNAc	6.8	2.7
NeuAc	1.0	0.4
Fuc	<0.1	<0.1
Total carbohydrate (%, w/w)...	3.7	1.4

recorded on a Bruker WM-500 spectrometer (SON NMR-facility, Nijmegen, The Netherlands) operating in the Fourier-transform mode at a probe temperature of 27 °C. Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulphonate (indirectly to acetone in $^2\text{H}_2\text{O}$: $\delta = 2.225$ p.p.m.). Resolution enhancement of the 500 MHz spectra was achieved by Lorentzian-to-Gaussian transformation.

RESULTS AND DISCUSSION

The carbohydrate compositions of β -*N*-acetylhexosaminidase A and B are shown in Table 1. The results suggest the occurrence of both oligomannoside- and *N*-acetyl-lactosamine-type *N*-glycosidic carbohydrate chains with a predominance of the former.

^1H -n.m.r. spectra (500 MHz) of the glycopeptides of both β -*N*-acetylhexosaminidase A (Fig. 1) and B were recorded. No significant differences were observed

between the preparations of the two isoenzymes. From the spectra it could be inferred that mainly (more than 90%) oligomannoside-type chains are present. In Fig. 2 their comprehensive structure is shown. The chains contain at least four, at most nine, mannose residues, with five or six residues on average. The mannose residues 3, 4, 4' and A that constitute the two branching points are present in each component of the mixture. Heterogeneity occurs mainly as to the degree of termination of the branches by $\alpha(1\rightarrow2)$ -linked mannose residues, denoted C, D₁, D₂ and D₃.

The chemical shifts of the anomeric protons of the mannose residues in the various components of the glycopeptide mixture are given in Table 2. The presence of mannose C and D influence some of the δ -values of the protons belonging to residues in the same branch (Vliegthart *et al.*, 1983; Marti *et al.*, 1984). In those cases, two or three values for the δ -value of that particular proton are listed. The signals in the spectra were assigned, as described for procollagen glycopeptides (Marti *et al.*, 1984).

In addition the spectra contained weak indications for the presence of *N*-acetyl-lactosamine-type chains in minor quantities (less than 10%). The branching pattern may be biantennary. In the complete branches, the terminal sialic acid ($\delta \text{H-3}_{\text{ax}} = 1.718$ p.p.m.) is bound to galactose in an $\alpha(2\rightarrow6)$ -linkage. Intersecting *N*-acetylglucosamine bound to the branching Man-3 residue could not be detected as is true for fucose attached to the chitobiose unit.

In recent studies (Takahashi *et al.*, 1983; Nakao *et al.*, 1984) ^1H -n.m.r. spectroscopy was used for the determination of the oligosaccharide structure of a lysosomal proteinase, namely, cathepsin D from porcine spleen. Several oligomannoside-type structures could be characterized. A few *N*-acetyl-lactosamine-type glycopeptides were also identified. One of those contained a fucose residue bound to the *N*-acetylglucosamine of the chitobiose part-linked to asparagine, whereas a very minor subspecies contained galactose (Takahashi *et al.*, 1983). Small quantities of phosphate were found by both groups (0.02 and 0.16 mol/mol respectively), held to be

Fig. 2. Comprehensive structure of the oligomannoside-type chains of β -*N*-acetylhexosaminidases A and B from bovine brain

Indices at square brackets indicate the relative abundance (± 0.03) of the mannose residue included by the brackets. These were deduced from the relative intensities of the corresponding signals in the n.m.r. spectrum (cf. Marti *et al.*, 1984).

Table 2. ^1H chemical shifts of structural reporter groups of the monosaccharides present in the glycopeptides from β -*N*-acetylhexosaminidases from bovine brain

Reporter group	Residue*	Chemical shift†	
		Terminal‡	Substituted
H-1	GlcNAc-1		$\approx 5.08\text{\S}$
	GlcNAc-2	4.61	4.595
	Man-3		≈ 4.78
	Man-4'	4.912¶	4.869
	Man-4	5.090	5.345(5.335)**
	Man-C	5.052	5.305
	Man-D	5.038	—
	Man-A	5.094	5.401
	Man-D ₂	5.052	—
	Man-B	4.908	5.142
	Man-D ₃	5.038	—
H-2	Man-3	4.251††	4.232
	Man-4'		4.145
	Man-4	4.065	4.112
	Man-C	4.065	4.112
	Man-D ₁	4.065	—
	Man-A	4.065	4.112
	Man-D ₂	4.065	—
	Man-B	3.99	4.02
NAC	GlcNAc-1		2.006§
	GlcNAc-2	2.073	2.059
H- α	Asn		4.44
H- β'' , β'	Asn		2.74; 2.84

* For complete structure and coding of monosaccharide residues, see Fig. 2.

† Chemical shifts were acquired at 500 MHz for neutral solutions in $^2\text{H}_2\text{O}$ at 27 °C.

‡ Centering δ -values between the two columns indicates that these chemical shifts are not sensitive to heterogeneity with regard to the Man-D residues.

§ Multiplicity of signal is due to heterogeneity of the peptide portion (cf. Vliegthart *et al.*, 1983).

|| The occurrence of the lower-intensity signals at δ 4.61 and 2.073 may either reflect some sort of heterogeneity in the oligomannoside-type glycopeptides or they point to *N*-acetyl-lactosamine-type glycans in the mixture (see the text).

¶ 'Terminal' in this case means 'in structures without Man-B-linked to Man-4'; 'Substituted' means here 'when Man-4' bears Man-B (in addition to Man-A)'.

** The chemical shift of H-1 of Man-4 is slightly influenced by substitution of Man-C by Man-D₁ (δ 5.345 $\rightarrow$ δ 5.335) (cf. Marti *et al.*, 1984).

†† 'Terminal' in this case means 'in structures with Man-4 in terminal position'; 'Substituted' means here 'when Man-4 bears Man-C.'

indicative for the presence of Man6P. Our glycopeptide preparations from purified β -*N*-acetylhexosaminidases A and B showed no signal with ^{31}P -n.m.r. spectroscopy. However, because of the limited sensitivity of this method, it is not possible to exclude definitely the presence of phosphate.

The carbohydrate moieties of the glycopeptides of β -*N*-acetylhexosaminidases A and B were further analysed by g.l.c.-m.s. after permethylation, methanolysis and acetylation. The oligomannoside-type structures proposed on the basis of the n.m.r. analyses could

Table 3. Relative amounts of methylated acetylated glycosides present in β -*N*-acetylhexosaminidases from bovine brain

Glycoside	Relative amount (%)
2,3,4,6-Man	18.1
3,4,6-Man	14.2
2,4,6-Man	6.5
2,3,4-Man	4.5
2,4-Man	27.7
3,6-GlcNAc	19.4
Others*	9.6

* The other derivatives that were detected in low amounts were 2,3,4-Gal; 2,4,6-Gal; 2,6-Gal; 4,6-Man; 2,3-Man; 4,7,8,9-NeuAc.

be confirmed by the results of the methylation analyses (Table 3).

As to the *N*-acetyl-lactosamine-type components, the presence of very small quantities of both 2,3,4-Gal and 2,4,6-Gal would indicate that, in the case of complete chains, sialic acid was bound in $\alpha(2-6)$ - or in $\alpha(2-3)$ -linkage to galactose respectively. The presence of the $\alpha(2-3)$ -linkage, however, could not be confirmed by ^1H -n.m.r. spectroscopy. The above can be ascribed by partial undermethylation, since there were also some lower peaks in the chromatograms of the methylated acetylated glycosides belonging to dimethylated mannosides that cannot be explained in terms of *N*-glycosidic glycan structures (e.g. 4,6Man).

Taking into account the M_r values for the native isoenzymes, their carbohydrate content (Table 1) and the mean M_r of a glycan chain (1250–1400), it can be concluded that, on average, each enzyme molecule of the A-form contains four oligosaccharide chains, whereas the B-form molecules bear one or two chains only. Since the β -*N*-acetylhexosaminidases are composed of subunits (hexosaminidase A of three: $\alpha\beta_2$; hexosaminidase B of four: $\beta_2\beta_2$), it is unlikely that all polypeptides of the B-isoenzyme bear a glycan chain. The distribution of the chains over the respective subunits and polypeptides can only be studied after isolation of the polypeptides concerned.

The glycan structures described here are in agreement with the generally proposed structures for lysosomal enzymes studied so far. Further studies are needed in which the detailed carbohydrate structures of β -*N*-acetylhexosaminidases from human tissues are compared with those from bovine brain and with those of some other lysosomal enzymes from human tissues.

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