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SPECIFICITY IN THE ENZYMIC TRANSFER OF SIALIC ACID TO THE OLIGOSACCHARIDE BRANCHES OF BI- AND TRIANTENNARY GLYCOPEPTIDES OF α_1-ACID GLYCOPROTEIN

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Summary. Partial in vitro sialylation of biantennary and triantennary glycopeptides of α_1-acid glycoprotein using colostrum β-galactoside α(2→6) sialyltransferase followed by high resolution ^{1}H-NMR spectroscopic analysis of the isolated products enabled the assignment of the Galβ(1→4)GlcNAcβ(1→2)Manα(1→3)Man branch as the most preferred substrate site for sialic acid attachment. The Galβ(1→4)GlcNAcβ(1→2)Manα(1→6)Man branch appeared to be much less preferred and the Galβ(1→4)GlcNAcβ(1→4)Manα(1→3)Man sequence of triantennary structures was of intermediate preference for the sialyltransferase. The specificity of the β-galactoside α(2→6) sialyltransferase is thus shown to extend to structural features beyond the terminal N-acetyllactosamine units on the oligosaccharide chains of serum glycoproteins.

Sialyltransferases have been recognized from the early investigations on as glycosyltransferases with a high specificity for the carbohydrate structure of the acceptor glycoconjugate or oligosaccharide (1, 2). Indeed with the highly purified sialyltransferases available at present, this high degree of specificity has been further substantiated (3, 4, 5). At least four different glycoprotein sialyltransferases have been described each of which is involved in the formation of a particular sialic acid-containing structure (3, 4, 5, 6). In some instances, however, the specificity of these enzymes towards low molecular weight acceptors seems to be relative rather than absolute (3, 4, 7, 8).

Of particular interest is the reported difference in the in vitro sialylation of transferrin and α_1-acid glycoprotein (9). Under conditions where the biantennary oligosaccharide chains of the former glycoprotein are readily sialylated to almost completion by bovine colostrum β-galactoside α(2→6) sialyltransferase,

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less than half of the acceptor sites on the tetraantennary chains of asialo-α_1-acid glycoprotein are sialylated. To obtain a higher degree of sialylation of the latter glycoprotein prolonged incubation times with much increased amounts of enzyme are required (9).

To investigate whether a preferred pattern of sialylation of the different branches of the oligosaccharide chains of serum glycoproteins exists, we have studied the *in vitro* sialylation of the biantennary and triantennary glycopeptides of α_1-acid glycoprotein (10) utilizing a partially purified preparation of the colostrum sialyltransferase. Following incubation the sites of sialic acid attachment on the glycopeptides were established by high resolution ^{1}H-NMR spectroscopy.

Materials and Methods. Biantennary and triantennary glycopeptides derived from asialo α_1-acid glycoprotein, GPII-6 and GPII-5 respectively, were prepared and characterized as described previously (10, 11). CMP-[^{14}C]AcNeu (1.68 Ci/mol) was purchased from New England Nuclear, Boston, Mass., and diluted with unlabeled CMP-AcNeu (12) to the desired specific radioactivity. β-Galactoside α(2→6) sialyltransferase was partially purified (2000 fold) from bovine colostrum using CDP-ethanolamine-Sepharose affinity chromatography (13). The activity of the sialyltransferase preparation was assayed in a system containing 1 mg asialo-α_1-acid glycoprotein (425 nmol theoretical acceptor sites), 0.126 µmol CMP-[^{14}C]AcNeu (0.79 Ci/mol) and 8 µmol Tris-maleate pH 6.8 in a volume of 80 µl. The amount of enzyme transferring 1 µmol of [^{14}C]AcNeu per min at 37°C is defined as one unit of sialyltransferase activity.

Sialylation of glycopeptides was carried out in 800 µl water containing 1.0-1.1 mg of a glycopeptide (not water and salt free), 20 mU β-galactoside α(2→6) sialyltransferase (specific activity 29 mU/mg protein), 800 µg bovine serum albumin, 80 µmol Tris-maleate at pH 6.8, and 2.93 µmol CMP-[^{14}C]AcNeu (0.34 Ci/mol). Incubations were conducted at 37°C for 45 min and 6 h for the biantennary and triantennary glycopeptide, respectively. These incubation periods were chosen in order to effect a partial sialylation of the available acceptor sites. The resulting mixtures were then applied to a column (1.5 x 83 cm) of Sephadex G-50 superfine in 0.05 M pyridinium acetate at pH 5.0. The materials were eluted with the same buffer at a flow rate of 23 ml per h and fractions of 1.7 ml were collected (Fig. 1). Appropriate radioactive fractions were pooled and lyophilized.

For ^{1}H-NMR spectroscopic analysis the [^{14}C]sialylated glycopeptides were repeatedly treated with D_2O (100% D; Aldrich, Beerse, Belgium) at room temperature with intermediate lyophilization. The 360-MHz ^{1}H-NMR spectra were recorded on a Bruker HX-360 spectrometer operating in the Fourier transform mode at probe temperatures of 25° and 42°C. Chemical shifts are given relative to sodium-2,2-dimethyl-2-silapentane-5-sulphonate (indirectly to acetone: 2.225 ppm).

Results. Incubation of the glycopeptides with β-galactoside α(2→6) sialyltransferase and CMP-[^{14}C]AcNeu resulted in the incorporation

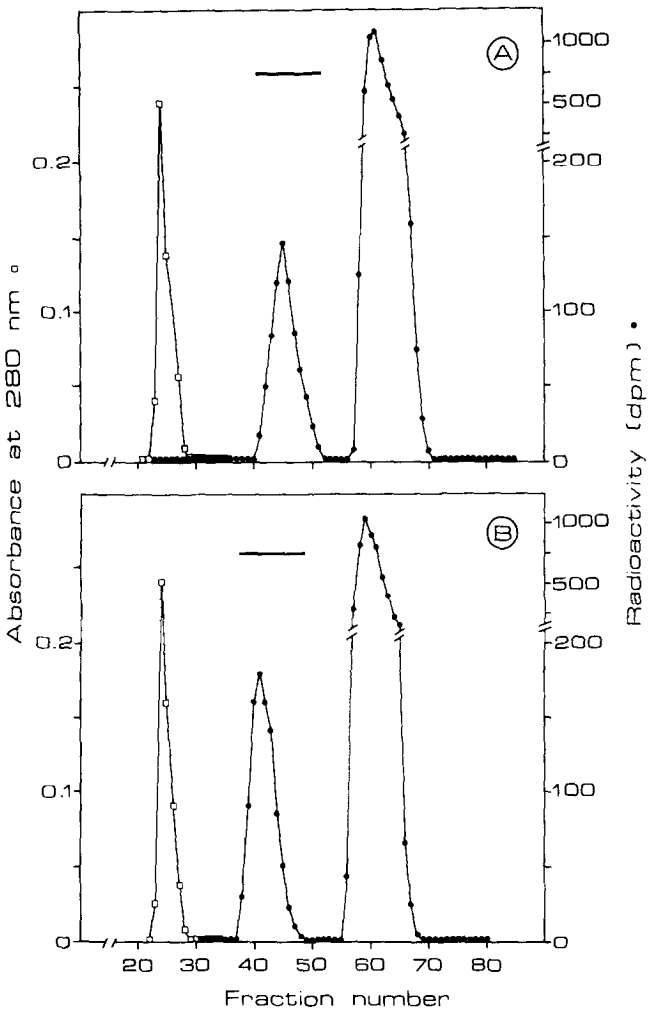

Figure 1. Sephadex gel filtration of the incubation mixtures of the biantennary glycopeptide GPII-6 (A) and the triantennary glycopeptide GPII-5 (B). The effluent was monitored for absorbance at 280 nm (□—□) and for ^{14}C radioactivity (●—●) using a 10 µl aliquot. The fractions indicated by the bars (A, fractions 41-51; B, fractions 38-48), containing the sialylated glycopeptides, were pooled.

of 337 and 446 nmol [^{14}C]AcNeu into the biantennary and triantennary structure, respectively. In agreement with the reported specificity of the enzyme (3), sialic acid was introduced to galactose of both glycopeptides in an α(2→6) linkage as evidenced by the sets of chemical shift values of the axial and equatorial H-3 protons of AcNeu (Table I) in the ^{1}H-NMR spectra of the sialylated glycopeptides (14). The NMR spectrum of the sialylated biantennary glycopeptide shows an increment in the chemical shift of H-1 of Man 4 as

Table I. - Chemical shift values of some characteristic protons of glycopeptides from α_1-acid glycoprotein before and after partial sialylation in vitro.

proton	chemical shift (ppm)			
	biantennary glycopeptides[§]		triantennary glycopeptides[§]	
	asialo	monosialylated	asialo	bisialylated
H-1 Man 4[§]	5.121	5.136	5.120	5.135
H-1 Man 4'	4.928	4.927	4.927	4.927
H-3ax AcNeu	-	1.727	-	1.721
H-3eq AcNeu	-	2.666	-	2.667

[§]For the structures of the glycopeptides and the numbering of the glycosyl residues see Fig. 2 and reference 10.

compared to the asialo compound, whereas the major resonance signal of H-1 of Man 4' remains unchanged (Table I). From this it can be deduced that the major product of in vitro sialylation of the biantennary glycopeptide is the monosialo derivative in which the sialic acid is attached to the terminal residue of the Galβ(1→4) GlcNAcβ(1→2)Manα(1→3)Man-branch (15). A small portion (<10%) of the anomeric protons of Man 4' resonates at a chemical shift of 4.942 ppm indicating that the bisialo derivative bearing an AcNeu at both Gal 6 and Gal 6' is a minor product (15). Regarding the triantennary glycopeptide, integration of the H-3eq AcNeu signal of the NMR spectrum of the sialylated product reveals the incorporation of somewhat less than two AcNeu residus per mole. One of these residues is linked to Gal 6 as is apparent from the chemical shift of H-1 of Man 4 (Table I). Since the chemical shift of H-1 of Man 4' is identical to that of the corresponding asialo compound, no sialic acid is attached to Gal 6'. Consequently, the major triantennary product possesses the second AcNeu moiety attached to Gal 8 (Fig.2).

As pointed out earlier (14), the chemical shifts of the anomeric protons of the Gal and GlcNAc residues in sialylated carbohydrate chains are different from those of the asialo chains. These differences, however, cannot be used for the assignment of the location of AcNeu, since the lactosamine units of different branches are at 360 MHz indistinguishable from each other.

Discussion. It has been shown earlier that N-acetyllactosamine and asialo glycoproteins containing this disaccharide unit at the non-reducing ends of their oligosaccharide chains are by far the

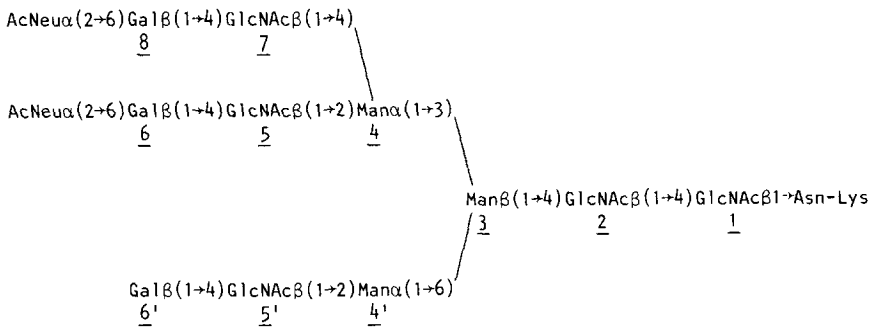

Figure 2. Structure of the *in vitro* partially sialylated triantennary glycopeptide of α_1-acid glycoprotein and the numbering of the glycosyl residues.

best acceptor substrates for the β-galactoside α(2→6) sialyltransferase (3). Our findings indicate that, in addition, structural features of the oligosaccharide acceptor chains beyond the terminal N-acetyllactosamine units are also recognized by the sialyltransferase. For the biantennary structure the linkage between the mannose residues appears to be of major importance. The N-acetyllactosamine unit linked to the Manα(1→3)Man part of the mannotriose branching point becomes fully sialylated upon incubation, whereas that attached to the Manα(1→6)Man part is poorly substituted. It is conceivable that the tetrasaccharide sequence of one branch as such shows a structure which is preferred to that of the other branch by the sialyltransferase. In addition the spatial arrangement and flexibility of the branches with respect to the rigid mannosido-di-N-acetylchitobiose core (16) might also be important factors in the process of sialylation.

A similar preference in sialylation of the branches of the triantennary structure is observed. Both N-acetyllactosamine units linked β(1→2) and β(1→4) to the Manα(1→3)Man part of the branching point are preferential substrate sites. The observations that Gal 6 is fully sialylated and Gal 8 for approximately 80%, however, suggests that the branch with the former Gal residue is sialylated at a higher rate than that with Gal 8. Whereas the N-acetyllactosamine unit at Manα(1→6)Man of the biantennary structure becomes sialylated for about 10% in 45 min, no detectable sialylation of this branch in the triantennary glycopeptide occurs in 6 h. This indicates that the third branch of the triantennary structure might have a negative effect on the rate of sialylation at Gal 6'.

It is not known whether partially sialylated structures of biantennary and triantennary glycopeptides and oligosaccharides obtained from natural sources reflect a preferred pattern of sialylation *in vivo* or are formed from fully sialylated forms by metabolic breakdown or degradation during isolation (14, 15). Therefore comparison of the structures of the products of the *in vitro* sialylations described in this study with these structures seems not very meaningful. It is noteworthy, however, that in cases where fully sialylated structures contain $\alpha(2\rightarrow3)$ as well as $\alpha(2\rightarrow6)$ linked sialic acid residues, as has been found for the N-glycosidically linked chains on fetuin (17, 18), equine pancreatic ribonuclease (19), thyroxine binding globulin (20) and for the oligosaccharides VI, VIII and X from the urine of a patient with sialidosis (14), in all but one structure (18) the Gal$\beta(1\rightarrow4)$GlcNAc $\beta(1\rightarrow2)$Man$\alpha(1\rightarrow3)$Man branch is sialylated $\alpha(2\rightarrow6)$. This suggests that tissue β-galactoside $\alpha(2\rightarrow6)$ sialyltransferases, such as that in liver (8, 21), show specificities identical to that of the colostrum enzyme. Further studies with serum-type glycoprotein $\alpha(2\rightarrow6)$ and $\alpha(2\rightarrow3)$ sialyltransferases are required to establish in full the preferred pattern of sialylation of the branches of bi-, tri- and tetraantennary oligosaccharide chains on glycoproteins.

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