THE STRUCTURE OF THE ASIALO-CARBOHYDRATE UNITS OF HUMAN SEROTRANSFERRIN AS PROVEN BY 360 MHz PROTON MAGNETIC RESONANCE SPECTROSCOPY

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1. Introduction

Human serotransferrin contains two identical carbohydrate chains [1–3] about the primary structure of which controversy exsists. Jamieson et al. [3] suppose a branched structure, wherein the branching mannose, glycosidically linked to *N*-acetylglucosamine β1→Asn, bears two chains: one consisting of a mannotriose, the other of an *N*-acetylglucosamine residue and both terminated by an *N*-acetylneuraminyl-*N*-acetyllactosamine unit. Spik et al. [1,2] propose a more symmetrical structure, built up from a mannotriosido-di-*N*-acetylchitobiose core substituted by two *N*-acetylneuraminyl-*N*-acetyllactosamine units. This structure, presented in fig.1 differs essentially from the foregoing structure since it has only 3 mannose residues instead of 4.

In this paper the investigation by high-resolution ^{1}H nuclear magnetic resonance spectroscopy of the structure of the asialoglycopeptide (asialo-glycan–Asn) isolated from serotransferrin is described. In particular the signals of the anomeric protons, the mannose-H-2 protons and the *N*-acetyl methyl groups were analysed. For the assignment of these signals, spectra of the corresponding asialo-agalacto-glycan–Asn–Lys, the glyco-amino acids GlcNAcβ1→Asn [4] and Manα(1→6)Manβ(1→4)GlcNAcβ(1→4) [Fucα(1→6)] GlcNAcβ1→Asn [5] and the trisaccharide Manα(1→3) Manβ(1→4) GlcNAc [6], representing partial structures of the asialo-glycan–Asn have been used as reference compounds. The monosaccharide units in these substances are numbered in correspondence to the numbering in the serotransferrin glycopeptides (see fig.1 and table 1).

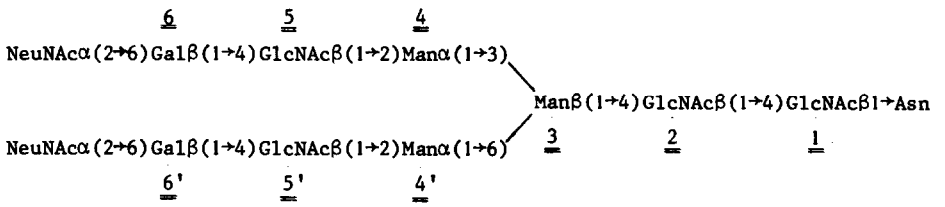

Fig.1. Structure of the carbohydrate units of human serotransferrin [1,2].

Table 1

^{1}H NMR data[a] of anomeric protons, mannose-H-2 protons and *N*-acetyl methyl protons[b] for glycopeptides isolated from human serotransferrin and reference compounds

Compound	H-1 of residue									H-2 of residue			NAc of residue			
	1	2	3	4	4′	5	5′	6	6′	3	4	4′	1	2	5	5′
Asialo-glycan–Asn(–Lys)	5.07 (9.6)	4.61 (7.6)	4.78 (0.9)[c]	5.12 (1.3)[c]	4.93 (1.7)[c]	4.58 (8.0)	4.58 (8.0)	4.47 (8.0)	4.47 (8.0)	4.24	4.18	4.11	2.01	2.08	2.05	2.05
Asialo-agalacto-glycan–Asn–Lys	5.07 (9.2)	4.63 (7.6)	4.77 (<1)	5.12 (∿1.5)	4.92 (∿1.5)	4.59 (7.8)	4.59 (7.8)			4.24	4.19	4.10	2.01	2.08	2.05	2.05
Manα(1→6)Manβ(1→4)GlcNAcβ(1→4) [Fucα(1→6)]GlcNAcβ1→Asn	5.07 (9.5)	4.69 (6.8)	4.77 (<1)		4.92 (1.2)					4.08		3.96	2.02	2.09		
GlcNAcβ1→Asn [4]	5.09 (9.8)												2.02			
Manα(1→3)Manβ(1→4)GlcNAc$^{\beta}_{\alpha}$		4.73 (7.8) 5.21 (2.6)	4.79 (<1)	5.12 (1.7)						4.25	4.09			2.04		

[a] Chemical-shifts δ are given in ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulphonate; the values in brackets are coupling constants $J_{1,2}$ in Hz

[b] The numbering of the monosaccharide units in the reference compounds corresponds to that in the asialo-glycan–Asn (fig.1)

[c] Values from a convolution difference spectrum [18]

2. Materials and methods

Human serotransferrin prepared according to Roop and Putnam [7] was submitted to pronase digestion and the two glycopeptides (glycan–Asn and glycan–Asn–Lys) were isolated applying previously described procedures [1,2,8,9]. Asialo-glycopeptides were prepared by digestion with neuraminidase (EC 3.2.1.18) from *Clostridium perfringens* and asialo-agalacto-glycopeptide by sequentional digestion with neuraminidase and β-galactosidase (EC 3.2.1.23) from jack-bean meal [1]. Glycopeptide Manα(1→6)Manβ(1→4) GlcNAcβ(1→4) [Fucα(1→6)] GlcNAcβ1→Asn and trisaccharide Manα(1→3)Manβ(1→4)GlcNAc were isolated from urine of fucosidosis [5] and mannosidosis [6] patients respectively.

The oligosaccharide and glycopeptides were repeatedly exchanged in D_2O [4]; 0.02–0.05 M solutions of the compounds in D_2O were used for spectral analysis. The 360 MHz ^1H NMR spectra were recorded on a Brucker HX-360 spectrometer, operating in the Fourier Transform mode at probe temperatures of 25°C or 60°C. Chemical shifts are given relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate (indirectly to acetone in D_2O: δ=2.225 ppm).

3. Results and discussion

The 360 MHz ^1H NMR spectrum of the asialo-glycan–Asn isolated from human serotransferrin is given in fig.2a. For the carbohydrate part of the asialo-glycan–Asn–Lys an identical spectrum was obtained.

The resonances in the anomeric region of the spectrum (4.4–5.2 ppm; fig.2b) are well resolved. At 25°C the signal of one anomeric proton is hidden under the HOD resonance (see dashed insert in fig.2), but it could be detected at 60°C. The increase in temperature affects only slightly the resonance positions of the anomeric protons ($\Delta\delta \leqslant 0.02$ ppm), whereas the HOD resonance is shifted from 4.8–4.3 ppm. The values of coupling constants $J_{1,2}$, specific for vicinal di-axial protons (6–8 Hz; β-D-Galp, β-D-GlcNAcp) or for vicinal di-equatorial and axial–equatorial protons (0–4 Hz; α- and β-D-Manp) have been used for the assignment of the signals of the anomeric protons [10].

The resonances of the mannose-H-2 protons, laying apart from the other non-anomeric carbohydrate protons (4.0–4.3 ppm, fig.2), could be interpreted using the spectra of the afore-mentioned reference compounds, together with selective irradiation of the mannose-H-1 resonance frequencies. The signals due to the *N*-acetyl methyl groups (2.0–2.1 ppm, fig.2) could also be identified by comparison with the data of the reference compounds. The spectral data of anomeric protons, mannose-H-2 protons and *N*-acetyl methyl protons for the glycopeptides and the reference compounds are compiled in table 1.

In the spectrum of the pentasaccharide-Asn the doublets at 5.07 ppm ($J_{1,2}$ = 9.5 Hz) and 4.69 ppm ($J_{1,2}$ = 6.8 Hz) represent the anomeric protons of GlcNAc $\underline{\underline{1}}$ and GlcNAc $\underline{\underline{2}}$ respectively; the coupling constant and the chemical shift of H-1 of GlcNac $\underline{\underline{1}}$ being in accordance with the data observed for GlcNAcβ1→Asn [4]. The anomeric protons of the mannose residues at 4.77 ppm at 4.92 ppm are coupled with the H-2 protons at 4.08 ppm and 3.96 ppm respectively, as could be confirmed by selective irradiation.

In the spectrum of the trisaccharide, H-1β of the reducing GlcNAc-residue resonates at 4.73 ppm ($J_{1,2}$ = 7.8 Hz, 0.35 proton) and the corresponding H-1α at 5.21 ppm ($J_{1,2}$ = 2.6 Hz, 0.65 proton). Selective irradiation experiments showed that the mannose-H-1 protons at 5.12 ppm at 4.79 ppm are coupled with the mannose-H-2 protons at 4.09 ppm and 4.25 ppm respectively. The chemical-shift of the *N*-acetyl methyl group (2.04 ppm) differs from that of GlcNAc $\underline{\underline{2}}$ in the pentasaccharide-Asn because the GlcNAc is in reducing position.

The anomeric region of the spectrum of the asialo-agalacto-glycan–Asn–Lys was essentially the same as observed for the asialo-glycan–Asn(–Lys); only the two doublets of Gal $\underline{\underline{6}}$ and Gal $\underline{\underline{6}}'$ (4.47 ppm, $J_{1,2}$ = 8.0 Hz) were absent.

On the basis of the data given in table 1 the signals of the anomeric protons, the mannose-H-2 protons and the *N*-acetyl methyl protons in the spectrum of the asialo-glycan–Asn have been assigned (see also fig.2b). The slightly different resonance position of the anomeric proton of GlcNAc $\underline{\underline{2}}$ in the penta-saccharide-Asn with respect to that in the asialo-glycan–Asn can be ascribed to the influence of the fucose residue attached to C-6 of GlcNAc $\underline{\underline{1}}$ in the

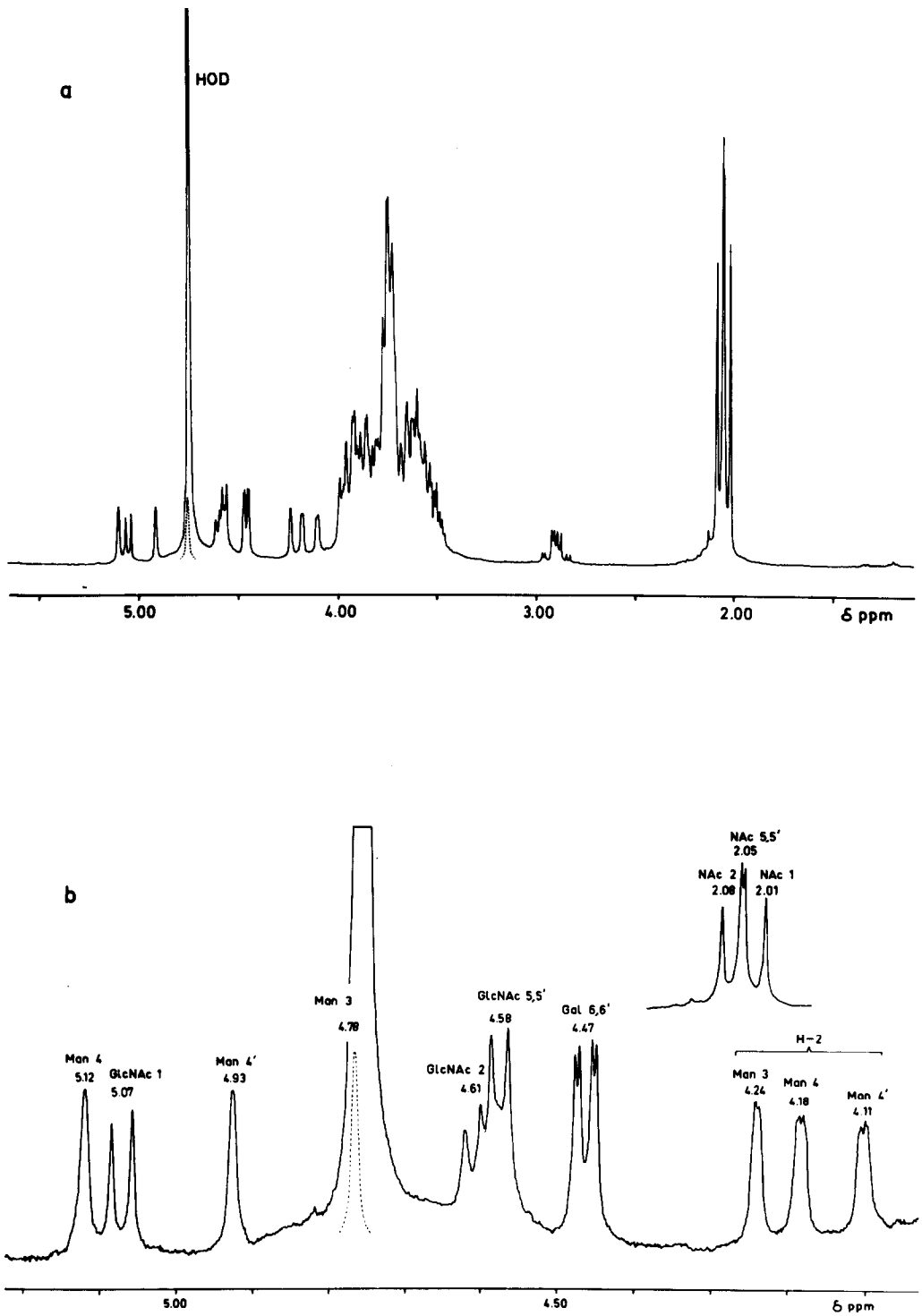

Fig.2. 360 MHz ^{1}H NMR spectrum of the asialo-glycan–Asn isolated from human serotransferrin.

pentasaccharide-Asn whereas a similar deviation in the trisaccharide stems from the reducing position of GlcNAc 2. The assignment of the signals for the H-1 and H-2 protons of the mannose residues 3, 4 and 4′, based on the spectrum correlations and the specific irradiation experiments is in agreement with the data observed for corresponding protons in the not interpreted spectra of two structurally related oligosaccharides published by Wolfe et al. [11]. (For comparison of the data use was made of the relation $\delta_{TMS\ external} - \delta_{DSS} = 0.46$ ppm.) It turns out that removal of the two *N*-acetyllactosamine units, linked to C-2 of the mannose residues 4 and 4′ introduces upfield shifts of 0.09 ppm and 0.15 ppm, respectively in the resonance positions of the H-2 protons of these mannoses but that the chemical shifts of the anomeric protons of 4 and 4′ are not significantly influenced. Removal of the mannose residue 4 (linked to C-3 of Man 3) from the mannotriosido-GlcNAc core results in an upfield shift of 0.17 ppm for H-2 of Man 3 whereas the presence of Man 4′ (linked to C-6 of Man 3) has no influence on the chemical shift of this H-2 proton. The small differences in chemical shift between the anomeric protons of Gal 6 and Gal 6′ and between the *N*-acetyl methyl groups of GlcNAc 5 and GlcNAc 5′ indicate a small but significant influence of the position of attachment of the *N*-acetyllactosamine–mannose chains either to C-3 or C-6 of Man 3.

The presence of an additional lysine residue in the glycopeptides (e.g., the asialo-glycan–Asn–Lys and the asialo-agalacto-glycan–Asn–Lys) does not affect the spectrum of the carbohydrate part of the molecules, but influences the chemical shifts of the β-CH_2 protons of asparagine (β-CH_2 Asn: 2.95 ppm and 2.86 ppm in glycan–Asn, see fig.2a; 2.70 ppm and 2.53 ppm in glycan–Asn–Lys [4,12]). Signals for the lysine residue are found at 1.41 ppm (γ-CH_2), 1.70 ppm (β-CH_2, δ-CH_2) and 2.99 ppm (ϵ-CH_2) [12].

4. Conclusions

From the presented NMR data for the asialo-glycan–Asn and the reference substances it can be concluded that the chemical shifts and the coupling constants of the anomeric protons in this type of compounds are characteristic for the type of monomer, i.e. the nature of the monomer, the type and configuration of the glycosidic bond and the position in the carbohydrate chain. The general rule that anomeric protons of pyranose residues in *O*-glycosidic α-linkages resonate at lower field than those in β-linkages [13] holds also for these complex structures. Obviously, the anomeric proton of β-GlcNAc 1, which is involved in an N-glycosidic bond resonates at relatively low field [4]. These observations make it possible to use high-resolution ^{1}H NMR spectroscopy as a fingerprint method, enabling a rapid characterization of common structural elements in various glycoconjugates. For this purpose the chemical shifts of the mannose-H-2 protons and the *N*-acetyl methyl protons have also to be taken into account. The three mannose-H-2 signals at the characteristic chemical shifts as indicated in fig.2 give a direct indication for the mannotriosido-branching region in the molecule, to which at least the GlcNAc units 2, 5 and 5′ are attached as mentioned in fig.1.

This NMR investigation of the asialo-glycan–Asn confirms the structure of the glycan chains of human serotransferrin as proposed by Spik et al. [1,2]. The presence of only 3 signals for mannose-anomeric protons clearly rules out the structure suggested by Jamieson et al. [3]. The 'bi-antennary' structure [14], composed of a mannotriosido-di-*N*-acetyl-chitobiose core, bearing two *N*-acetylneuraminyl–*N*-acetyl lactosamine units is a frequently occurring structural element of glycan chains in glycoproteins and is also found in glycans isolated from human lactotransferrin [1,15], human and bovine immunoglobulins [16,17].

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