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Investigation by 360-MHz ^{1}H-Nuclear-Magnetic-Resonance Spectroscopy and Methylation Analysis of the Single Glycan Chain of Chicken Ovotransferrin

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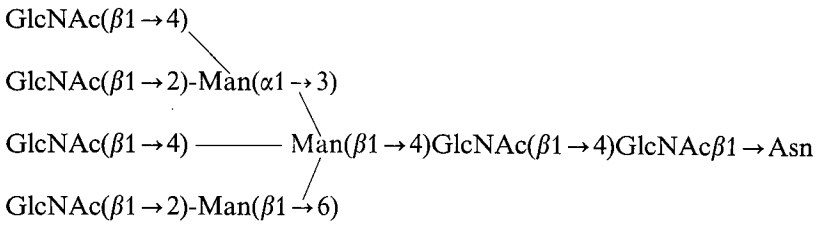
The primary structure of two glycopeptides obtained by pronase digestion of chicken ovotransferrin has been investigated by 360-MHz proton nuclear magnetic resonance (NMR) spectroscopy and methylation analysis. The two glycopeptides differ in amino acid composition but contain the same carbohydrate moiety, viz:



Using the NMR data of some reference compounds the chemical shifts of the anomeric protons and mannose H-2 protons could be predicted with an accuracy of 0.01 ppm.

Ovotransferrin, serotransferrin and lactotransferrin are iron-binding glycoproteins with similar molecular weight and amino acid compositions [1,2]. The primary structure of the N-terminal sequences of the polypeptide chain of these three transferrins shows a close homology [3] and an internal homology of human lactotransferrin and human serotransferrin has recently been described [4]. However, their carbohydrate moieties differ in molar composition, in number as well as in location on the polypeptide chain [2,5–8]. In order to compare the primary structure of ovotransferrin glycan with those of human serotransferrin and lactotransferrin that we have previously determined [9–12] we have undertaken the determination of the ovotransferrin glycan structure. In a preliminary investigation [13] it was concluded that two possibilities had to be considered for the carbohydrate structure viz. that given in Fig. 3 and an isomeric structure having GlcNAc-7 attached to Man-4′ instead of Man-4. The present paper deals with the determination of the structure by 360-MHz ^{1}H-NMR spectroscopy and methylation analysis.

Abbreviations. GlcNAc, *N*-acetylglucosamine; NMR, nuclear magnetic resonance.

MATERIALS AND METHODS

Iron-free ovotransferrin (type I) from chicken egg white was obtained from Sigma. It consisted of a major and a minor component as was apparent from disc gel electrophoresis. After exhaustive pronase digestion [14] the glycopeptide fraction was purified by gel filtration through a Biogel P 30 column (100 × 2 cm) equilibrated in water and then fractionated by paper electrophoresis in 1 M acetic acid, pH 2.4 at 7 V/cm for 18 h. Qualitative and quantitative carbohydrate analyses of the iron-free ovotransferrin and of the glycopeptides were carried out by colourimetry [15] and by gas-liquid chromatography [16].

Permethylation of the glycopeptides was performed according to Hakomori [17] and the partially methylated monosaccharides were identified according to Fournet et al. [18,19].

The amino acid composition of hydrolyzed glycopeptides (5.6 M HCl, 24 h, 105 °C under vacuum) was determined with a Beckman Multichrom analyser. The Edman degradation was carried out according to Konigsberg and Hill [20] and Light and Greenberg [21].

Table 1. *Carbohydrate and amino acid compositions of the ovotransferrin glycopeptides. The percentage sugar composition was based on colourimetry*
The molar sugar composition was calculated on the basis of three residues of mannose

Constituent	Ovotransferrin	Ovotransferrin glycopeptides	
		GP-I	GP-II
	%		
Mannose	0.85	27.0	23.3
N-Acetylglucosamine	1.78	62.2	58.5
	mol		
Mannose	3	3	3
N-Acetylglucosamine	5.60	5.76	6.02
Aspartic acid	–	1	1
Arginine	–	0	1

Table 2. *Identification and determination of the methylated monosaccharides obtained from permethylated ovotransferrin glycopeptides*
Methylated derivatives were assayed on the basis of one 2-mono-*O*-methylmannose residue. The nearest integral numbers are given in brackets

Methylated derivatives	Glycopeptides	
	GP-I	GP-II
3,4,6-Tri-*O*-methylmannose	0.70 (1)	1.2 (1)
3,6-Di-*O*-methylmannose	0.75 (1)	0.69 (1)
2-Mono-*O*-methylmannose	1 (1)	1 (1)
3,4,6-Tri-*O*-methyl-*N*-methylglucosamine	3.7 (4)	3.8 (4)
3,6-Di-*O*-methyl-*N*-methylglucosamine	1.6 (2)	1.4 (2)

For ^{1}H-NMR analysis the glycopeptides were repeatedly exchanged in ^2H_2O. The 360-MHz ^{1}H-NMR spectra of 5–10 mM solutions of the compounds in ^2H_2O were recorded on a Bruker 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 ^2H_2O: δ = 2.225 ppm).

RESULTS

10 g iron-free ovotransferrin were exhaustively digested with pronase. To isolate glycopeptides the digest was fractionated by two successive filtrations on Biogel P 30. The glycopeptide fraction was separated by paper electrophoresis into glycopeptide GP-I (85 mg) and glycopeptide GP-II (48 mg). The carbohydrate and amino acid compositions of these glycopeptides and the carbohydrate composition of the iron-free ovotransferrin are summarized in Table 1. From the colourimetric analysis and on the basis of a molecular weight of 80000, it may be concluded that ovotransferrin contains only one glycan chain. By gas-liquid chromatography analysis, on the basis of three residues of mannose six *N*-acetylglucosamine residues are found in ovotransferrin and in the two ovotransferrin glycopeptides. The compositions of the glycopeptides GP-I and GP-II differ only in the arginine content, explaining the difference in electrophoretic behaviour. The Edman degradation carried out on both glycopeptides showed for GP-I the presence of the phenylthiohydantoin derivative of the glycoamino acid, which gave after hydrolysis with hydrochloric acid the aspartic acid phenylthiohydantoin. In the case of GP-II the phenylthiohydantoin derivative of the glycopeptide and free arginine were characterized by paper electrophoresis indicating that arginine is situated in C-terminal position. The results of methylation analysis of the glycopeptides GP-I and GP-II are given in Table 2. These data demonstrate that in both glycopeptides four *N*-acetylglucosamine residues occupy terminal positions and that two *N*-acetylglucosamine residues are substituted at position 4. One mannose residue is substituted at position 2, another mannose at position 2 and 4 and the third mannose is substituted at positions 3, 4 and 6.

The glycopeptides GP-I and GP-II were analyzed by 360-MHz ^{1}H-NMR spectroscopy. The essential parts of the spectrum of GP-I are presented in Fig. 1. The signals which stem from the carbohydrate moiety have the same chemical shifts for both glycopeptides. The presence of arginine in GP-II gives rise to the corresponding extra signals [22]. Relevant NMR data are summarized in Table 3. The assignment of the signals was made by using the ^{1}H-NMR data of two glycopeptides from α_1-acid glycoprotein (GP-A and GP-B) and of a heptasaccharide obtained from the urine of a patient with Sandhoff's disease (OL-C) [24]. The structures of the reference compounds are given in Fig. 2 and the NMR data in Table 3.

Spectral integration of the anomeric protons and of the protons of the *N*-acetyl groups demonstrates that the carbohydrate moiety consists of three mannose and six *N*-acetylglucosamine residues. The anomeric signals of the GlcNAc residues 1 and 2 (δ = 5.070 ppm, $J_{1,2}$ = 9.6 Hz and δ = 4.612 ppm, $J_{1,2}$ = 7.9 Hz respectively) can directly be assigned by comparison with the data of GP-A and GP-B. The resonances of the anomeric protons of the mannose residues 3, 4 and 4′ differ significantly from those found for GP-A and GP-B but are almost identical to those of OL-C. This suggests that the characteristic structural feature of OL-C, viz. the occurrence of GlcNAc-9 $\beta1\rightarrow4$ linked to Man-3 of the mannotriosido branching core, also holds for GP-I and GP-II. Selective irradiation of the anomeric proton of Man-4 at

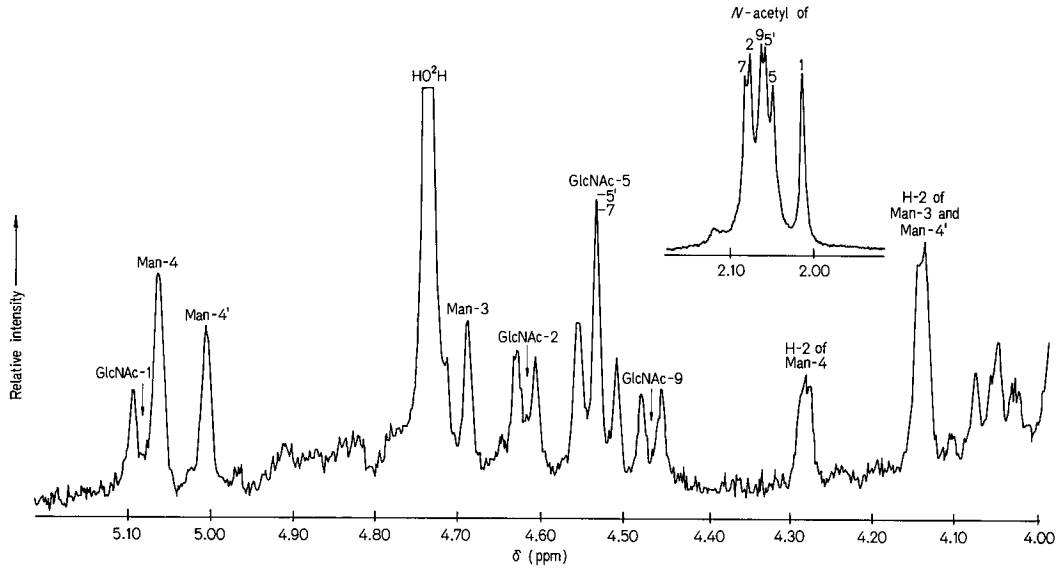


Fig. 1. *Regions of the anomeric, mannose H-2 and* N-*acetyl protons of the* ^{1}*H-NMR spectrum of GP-I at 360 MHz.* The relative intensity scale of the insertion with the *N*-acetyl proton region differs from that of the region with the anomeric and mannose H-2 protons

Table 3. ^{1}*H-NMR chemical shifts of anomeric protons, mannose H-2 protons and* N-*acetyl protons for the reference compounds GP-A, GP-B and OL-C (see Fig. 2) and the glycopeptides GP-I and GP-II isolated from ovotransferrin*

Proton	GP-A	GP-B	OL-C	GP-I	GP-II
	ppm				
H-1 of					
GlcNAc-1	5.072	5.076	–	5.070	5.072
GlcNAc-2	4.616	4.616	5.208 (α) 4.722 (β)	4.612	4.608
Man-3	4.764	4.755	4.697	4.684	4.683
Man-4	5.119	5.118	5.062	5.056	5.056
Man-4′	4.926	4.922	5.004	4.997	4.995
GlcNAc-5	4.581	4.580	4.555	4.540	4.540
GlcNAc-5′	4.581	4.580	4.555	4.540	4.540
Gal-6	4.470	4.471	–	–	–
Gal-6′	4.470	4.471	–	–	–
GlcNAc-7	–	4.548	–	4.516	4.512
Gal-8	–	4.448	–	–	–
GlcNAc-9	–	–	4.471	4.465	4.462
H-2 of					
Man-3	4.247	4.213	4.184	4.143	4.141
Man-4	4.188	4.213	4.250	4.281	4.279
Man-4′	4.109	4.108	4.151	4.143	4.141
N-Acetyl protons of					
GlcNAc-1	2.009	2.020	–	2.013	2.004
GlcNAc-2	2.078	2.077	2.555	2.076[b]	2.075[d]
GlcNAc-5	2.050[a]	2.048	2.555	2.048[c]	2.046[e]
GlcNAc-5′	2.047[a]	2.048	2.555	2.057[c]	2.057[c]
GlcNAc-7	–	2.077	–	2.082[b]	2.080[d]
GlcNAc-9	–	–	2.063	2.062	2.060

[a,b,c,d,e] Assignments may be interchanged.

δ = 5.056 ppm shows that this proton is coupled with the resonance signal at δ = 4.281 ppm, thus being the H-2 of Man-4. Compared with OL-C the H-2 of Man-4 is shifted downfield. As shown before [25] the differences in chemical shift between the sets of H-2 protons of the mannose residues in GP-A and GP-B stem from the presence of GlcNAc residue 7. Similar differences in chemical shift are observed for the set of H-2 protons of the mannoses in GP-I and GP-II in comparison to OL-C. This leads to the conclusion that in GP-I and GP-II an additional *N*-acetylglucosamine residue is present in position 4 of Man-4. The anomeric signals at δ = 4.540 ppm are assigned to the *N*-acetylglucosamine residues 5 and 5′ $\beta 1 \rightarrow 2$ linked to the mannose residues 4 and 4′ respectively. This identification was carried out by comparison with the data of OL-C. Based on methylation analysis and ^{1}H-NMR spectroscopy the following primary structure for the glycopeptides GP-I and GP-II can be presented (Fig. 3).

DISCUSSION

A novel type of carbohydrate structure has been derived for the glycopeptides GP-I and GP-II from ovotransferrin. It is remarkable that the glycan chain ends with *N*-acetylglucosamine residues and is completely devoid of galactose and sialic acid as constituents. The substitution pattern of Man-4 has also been observed in the triantennary structures occurring in α_1-acid glycoprotein [23] and fetuin [26]. The tribranched Man-3 is known to occur in the glycan chains of human myeloma immunoglobulins IgA_1 and IgG [27,28], ovomucoid [29] and ovalbumin [30,31]. However the finding of a triantennary structure with an additional GlcNAc attached to Man-3 is new.

The two glycopeptides of ovotransferrin which have been separated by paper electrophoresis differ in amino acid composition, since arginine was found

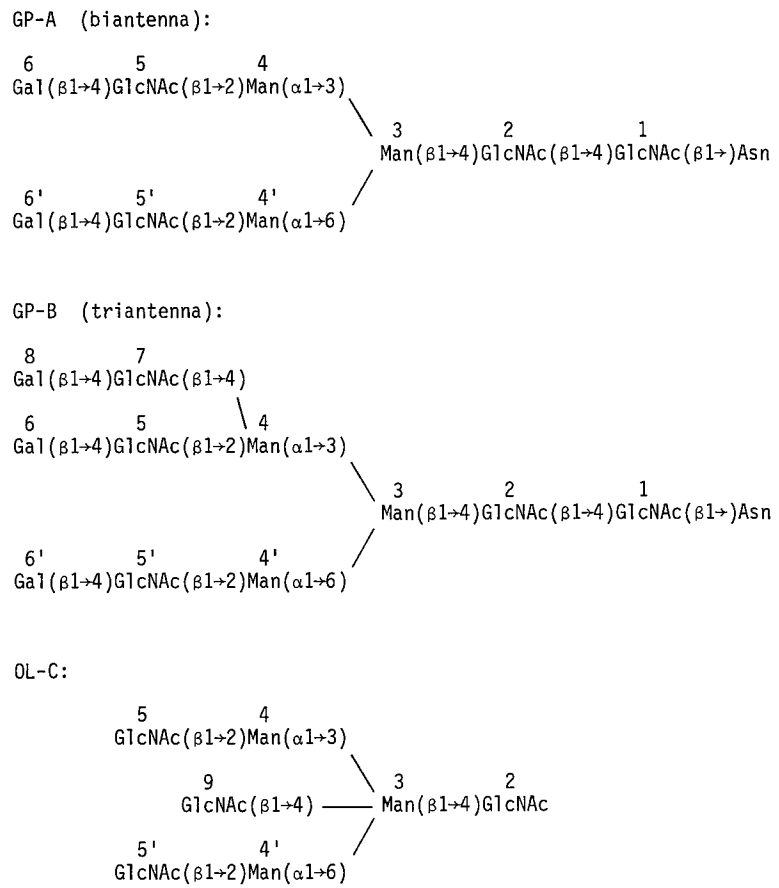


Fig. 2. *Structures of the reference glycopeptides GP-A and GP-B and the reference oligosaccharide OL-C*

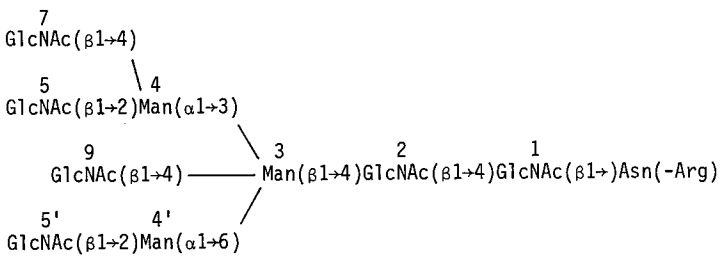


Fig. 3. *Primary structure of the glycopeptides GP-I (CHO-Asn) and GP-II (CHO-Asn-Arg) from ovotransferrin*

to be in C-terminal position in GP-II. This result agrees with a polypeptide structure proposed by Williams [7]: Ile-His-Asp(CHO)-Arg-Thr-Gly-Thr-Cys, where CHO = carbohydrate.

On the basis of the colourimetric method and a molecular weight of ovotransferrin of 80000 we can conclude that only one oligosaccharide is present in the molecule, which is in agreement with the results of Williams [7]. The molar ratio of mannose: *N*-acetylglucosamine of 3:6 that we have found differs from the values of 4:8 given by Williams [7] and 3:5 given by Iwase and Hotta [32]. The presence of galactose and the presence of a heterogeneity of the glycan, which was noticed by Iwase and Hotta [32] after fractionation of ovotransferrin on a concanavalin-A–Sepharose column were not confirmed in this study. The carbohydrate group of ovotransferrin has not yet been found in other glycoproteins. In particular, the glycans of ovomucin [33], ovalbumin [30] and ovomucoid [29], which are three glycoproteins synthesized as ovotransferrin by the hen oviduct tract, are different. Significance of the differences which also exist between the structure of the glycans of human [9–11] and rabbit serum transferrin [34], human [12] and bovine [35] lactotransferrin and those of hen ovotransferrin are still subject of investigation.

A close inspection of the NMR data, summarized in Table 3, revealed that the chemical shifts of the anomeric protons and of the H-2 protons of mannose of GP-I and GP-II could be predicted from the reference data. Taking the data of GP-A as starting point, shift increments can be calculated for the structures

Table 4. *Shift increments for the anomeric protons and mannose H-2 protons due to changes in the biantennary structure GP-A; predicted and observed chemical shifts for the glycopeptides GP-I and GP-II of ovotransferrin*
Shift increments smaller than 0.005 ppm are ignored. Structures of reference compounds are shown in Fig. 2

Proton	Shift increments			Chemical shifts		
	GP-A→GP-B	GP-A→OL-C	sum	predicted	observed	
					GP-I	GP-II
	ppm					
H-1 of						
GlcNAc-1	0	–	0	5.072	5.070	5.072
GlcNAc-2	0	–	0	4.616	4.612	4.608
Man-3	−0.009	−0.067	−0.076	4.688	4.684	4.683
Man-4	0	−0.057	−0.057	5.062	5.056	5.056
Man-4′	0	+0.078	+0.078	5.004	4.997	4.995
GlcNAc-5	0	−0.026	−0.026	4.555	4.540	4.540
GlcNAc-5′	0	−0.026	−0.026	4.555	4.540	4.540
Gal-6	0	–	–	–	–	–
Gal-6′	0	–	–	–	–	–
GlcNAc-7	–	–	−0.026	4.522	4.516	4.512
Gal-8	–	–	–	–	–	–
GlcNAc-9	–	–	–	4.471	4.465	4.462
H-2 of						
Man-3	−0.034	−0.063	−0.097	4.150	4.143	4.141
Man-4	+0.025	+0.062	+0.087	4.275	4.281	4.279
Man-4′	0	+0.042	+0.042	4.151	4.143	4.141

of GP-B and OL-C (Table 4). Addition of the sum of these increments to the chemical shifts of GP-A yields the predicted set of data for GP-I and GP-II. The agreement between the predicted and observed values in the order of 0.01 ppm. The observation that shift increments, which arise as a result of structural variations, can be handled independently of each other cannot easily be explained on theoretical grounds. However, these shift increments can efficiently be used to deduce structures, since the magnitude and sign of these increments correlate with differences in structure. On this basis it can be expected that the chemical shifts of the H-1 protons and mannose H-2 protons of complex carbohydrate chains can be predicted on the basis of the data of predecessors in complexity.

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