STRUCTURE DETERMINATION OF THE SINGLE GLYCAN OF RABBIT SEROTRANSFERRIN BY METHYLATION ANALYSIS AND 360 MHz ^{1}H NMR SPECTROSCOPY

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1. Introduction

Transferrins are glycoproteins capable of reversibly binding 2 iron atoms/molecule. They are responsible for iron transfer to reticulocytes for the synthesis of hemoglobin [1,2]. The interaction between serotransferrin (STF) and reticulocytes has been most studied in the heterologous system human STF–rabbit reticulocytes [3–5]. However, the affinity of human STF for rabbit reticulocytes is 2-times lower than that of rabbit STF [1,6]. This stronger binding in the homologous system might be due to differences in the structure of the carbohydrate moiety or in the position of its attachment to the polypeptide backbone.

To make a comparison with the glycan units of human STF [7–9] we investigated the glycopeptide fraction of rabbit STF applying an original method for the determination of glycan primary structure combining monosaccharide determination, permethylation and 360 MHz ^{1}H NMR [9–12].

From the results obtained it has been concluded:

(i) That the highly purified rabbit transferrin contains only 1 glycan chain/molecule.

(ii) That a heterogeneity of the glycan moiety in the sialic acid residues was observed on isolation by paper electrophoresis of a disialylglycopeptide G-1 and a monosialylglycopeptide G-2.

(iii) That the primary structure of glycopeptide G-1 deduced on the basis of the data of carbohydrate composition, permethylation analysis and 360 MHz ^{1}H NMR spectroscopy is identical to the primary structure of human serotransferrin glycan and that the glycopeptide G-2 was shown, by ^{1}H NMR spectroscopy, to be mixture of two isomeric monosialylglycopeptides.

2. Materials and methods

Rabbit serum was collected in a local slaughter house and STF was isolated from the heparinized plasma by rivanol and ammonium sulphate precipitations as in [13]. Further purification was performed by chromatography on a DEAE Sephadex A-50 column under the conditions described in fig.1 legend. Peak B of this fractionation was submitted to a chromatography on a SP-Sephadex C-50 column using a slight modification of the procedure in [14] (see fig.2 legend). Peak II of fig.2 was finally rechromatographied on SP-Sephadex C-50 column under the same conditions. The homogeneity of this preparation was checked by polyacrylamide gel electrophoresis [15] and by immunoelectrophoresis with a goat anti-

rabbit serum (Miles Labs). Contamination by hemopexin was detected by specific staining with *O*-dianisidine. The molecular weight of the STF was determined by sedimentation equilibrium analysis using the high-speed method in [16] as modified [17].

STF was digested with pronase [18] and the glycopeptides were obtained by preparative paper electrophoresis in 1 M acetic acid, pH 2.4 at 10 V/cm for 18 h followed by high-voltage electrophoresis in pyridine/acetic acid/water (25 : 1 : 224), pH 6.5 at 70 V/cm for 1.5 h, using a Technik apparatus. Total removal of sialic acid from the glycopeptide fraction was carried out by digestion with neuraminidase (EC 3.2.1.18) from *Clostridium perfringens* (0.25 U/15 mg glycopeptide in sodium acetate 0.1 M buffer, pH 5.9 at 37°C during 4 h) [19].

Qualitative and quantitative carbohydrate analysis of the Apo-STF and of the glycopeptides was carried out by colorimetry [20] and gas–liquid chromatography as in [21].

Permethylation of glycopeptides was performed as in [22]. Partially methylated monosaccharides were identified as in [23].

The amino acid composition of hydrolysed glycopeptides (5.6 M HCl, 24 h, 105°C under vacuum) was determined with a Beckman Multichrom analyser. Glycopeptides were dansylated as in [24].

For NMR analysis, glycopeptides were repeatedly exchanged in D_2O. The 360 MHz ^1H NMR spectra of 5–10 mM solutions of the compounds in D_2O were recorded on a Brucker HX-360 spectrometer, operating in the Fourier Transform mode at probe temperatures of 25°C and 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

3.1. *Preparation of rabbit STF*

The fraction obtained after rivanol and ammonium sulphate precipitations showed upon polyacrylamide gel electrophoresis and immunoelectrophoresis the presence of transferrin, hemoglobin, hemopexin and immunoglobulin IgG. By chromatography on DEAE–Sephadex A-50 (fig.1) 4 fractions (A–D) were isolated, each containing STF but contaminated by different

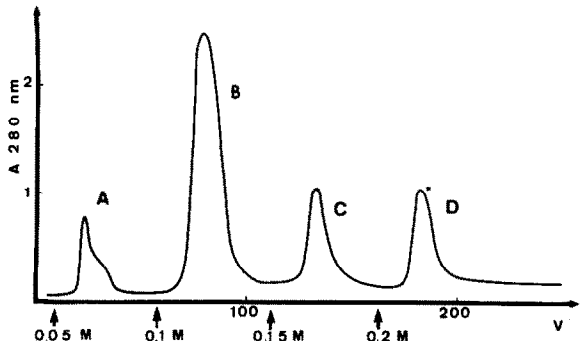


Fig.1. Fractionation of rabbit serotransferrin on DEAE Sephadex A-50 column (7.5 × 45 cm) eluted with a discontinuous gradient of 0–0.2 M NaCl in 0.05 M Tris–HCl buffer, pH 8.5, at 25 ml/h flow rate. The effluent was monitored at 280 nm and 8 ml fractions were collected.

amounts of the above-mentioned materials. The major peak B, showed upon polyacrylamide gel electrophoresis only STF and hemopexin in a ratio of about 9:1. The complete elimination of hemopexin was obtained by two chromatography runs on SP–Sephadex columns. From 500 g lyophilized rabbit plasma, the final recovery of pure STF was 5.8 g. STF was estimated to be mol. wt 75 500 as determined by ultracentrifugation. The carbohydrate composition of the rabbit STF is given in table 1. The centesimal carbohydrate composition (table 1) corresponds to 50% of the value determined for human STF [25,26]. These results indicate that rabbit STF may contain only one glycan moiety.

Table 1
Centesimal and molar carbohydrate compositions of rabbit STF and the rabbit STF glycopeptides

Centesimal composition	Rabbit STF	Glycopeptides	
		G1	G2
Hexoses	1.2	32.2	38.4
N-Acetylglucosamine	1.2	35.7	40.2
N-Acetylneuraminic acid	0.8	26.5	15.9
Molar composition[a]			
Galactose	2.2	1.8	2.0
Mannose	3	3	3
N-Acetylglucosamine	4.2	4.2	4.2
N-Acetylneuraminic acid	2.0	2.2	1.1

[a] On the basis of mol. wt 75 500 of rabbit STF

Table 2
Identification and determination of methylated monosaccharides obtained from permethylated glycopeptides G-1 and G-2

Glycopeptides	Methylated monosaccharides[a]				
	2,3,4,6-Gal	2,3,4-Gal	3,4,6-Man	2,4-Man	3,6-GlcNAc
G-1	0.12 (0)	1.76 (2)	1.85 (2)	1	3.72 (4)
G-2	1 (1)	1.13 (1)	1.70 (2)	1	3.69 (4)

[a] On the basis of one 2,4-di-*O*-methylmannose residue
The nearest integral numbers are given in brackets

Table 3
^{1}H NMR chemical shifts of anomeric protons, mannose H-2 protons, sialic acid H-3 protons and *N*-acetyl protons for the sialo and asialo-glycopeptides G-1 and for the mixture of monosialoglycopeptides G-2a and G-2b isolated from rabbit STF

	G-1		G-2	
H-1 of	Bisialoglycan	Asialoglycan	G-2a[a]	G-2b[a]
GlcNAc 1	5.072	5.070	5.073	5.073
GlcNAc 2	4.622	4.617	4.620	4.620
Man 3	4.775	4.765	4.769	4.769
Man 4	5.134	5.121	5.138	5.120
Man 4'	4.946	4.929	4.928	4.944
GlcNAc 5	4.600	4.581	4.599	4.577
GlcNAc 5'	4.600	4.581	4.577	4.599
Gal 6	4.445	4.471	4.445	4.471
Gal 6'	4.445	4.471	4.471	4.445
H-2 of				
Man 3	4.254	4.248	4.251	4.251
Man 4	4.195	4.191	4.192	4.192
Man 4'	4.114	4.110	4.112	4.112
H-3 of				
NeuNAc (H_3 eq)	1.715	–	1.716	1.716
NeuNAc (H_3 ax)	2.671	–	2.670	2.670
N-acetyl protons of				
GlcNAc 1	2.007	2.009	2.005	2.005
GlcNAc 2	2.080	2.078	2.078	2.078
GlcNAc 5	2.030	2.050	2.030	2.048
GlcNAc 5'	2.030	2.047	2.048	2.030
NeuNAc	2.067	–	2.068	2.068

[a] G-2a, sialic acid located in the upper branch; G-2b, sialic acid located in the lower branch

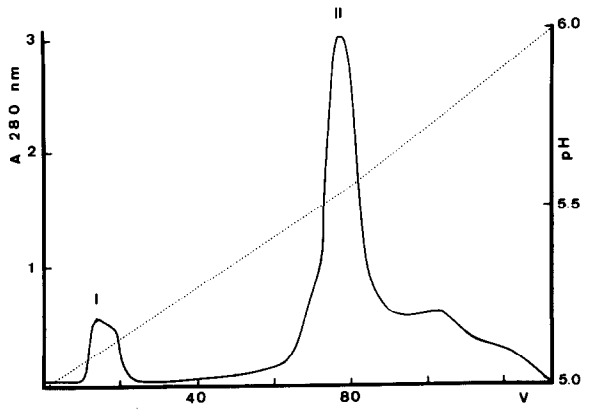


Fig.2. Fractionation of peak B (fig.1) on SP–Sephadex C-50 column (2.5 × 35 cm) equilibrated with 0.02 M sodium citrate buffer, pH 5.1, and eluted with a linear gradient of 0.02 M sodium citrate buffer pH 5.1–6.0 at 30 ml/h flow rate. The effluent was monitored at 280 nm and 15 ml fractions were collected.

3.2. *Preparation of the glycopeptides and structure determination*

After pronase digestion of 3 g rabbit STF followed by electrophoretic purification, 40 mg glycopeptide G-1 was obtained besides 10 mg glycopeptide G-2. Hydrolysates of both glycopeptides contained aspartic acid and serine in a molar ratio of 1 : 1. After dansylation, dansyl-Asp has been characterized. The carbohydrate compositions of G-1 and G-2 are summarized in table 1. The two glycopeptides differ only in the sialic acid content.

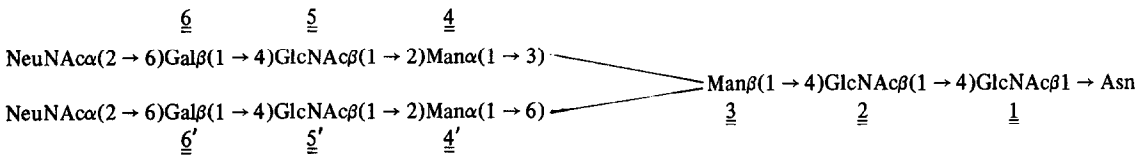
The results of methylation analysis of G-1 and G-2 are presented in table 2. They show that the sialic acid residues in both compounds are linked to position 6 of galactose, and are in good agreement with the existence of the classical mannotriosido branching trisaccharide in the molecule.

The disialylglycopeptide G-1, the monosialylglycopeptide G-2 and the asialoglycopeptide G-1 obtained after removal of the sialic acid residues by neuraminidase treatment were analyzed by 360 MHz ^{1}H NMR spectroscopy. Relevant NMR data are summarized in table 3.

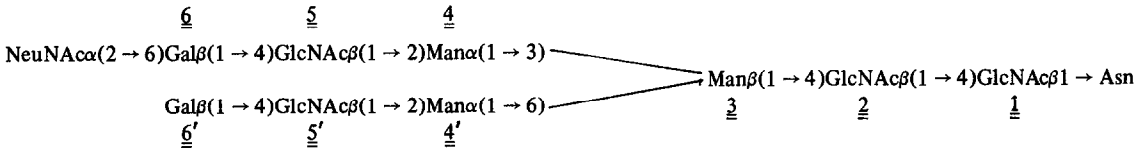
The primary structures of glycopeptides G-1 and G-2 are given in table 4. They were deduced on the basis of the data of carbohydrate composition (table 1), permethylation analysis (table 2) and

Table 4
Primary structure of the glycopeptides isolated from rabbit serum transferrin

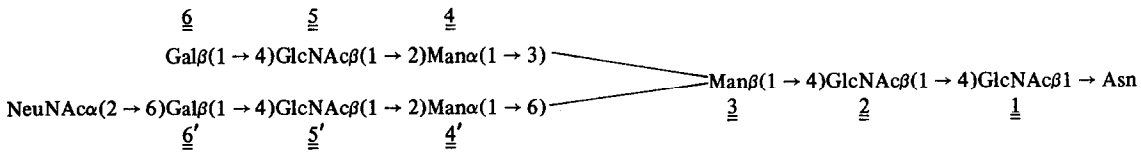
Disialylglycopeptide G-1



Monosialylglycopeptide G-2a



Monosialylglycopeptide G-2b



360 MHz ^{1}H NMR spectroscopy (table 3). The ^{1}H NMR spectrum of G-1 is in agreement with that of the sialo glycan structure of human STF [11].

4. Discussion

The present data show that the glycan moiety of rabbit STF as determined for the isolated glycopeptide G-1 has the complex biantennary type of structure, built up from a mannotriosido-di-*N*-acetylchitobiose core substituted by two *N*-acetylneuraminyl-α-(2 → 6)-*N*-acetyllactosamine moieties (table 4). This structure is identical to that of glycan chains of human STF [8,9,11]. On the basis of the molar carbohydrate composition (table 1) it has to be concluded that the glycoprotein contains only 1 glycan chain/molecule, which is in contrast to human STF containing 2 glycan chains/molecule of glycoprotein. This result disagrees with [27], where two heteropolysaccharide units in rabbit transferrin were claimed. This discrepancy could be explained by the presence in the preparations [27] of some contaminating hemopexin which contains 20% carbohydrate [28].

The glycan chains of both rabbit and human STF, [25,26] are linked by asparaginyl-*N*-acetylglucosamine bonds to the protein. However the sequence around the carbohydrate bearing Asn residues is different for both proteins: Asn–Ser in the case of rabbit STF and Ser–Asn for human STF [25,26]. The presence of two sialic acid residues in the rabbit transferrin is in accordance with [29,30]. It is significant to note that one of the glycopeptides (G-2) contains only one sialic acid residue, and that a lack of one sialic acid residue was also noticed [30] on the whole serum transferrin molecule.

From the high resolution ^{1}H NMR data of G-2 (table 3) it can be concluded that G-2 is a mixture of two isomeric monosialoglycopeptides (G-2a and G-2b), one having the sialic acid residue in the upper branch, the other having it in the lower branch (table 4). The presence of these two isomers could not be established by the conventional analysis techniques (chromatography, methylation analysis, Smith degradation). It has to be noted that the attachment to both branches of the biantennary structure of sialic acid in a (2 → 6) linkage to galactose gives rise to small but significant shift increments for the anomeric protons of mannose 4 and 4′, *N*-acetylglucosamine 5 and 5′ and galactose 6 and 6′ as is evident from table 3. The monosialoglycan structure can stem from a native microheterogeneity or from loss of a sialic acid residue during the isolation procedure by a partial neuraminidase hydrolysis. This question has to be resolved because the monosialoglycan chains could be of importance for the molecular interaction of transferrin with reticulocytes.

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