Glycoconjugate Journal (1993) **10**:99–108

Enzymatic characterization of CMP-NeuAc:Galβ1-4GlcNAc-R α(2-3)-sialyltransferase from human placenta

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Received 3 November 1992

In this report we present the enzymatic characterization of CMP-NeuAc:Galβ1-4GlcNAc-R α(2-3)-sialyltransferase from human placenta using placenta membranes as an enzyme preparation. This sialyltransferase is highly sensitive to detergents and prefers type 2 chain (Galβ1-4GlcNAc) over type 1 chain (Galβ1-3GlcNAc) acceptors. Oligosaccharides and glycopeptides were better acceptor substrates than glycoproteins. Of the branched oligosaccharides, those with a bisected *N*-acetylglucosamine (GlcNAc) structure appeared to be poorer substrates, while triantennary structures containing a Galβ1-4GlcNAcβ1-$\underline{4}$Manα1-3Man branch were preferred. Product characterization, using 400 MHz ^{1}H-NMR spectroscopy, confirmed that sialic acid was introduced into the Galβ1-4GlcNAc-R units of the acceptor substrates in an α(2-3) linkage, and revealed that this sialyltransferase does not prefer either of the two branches of a complex type diantennary glycopeptide acceptor for sialic acid attachment. These properties distinguish this enzyme from all other sialyltransferases characterized to date.

Keywords: Acceptor specificity, branch specificity, human placenta, sialylation, α3-sialyltransferase.

Abbreviations: NeuAc, *N*-acetylneuraminic acid; CMP-NeuAc, cytidine 5′-monophospho-*N*-acetylneuraminic acid; GP-F2 and GP-F4, diantennary complex type glycopeptides from desialylated fibrinogen; GP-Trf, diantennary complex type glycopeptide from desialylated transferrin; LNT, Galβ1-3GlcNAcβ1-3Galβ1-4Glc (lacto-*N*-tetraose); α6-sialyltransferase, CMP-NeuAc:Galβ1-4GlcNAc-R α(2-6)-sialyltransferase; α3-sialyltransferase *O*, CMP-NeuAc:Galβ1-3GalNAc-R α(2-3)-sialyltransferase; α3-sialyltransferase I, CMP-NeuAc:Galβ1-3(4)GlcNAc-R α(2-3)-sialyltransferase; α3-sialyltransferase II, CMP-NeuAc:Galβ1-4GlcNAc-R α(2-3)-sialyltransferase

Introduction

Sialic acid, occurring in different linkage types on a variety of carbohydrate structures, has been described on many glycoproteins, glycolipids and oligosaccharides [1]. Synthesis of sialylated glycans involves sialyltransferases, which constitute a class of glycosyltransferases that are capable of catalysing the transfer of sialic acid (NeuAc) from CMP-NeuAc to distinct glycan acceptor structures [2]. These enzymes are highly specific for the linkage that is formed (α2-3, α2-6, α2-8), as well as for the primary structure and the spatial conformation of the acceptor [2]. Based on their specificities, at least twelve different mammalian sialyltransferases can be distinguished, eight of which are involved in the sialylation of oligosaccharide chains of glycoproteins (Table 1). Some of these enzymes have been purified to homogeneity (1, 3, 5, and 6, Table 1), but to date the cDNAs of only three sialyltransferases (1, 3 and 5, Table 1) have been cloned [3–5].

The presence of sialic acid in terminal positions of *N*- and *O*-linked glycans in glycoproteins has been shown to be an important factor in determining the half-life of these macromolecules in the circulation by preventing the interaction between cellular receptors and the subterminal sugar residues [6]. In addition, the biological activity of certain glycoproteins, such as human chorionic gonadotropin (hCG), is dependent on the presence of terminal sialic acid residues at their carbohydrate chains [7, 8].

It has been shown that human placenta contains two α(2-3)-sialyltransferases which are able to catalyse the sialylation of galactosyl residues on *N*-linked (2, Table 1) [9, 10] and *O*-linked (5, Table 1) [11] carbohydrate chains, respectively, of placental glycoproteins such as hCG. While the latter enzyme, a β-galactoside α(2-3)-sialyltransferase acting on *O*-linked glycans, has been purified and its physical and enzymatic properties have been examined [11], the

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Table 1. Sialyltransferases involved in the sialylation of protein-linked oligosaccharides.

Sialyltransferases as indicated by the structure formed	*References*
1. NeuAcα2-6Galβ1-4GlcNAcβ-R	30, 43
2. NeuAcα2-3Galβ1-4GlcNAcβ-R	9, 10
3. NeuAcα2-3Galβ1-3(4)GlcNAcβ-R	31, 32
4. [NeuAcα2-3Galβ1-3]NeuAcα2-6-GlcNAcβ-R	32, 44
5. NeuAcα2-3Galβ1-3GalNAcα-R	11, 45
6. NeuAcα2-6GalNAcα-R	46, 47
7. [NeuAcα2-3Galβ1-3]NeuAcα2-6-GalNAcα-R	47, 48
8. NeuAcα2-8NeuAcα2-8NeuAc-R	49

former has not been characterized to date because attempts to purify the enzyme have failed [11]. In this report we have studied the acceptor specificity and some other properties of the CMP-NeuAc:Galβ1-4GlcNAc-R α(2-3)-sialyltransferase (II) (2, Table 1) using membranes from human placenta as an enzyme preparation. These studies indicate that this enzyme has to be distinguished from a CMP-NeuAc:Galβ1-3(4)GlcNAc-R α(2-3)-sialyltransferase (I) (3, Table 1) previously described in rat liver [12]. Product characterization by 400 MHz ^{1}H-NMR spectroscopy revealed that, unlike the α6-sialyltransferase from bovine colostrum [13], placenta α3-sialyltransferase II does not show a preference for either branch of a diantennary glycopeptide substrate.

Materials and methods

Materials

Human placenta was obtained from the Academisch Ziekenhuis der Vrije Universiteit, Amsterdam. α_1-Acid glycoprotein was prepared as described by Hao and Wickerhauser [14], and human transferrin was purchased from Sigma (St Louis, MO, USA). Fibrinogen was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam. Asialo-α_1-acid glycoprotein, asialotransferrin and asialofibrinogen were prepared by mild acid hydrolysis (0.1 M trifluoroacetic acid or 0.1 M sulfuric acid, 1 h, 80 °C) of the corresponding native glycoproteins.

CMP-[^{14}C]NeuAc, specific radioactivity 4.1 Ci mol^{-1} (15.2×10^{10} Bq mol^{-1}) was obtained from New England Nuclear (Boston, MA, USA) and diluted with unlabelled CMP-NeuAc [15] to a specific activity of 1.05 Ci mol^{-1}.

The oligosaccharides in Tables 2–5 were tested as acceptors for the α3-sialyltransferase II from human placenta. Compounds 1, 15, 16 and 17 were donated by Drs S. David, J. Alais and A. Veyrières (Université Paris-Sud, Orsay, France); Galβ1-3GalNAc was a kind gift of Dr R. W. Jeanloz (Massachusetts General Hospital, Boston, MA, USA); Galβ1-3GlcNAcβ1-3Galβ1-4Glc (LNT) was obtained from Dr V. Ginsburg (National Institutes of Health, Bethesda, MD, USA); compounds 2, 3, and the branched oligosaccharides 5, 7, 9, 11 and 13 were kindly provided by Dr J. Lönngren (University of Stockholm, Stockholm, Sweden); compound 18 was donated by Dr H. Lönn (BioCarb, Lund, Sweden) and compounds 4, 6, 8, 10, 12 and 14 were generously provided by Drs G. Strecker and J. C. Michalski (Université de Lille, Villeneuve d'Ascq, France).

Preparation of glycopeptides

The glycopeptides GP-F2 and GP-F4 from asialofibrinogen and the glycopeptide from asialotransferrin were prepared by Pronase digestion. Typically, 5 g of desialylated glycoprotein were dissolved in 500 ml 0.1 M Tris-HCl, pH 8.0, containing 35 mM NaCl and 35 mM $CaCl_2$. After the addition of 50 mg Pronase and 100 µl toluene, the mixture was incubated at 37 °C for 96 h. During this period the pH was kept at 8.0 with 4 M NaOH and, each 24 h, 25 mg Pronase, together with 100 µl toluene, were added (to a total of 125 mg Pronase). After lyophilization, the peptides were dissolved in 10 ml H_2O and desalted on a column (2.5 cm × 50 cm) of Bio-Gel P-4 (100–200 mesh) equilibrated and eluted with 50 mM ammonium acetate at pH 5.2 and room temperature, at a flow rate of 70 ml h^{-1}. Fractions of 6 ml were collected and assayed for protein (ninhydrin reagent), hexose contents (phenol/sulfuric acid reagent [16]) and NaCl (precipitation with $AgNO_3$). Fractions positive for both protein and hexose were pooled and lyophilized.

After repeating this gel filtration step once, the products were separated on a column (1.6 cm × 200 cm) of Bio-Gel P-6 (200–400 mesh), equilibrated, and eluted with 50 mM ammonium acetate at pH 5.2 and room temperature, at a flow rate of 8 ml h^{-1}. Fractions of 4 ml were collected and assayed for hexose contents [16]. The top fractions of the two major peaks from the digestion of fibrinogen (designated GP-F2 and GP-F4) were pooled and analysed by 400 MHz ^{1}H-NMR spectroscopy (the ^{1}H-NMR data of GP-F2 are shown in Table 6). The two materials were identified as diantennary complex type glycans differing in the number of amino acids in the peptide portion, GP-F2 comprising several amino acids less than GP-F4. The product from the digestion of transferrin, designated GP-Trf, was identified as a complex type diantennary glycan with several amino acids attached.

Preparation of membranes

Human placenta was cut in small pieces, washed in a saline solution (1–2%) and stripped off the connective tissue. The material was homogenized in 1–2 volumes (ml per g tissue) of 0.25 M sucrose with the aid of a Potter–Elvehjem homogenizer (3 or 4 strokes of 15 s) at 0–4 °C. The homogenate was filtered through a fine cheese-cloth to remove debris and most of the nuclei, and membrane

fractions were prepared by centrifugation of the filtrate for 1.2 h at 80000 × g. The membranes were stored as such in aliquots at −20 °C.

Acceptor specificity studies

Incubation mixtures contained in a volume of 50 µl: 50 nmol acceptor substrate (theoretical acceptor sites), 25.5 nmol CMP-[^{14}C]NeuAc (1.05 Ci mol^{-1}), 5.0 µmol sodium cacodylate, pH 6.7, Triton X-100 (final concentration 0.1%), and 20 mg of the membrane pellet (2 mg protein). The mixtures were incubated at 37 °C for 30–45 min, after which the incubations were stopped by cooling on ice. After the addition of 100 µl ice-cold H_2O, the mixtures were centrifuged for 15 min in an Eppendorf centrifuge at top speed. The pellets were washed twice with 500 µl H_2O (0–4 °C) and the pooled supernatant fractions were applied to columns (1.6 cm × 200 cm or 0.7 cm × 45 cm) of Bio-Gel P-4 (200–400 mesh) equilibrated and eluted with 50 mM ammonium acetate at pH 5.2 and 37 °C, at a flow rate of 8 ml h^{-1} or 15 ml h^{-1}, respectively, to separate the product from the excess CMP-[^{14}C]NeuAc. Fractions (2.0 ml or 0.6 ml, respectively) were collected and assayed for ^{14}C radioactivity by liquid scintillation counting. In the cases where glycoproteins were used as acceptor substrates, the incorporation of [^{14}C]NeuAc was assayed by precipitation and counting of the dissolved pellet, as described previously [17].

Large scale incubation

Glycopeptide GP-F2 was incubated in a reaction mixture of 2.6 ml containing: 12.4 µmol glycopeptide substrate (in terms of theoretical acceptor sites), 13.9 µmol CMP-[^{14}C]NeuAc (1.05 Ci mol^{-1}), 0.260 mmol sodium cacodylate, pH 6.7, 2.6 µl Triton X-100 (final concentration 0.1%), and 871 mg membranes (87.1 mg protein). The mixture was incubated at 37 °C for 3 h and was occasionally vortexed (approximately each 30 min). After incubation the mixture was centrifuged for 10 min in an Eppendorf centrifuge at top speed. The pellet was washed twice with 300 µl H_2O and the pooled supernatants were subjected to chromatography on a column (1.6 cm × 200 cm) of Bio-Gel P-6 (200–400 mesh) equilibrated and eluted with 50 mM ammonium acetate at pH 5.2 and 45 °C, at a flow rate of 8 ml h^{-1} Fractions of 2 ml were collected and assayed for ^{14}C radioactivity and for hexose contents [16]. Fractions containing the monosialylated product were pooled and lyophilized.

400 MHz ^{1}H-NMR spectroscopy

Prior to ^{1}H-NMR spectroscopic analysis the samples were desalted on a column (0.7 cm × 45 cm) of Bio-Gel P-4 (200–400 mesh) run in H_2O. Samples were repeatedly treated with ^2H_2O (99.75 atom%) at p^{2}H 7 and room temperature. After each exchange treatment the samples were lyophilized. Finally, each sample was redissolved in 360 µl ^2H_2O (99.95 atom%; Aldrich, Milwaukee, WI, USA). ^{1}H-NMR spectroscopy was performed at 400 MHz on a Bruker MSL-400 spectrometer (facility of the Department of Physics, Vrije Universiteit, Amsterdam) operating in the Fourier transform mode. The probe temperature was kept at 300 K. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation. Chemical shifts are expressed downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ = 2.225 ppm) with an accuracy of 0.002 ppm.

Results

Preparation of glycopeptides

Glycopeptides from human fibrinogen (GP-F2 and GP-F4) and from human transferrin (GP-Trf) were prepared from the desialylated glycoproteins by extensive Pronase digestion. The products were identified by 400 MHz ^{1}H-NMR spectroscopy as complex type, asialo-diantennary glycopeptides, differing only in the amount of the attached amino acids (^{1}H-NMR data of GP-F2 shown in Table 6).

Incubation conditions

The sialyltransferase in the placental membranes catalysing the transfer of *N*-acetylneuraminic acid into asialo-α_1-acid glycoprotein (containing type 2 chains only), required the presence of Triton X-100 for optimal activity (Fig. 1). Too

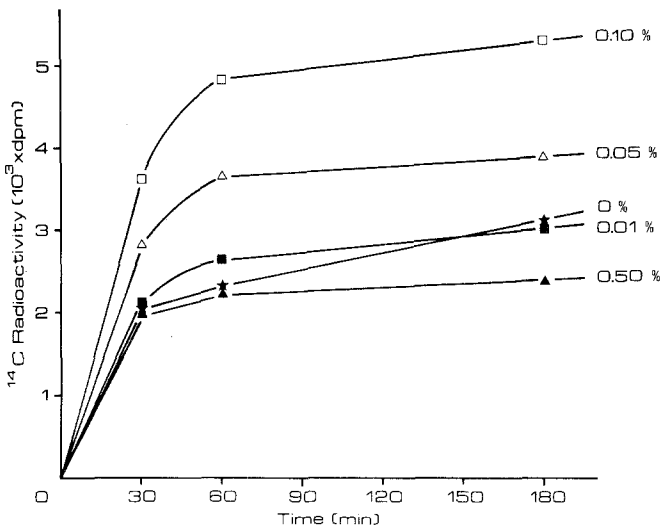

Figure 1. Effect of Triton X-100 on α3-sialyltransferase II activity. Asialo-α_1-acid glycoprotein was sialylated using CMP-[^{14}C]-NeuAc and human placenta membranes as a preparation of α3-sialyltransferase II. Incubation mixtures were prepared with various concentrations of the detergent as indicated (final % (by vol) of Triton X-100 in the incubation mixture). At intervals, aliquots were taken from the reaction mixture and incorporation of [^{14}C]NeuAc into asialo-α_1-acid glycoprotein was assayed by precipitation, as described in the Materials and methods section.

Table 2. Sialyltransferase activities of human placenta membranes, rat liver α3-sialyltransferase I and human placenta α3-sialyltransferase *O* with three different oligosaccharide substrates. Activities were determined using the assay methods described in the Materials and methods section.

Acceptor structure	*Activity of*		
	Human placenta membranes (%)	*α3-Sialyltransferase I (rat liver)* (%)	*α3-Sialyltransferase O (human placenta)* (%)
Galβ1-4GlcNAc (type 2)	100	15[a]	0[b]
Galβ1-3GlcNAcβ1-3Galβ1-4Glc (LNT) (type 1)	61	100[a]	10[b]
Galβ1-3GalNAc	145	9[a]	100[b]

[a] Calculated from data in [4] and [12].
[b] Taken from [11].

high a concentration of Triton X-100 or prolonged incubation in the presence of this detergent, however, appeared to inhibit the sialyltransferase activity (Fig. 1). Therefore the sialyltransferase assays were conducted at a final concentration of Triton X-100 of 0.1% by vol, and the incubation time was 30–45 min.

Sialyltransferase acceptor specificity

In order to discriminate between the different α3-sialyltransferases that may be present in human placenta membranes, incubations were performed with Galβ1-4GlcNAc, Galβ1-3GlcNAcβ1-3Galβ1-4Glc (LNT) and Galβ1-3GalNAc as acceptor substrates (Table 2). The membranes showed a high activity with Galβ1-3GalNAc, reflecting the presence of α3-sialyltransferase *O* in human placenta [11]. The activity of the membranes with Galβ1-4GlcNAc (type 2 chain acceptor) was higher than with LNT (type 1 chain acceptor). As a part of the incorporation into LNT can be ascribed to the action of α3-sialyltransferase *O* (Table 2), the preference for type 2 acceptors of the sialyltransferase acting on Galβ1-4GlcNAc appears to be even more pronounced. This preference is opposite to that of the Galβ1-3(4)GlcNAc-R α(2-3)-sialyltransferase (I) of rat liver (Table 2), which enzyme highly prefers type 1 chain acceptors [12]. Previously, placenta Galβ1-4GlcNAc-R sialyltransferase has been demonstrated to catalyse the formation of >97% of NeuAcα2-3Gal linkages [10]. Because of its acceptor preference it is referred to as Galβ1-4GlcNAc-R α(2-3)-sialyltransferase II.

The acceptor specificity of α3-sialyltransferase II was studied with linear and branched oligosaccharides that form part of *N*-linked glycans (Table 3), oligosaccharides that are structurally related to blood group I- and i-active polylactosaminoglycans (Table 4) and glycopeptide and glycoprotein acceptors (Table 5). It appeared that all substrates containing terminal Galβ1-4GlcNAc units were effective as an acceptor. Linear oligosaccharides showed somewhat better acceptor properties than branched structures. Minor differences were observed between acceptors with or without a terminal reducing *N*-acetylglucosamine residue (Table 3). Of the branched oligosaccharides the triantennary substrates possessing a Galβ1-4GlcNAcβ1-4Manα1-3Man branch (compounds 9 and 10) appeared to have the best acceptor properties, while those containing a bisected *N*-acetylglucosamine residue (compounds 7 and 8) showed the lowest activities. Little difference was found for the activities with diantennary structures whether they were linked to a small peptide or not (compare compounds 5 and 6, Table 3, with the glycopeptides, Table 5). Extension of the peptide portion, however, to a larger peptide or to protein resulted in a reduction of the activity (Table 5). This might be due to steric hindrance, or to impaired accessibility in the assay using membranes at a relative low concentration of Triton X-100.

Branch specificity

The product obtained with human placenta membranes and asialo-α_1-acid glycoprotein or a linear oligosaccharide as acceptor has been characterized previously [9, 10]. In order to define further the specificity of the enzyme, an α3-monosialylated preparation of the diantennary glycopeptide from fibrinogen, GP-F2, was prepared by incubating this glycopeptide and CMP-[^{14}C]NeuAc with human placenta membranes. After incubation the product was isolated by gel filtration on Bio-Gel P-6 (Fig. 2, yield 366 nmol) and analysed by 400 MHz ^{1}H-NMR spectroscopy.

The chemical shifts of the structural reporter groups of both the acceptor glycopeptide GP-F2 and the monosialylated products are summarized in Table 6, while the relevant structural reporter group regions of the ^{1}H-NMR spectrum of the product are presented in Fig. 3. The signals of the asialo substrate GP-F2 could be assigned by comparison with the chemical shift data of a diantennary, asialo glycopeptide (bearing the amino acids Asn-Lys at its reducing end) [18]. The chemical shifts of the signals of both compounds are virtually identical, the only difference

Table 3. Acceptor specificity of human placenta α3-sialyltransferase II: oligosaccharide acceptors that form part of *N*-linked glycans. The activity of α3-sialyltransferase II with the acceptors was assayed using human placenta membranes in the standard incubation mixture as described in the Materials and methods section. Acceptors were present at an amount of 50 nmol theoretical acceptor sites (1 mM terminal galactose residues). The mixtures were incubated at 37 °C for 45 min and incorporation of [^{14}C]NeuAc into the acceptor substrates was determined as described in the Materials and methods section. Relative rates for each acceptor are expressed as a percentage of the incorporation obtained with the diantennary oligosaccharide 6, which amounted to 1.52 nmol per mg protein per h.

Oligosaccharide	*Relative rate of sialylation* (%)
1. Galβ1-4GlcNAc	135
2. Galβ1-4GlcNAcβ1-2Man	157
3. Galβ1-4GlcNAcβ1-6Man	153
4. Galβ1-4GlcNAcβ1-2Manα1-6Manβ1-4GlcNAc	127
5. Galβ1-4GlcNAcβ1-2Manα1 ↘6 Man ↗3 Galβ1-4GlcNAcβ1-2Manα1	116
6. Galβ1-4GlcNAcβ1-2Manα1 ↘6 Manβ1-4GlcNAc ↗3 Galβ1-4GlcNAcβ1-2Manα1	100
7. Galβ1-4GlcNAcβ1-2Manα1 ↘6 GlcNAcβ1→4Man ↗3 Galβ1-4GlcNAcβ1-2Manα1	66
8. Galβ1-4GlcNAcβ1-2Manα1 ↘6 GlcNAcβ1→4Manβ1→4GlcNAc ↗3 Galβ1-4GlcNAcβ1-2Manα1	48
9. Galβ1-4GlcNAcβ1→2Manα1 ↘6 Man ↗3 Galβ1-4GlcNAcβ1→2Manα1 ↗4 Galβ1-4GlcNAcβ1	122
10. Galβ1-4GlcNAcβ1→2Manα1 ↘6 Manβ1-4GlcNAc ↗3 Galβ1-4GlcNAcβ1→2Manα1 ↗4 Galβ1-4GlcNAcβ1	132
11. Galβ1-4GlcNAcβ1 ↘6 Galβ1-4GlcNAcβ1→2Manα1 ↘6 Man ↗3 Galβ1-4GlcNAcβ1→2Manα1	75
12. Galβ1-4GlcNAcβ1 ↘6 Galβ1-4GlcNAcβ1→2Manα1 ↘6 Manβ1-4GlcNAc ↗3 Galβ1-4GlcNAcβ1→2Manα1	89
13. Galβ1-4GlcNAcβ1 ↘6 Galβ1-4GlcNAcβ1→2Manα1 ↘6 Man ↗3 Galβ1-4GlcNAcβ1→2Manα1 ↗4 Galβ1-4GlcNAcβ1	68
14. Galβ1-4GlcNAcβ1 ↘6 Galβ1-4GlcNAcβ1→2Manα1 ↘6 Manβ1-4GlcNAc ↗3 Galβ1-4GlcNAcβ1→2Manα1 ↗4 Galβ1-4GlcNAcβ1	83

(*continued*)

being the H-1 and NAc signals at GlcNAc-1 (GlcNAc-1 H-1 and NAc of GP-F2 $\delta = 5.043$ and 2.010, respectively; GlcNAc-1 H-1 and NAc of the reference compound $\delta = 5.094$ and 2.004, respectively). These discrepancies are caused by differences in the peptide portion of these compounds.

The intensity ratio of *N*-acetylneuraminic acid reporter group signals to those of other residues in the spectrum of the products (Fig. 3) reveals that this material contains only 1 mol *N*-acetylneuraminic acid per mol glycopeptide. The *N*-acetylneuraminic acid residue occurs in α(2-3) linkage to a galactose residue in a Galβ1-4GlcNAc disaccharide unit, located on the Manα1-3Man and on the Manα1-6Man branch, as appears from the resonances at $\delta = 1.797/1.801$ ppm (H-3a of *N*-acetylneuraminic acid located on the (1-3) and on the (1-6) branch, respectively) and $\delta = 2.758/2.760$ ppm

Table 4. Acceptor specificity of human placenta α3-sialyltransferase II: oligosaccharide acceptors that are structurally related to blood group I- and i-active polylactosaminoglycans. The activities were determined using the assay method as described in the Materials and methods section. Relative rates for each acceptor are expressed as a percentage of the incorporation obtained with the diantennary oligosaccharide 6 (Table 3), which amounted to 1.52 nmol per mg protein per h.

Oligosaccharide	*Relative rate of sialylation (%)*
15. Galβ1-4GlcNAcβ1-3Gal	161
16. Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc	133
17. Galβ1-4GlcNAcβ1 (6) Gal (3) Galβ1-4GlcNAcβ1	78
18. Galβ1-4GlcNAcβ1 (6) Galβ1-4Glc (3) Galβ1-4GlcNAcβ1	109

Table 5. Acceptor specificity of human placenta α3-sialyltransferase II: glycopeptide and glycoprotein acceptors. The activities were assayed as described in the Materials and methods section. Relative rates for each acceptor are expressed as a percentage of the incorporation obtained with the diantennary oligosaccharide 6 (Table 3), which amounted to 1.52 nmol per mg protein per h.

Acceptor	*Branching*	*Relative rate of sialylation (%)*
Glycopeptides		
GP-F2	diantennary	102
GP-F4	diantennary	74
GP-Trf	diantennary	109
Glycoproteins		
asialo-transferrin	mainly diantennary	33
asialo-α_1-acid glycoprotein	di-, tri- and tetra-antennary	25

(H-3e of *N*-acetylneuraminic acid on the (1-3) and on the (1-6) branch, respectively), $\delta = 4.544/4.549$ ppm (H-1 of α3-sialylated galactose; (1-3) branch and (1-6) branch, respectively), $\delta = 4.576$ ppm (H-1 of both GlcNAc-5 and 5′ in the sialylated branches) and $\delta = 2.046/2.042$ ppm (NAc, of GlcNAc-5/5′ in the sialylated branches), as well as the absence of resonances at $\delta \approx 1.716$ and 2.670, which

Table 6. 400 MHz ^{1}H-NMR chemical shift values of structural reporter group protons of the constituent monosaccharides of the diantennary glycans of GP-F2 and α3-monosialylated GP-F2. Preparation of the glycopeptide GP-F2 from asialofibrinogen, α3-sialylation of GP-F2, isolation of the products and the analysis by ^{1}H-NMR spectroscopy are described in the Materials and methods section. Chemical shifts are given in ppm at 300 K by reference to internal acetone ($\delta = 2.225$). For the full structures of α3-monosialylated GP-F2 see Fig. 3.

Reporter group	*Residue*[a]	*GP-F2*	*Monosialylated GP-F2 (NeuAc to α(1-3) branch)*	*Monosialylated GP-F2 (NeuAc to α(1-6) branch)*
H-1	GlcNAc-1	5.043	5.042	5.042
	GlcNAc-2	4.615	4.615	4.615
	Man-3	4.762	4.762	4.762
	Man-4	5.118	5.118	5.118
	Man-4′	4.927	4.925	4.925
	GlcNAc-5	4.581	4.576	4.583
	GlcNAc-5′	4.581	4.583	4.576
	Gal-6	4.466	4.544	4.467
	Gal-6′	4.472	4.473	4.549
H-2	Man-3	4.246	4.247	4.247
	Man-4	4.190	4.193	4.193
	Man-4′	4.110	4.113	4.113
H-3a	NeuAc	–	1.797	–
	NeuAc′	–	–	1.801
H-3e	NeuAc	–	2.758	–
	NeuAc′	–	–	2.760
NAc	GlcNAc-1	2.010	2.013	2.013
	GlcNAc-2	2.078	2.078	2.078
	GlcNAc-5	2.050	2.046	2.051
	GlcNAc-5′	2.045	2.046	2.042
	NeuAc	–	2.031	–
	NeuAc′	–	–	2.031

[a] For numbering of the monosaccharide residues, see Fig. 3.

are the corresponding diagnostic chemical shifts for an *N*-acetylneuraminic acid residue present in an α(2-6)-linkage on a diantennary glycopeptide (Table 6, Fig. 3) [18]. The *N*-acetylneuraminic acid is present on either the (1-3) or on the (1-6) branch, as is evident from the chemical shift values of H-3a and H-3e of *N*-acetylneuraminic acid, H-1 of Gal-6/6′ and NAc of GlcNAc-5/5′ as indicated above. The ratio of sialylation of the two branches is approximately 1/1, as appears from the ratios of the Gal-6/Gal-6′ and the Gal-6*/Gal-6′* signals (Fig. 3). Hence it appears that the monosialylated product from the incubation of GP-F2 with human placenta membranes consists of two isomeric, monosialylated diantennary glycopeptides, both carrying *N*-acetylneuraminic acid in an α(2-3) linkage to galactose, which differ only in the attachment of this residue to the (1-3) or to the (1-6) branch, and are present in approximately equal amounts.

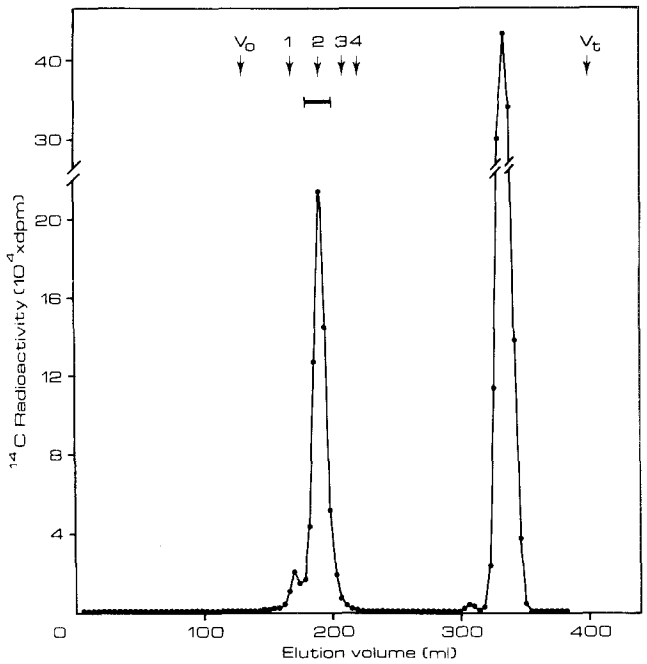

Figure 2. Isolation of the product of α3-sialylation of the diantennary glycopeptide GP-F2. GP-F2 was incubated with CMP-[^{14}C]NeuAc and human placenta membranes as a preparation of α3-sialyltransferase II. After centrifugation and washing of the pellet, the pooled supernatants were applied to a column (1.6 cm × 200 cm) of Bio-Gel P-6 (200–400 mesh) run in 50 mM ammonium acetate, pH 5.2, at a flow rate of 8 ml h^{-1}. Fractions of 2 ml were collected and monitored for ^{14}C radioactivity (●). The bar indicates the fractions that were pooled for analysis by ^{1}H-NMR. The elution positions of the following reference compounds [42] are indicated: 1, bisialo-diantennary oligosaccharide; 2, monosialo-diantennary oligosaccharide; 3, monosialo-monoantennary oligosaccharide; 4, diantennary glycopeptide substrate (GP-F2). The peak eluting at 170 ml represents a bisialylated form of GP-F2 and fractions at 330–340 ml could be identified as [^{14}C]NeuAc, which is a degradation product of the excess of CMP-[^{14}C]NeuAc.

Discussion

Many glycoproteins bearing complex-type asparagine-linked glycans have been found to contain sialic acid α(2-3) linked to the terminal Galβ1-4GlcNAc-R sequence [19–29]. The occurrence of these structures implies the existence of a Galβ1-4GlcNAc-R α(2-3)-sialyltransferase (α3-sialyltransferase I or II; 2, 3, Table 1). Previously, an α3-sialyltransferase has been identified in fetal calf liver, embryonic chicken brain and human placenta, using tritiated asialo-α_1-acid glycoprotein as an acceptor [9]. Attempts to purify this sialyltransferase from the latter tissue, however, have failed because of the instability of this enzyme upon extraction by detergents [11]. Therefore we used human placenta membranes as a preparation of α3-sialyltransferase for the characterization of the acceptor specificity of this enzyme.

Previous studies have shown that α3-sialyltransferase II, although abundantly expressed in human placenta [9], could not be detected in rat liver, using type 2 acceptor substrates and methylation analysis [9, 30]. Nevertheless, an α3-sialyltransferase that can act on Galβ1-4GlcNAc-R acceptors has been purified and cloned from this tissue [4, 31]. The latter enzyme, however, preferentially acts on Galβ1-3GlcNAc-R termini and has therefore been classified as a Galβ1-3(4)GlcNAc-R α(2-3)-sialyltransferase (α3-sialyltransferase I) [12]. Because of the abundant α6-sialyltransferase activity in rat liver, the methylation analysis method might not have been sufficiently sensitive to reveal the action of α3-sialyltransferase I on type 2 glycans. On the other hand, by the use of type 1 (Galβ1-3GlcNAc-R) oligosaccharides as acceptors, it was found that rat liver and fetal calf liver were relatively rich in α3-sialyltransferase activity, but that human placenta contained much less of it [32]. Our observation that human placenta α3-sialyltransferase II is highly sensitive to Triton X-100 while the α3-sialyltransferase from rat liver is Triton X-100 tolerant and actually requires detergent for its purification [31], along with the preference of the placenta enzyme for Galβ1-4GlcNAc over Galβ1-3GlcNAcβ1-3Galβ1-4Glc as an acceptor, and the difference in tissue and species distribution of these enzymes [12, 32], suggests that α3-sialyltransferase I and II activities have to be attributed to two different enzyme species. However, they resemble each other in recognizing the same donor substrate, CMP-NeuAc, and in having partial overlapping acceptor specificities.

In view of the similarities and differences of these sialyltransferases it is interesting to speculate about the molecular basis for the occurrence of these enzymes. The genes of the two α3-sialyltransferases might be related but non-allelic and might occur linked on the same chromosome. Such a situation would be analogous to that for two [Galβ1-4]GlcNAc-R α3/(4)-fucosyltransferases, the Lewis and the plasma enzyme, the genes of which show a high degree of identity and have been demonstrated to be syntenic but non-allelic [33]. Similar to the findings for α3-sialyltransferase I and II, the Lewis enzyme acts preferentially on type 1 acceptors (α4-activity), but is also capable of acting on type 2 chains (α3-activity), while the plasma enzyme exclusively interacts with type 2 acceptor structures [33, 34]. Alternatively, the two α3-sialyltransferases might be protein isoforms generated from a single gene locus by alternative splicing or alternative promoter utilization. In rat, three α(2-6)-sialyltransferase isoforms, that exhibit tissue-specific expression, are predicted to be generated in this way [35–37]. Also four different mRNAs encoding a mouse α(1-3)-galactosyltransferase have been found to be produced by alternative splicing of a murine pre-mRNA [38]. A definite conclusion, however, can be drawn only after cloning and sequencing of the corresponding α3-sialyltransferase genes.

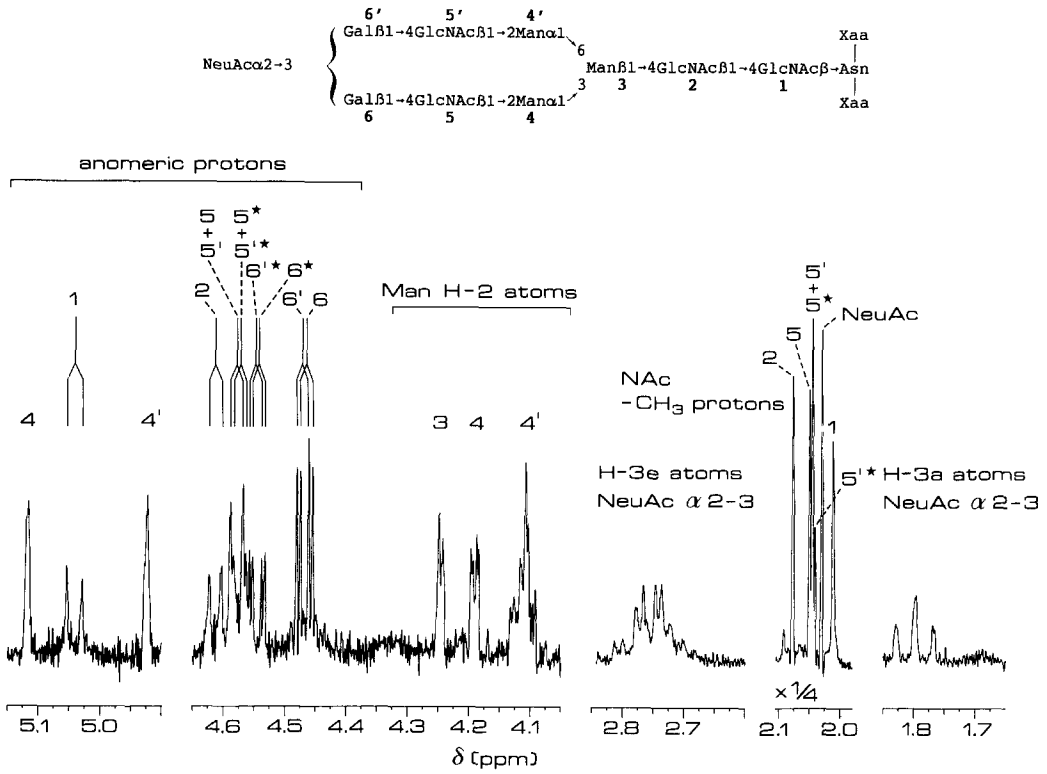

Figure 3. Structural reporter group regions of the 400 MHz ^{1}H-NMR spectrum of α3-monosialylated GP-F2. α3-Monosialylated GP-F2 was obtained from the incubation of asialo-GP-F2 with human placenta membranes as described in the Materials and methods section. The numbers in the spectrum refer to the corresponding monosaccharide residues of the oligosaccharide structure, according to Vliegenthart *et al.* [18]. The asterisks denote residues in a branch to which *N*-acetylneuraminic acid is attached.

The membranous preparation used for the acceptor specificity studies of human placenta α3-sialyltransferase II contained in addition α3-sialyltransferase *O* activity, which enzyme acts preferentially on *O*-linked glycan Galβ1-3GalNAc-R core structures [11]. The latter enzyme, however, did not influence the results of the acceptor studies with Galβ1-4GlcNAc-R based acceptors, because it does not act on such substrates (Table 2) [11]. On the other hand, human placenta appears to contain α3-sialyltransferase I activity, since the incorporation into the type 1 chain acceptor LNT cannot be accounted for entirely by the activity of α3-sialyltransferase *O*. Whether this is due to a side activity of α3-sialyltransferase II cannot be assessed from the current data. It could, however, be estimated that the contribution of an α3-sialyltransferase I to the activity assayed for α3-sialyltransferase II would not exceed 7%.

Studies with various acceptor substrates which contained a terminal Galβ1-4GlcNAc-R unit revealed that the specificity of α3-sialyltransferase II is only weakly influenced by structural features beyond the terminal *N*-acetyllactosamine unit. This is in contrast to α(2-6)-sialyltransferase from bovine colostrum [13, 39], which enzyme depends on the presence of at least one core *N*-acetylglucosamine residue in branched complex type oligosaccharides for high activity and full expression of branch specificity [13]. The α6-sialyltransferase is also much more sensitive to the degree and the type of branching of the substrates [39, 40]. Interestingly, the presence of a Galβ1-4GlcNAcβ1-4Manα1-3Man branch in the acceptor substrate results in increased activity of α3-sialyltransferase II while α6-sialyltransferase shows reduced activity [39]. As a consequence prior action of *N*-acetylglucosaminyltransferase IV, yielding such a branch [41], therefore will result in a shift from α6- to α3-sialylation, when both sialyltransferases are expressed in the cell. By contrast, prior action of *N*-acetylglucosaminyltransferase III, yielding bisected structures [41], will suppress α3- and α6-sialylation, since both these sialyltransferases act on bisected substrates at a reduced rate (this study, [40]).

Characterization of the product of sialylation of the diantennary complex type glycopeptide GP-F2 by 400 MHz ^{1}H-NMR spectroscopy confirmed the observation of previous studies [9, 10] that sialic acid was attached in an α(2-3) linkage to a Galβ1-4GlcNAc-R unit. Furthermore, ^{1}H-NMR spectroscopy of monosialylated GP-F2 revealed, that both branches of the diantennary glycan were α3-sialylated to a virtually equal extent. This indicates that α3-sialyltransferase II from human placenta has no outspoken preference for either branch of GP-F2. This again is in contrast to the specificity of the α6-sialyltransferase from bovine colostrum, which strongly prefers the Galβ1-4GlcNAc-R unit on the Manα1-3Man branch of a

diantennary acceptor substrate for the attachment of the first sialic acid residue [13, 17, 39, 42]. Whether α3-sialyltransferase II shows branch specificity with tri- and tetra-branched complex-type glycans is the subject of a current investigation.

The acceptor substrate specificity of α3-sialyltransferase-II suggests a function of the enzyme in the biosynthesis of the α3-sialylated *N*-linked oligosaccharide chains present on placental glycoproteins, such as human chorionic gonadotropin, β-glucocerebrosidase, transferrin receptor, and α-glucosidase [19, 20, 26–29]. Although the susceptibility to detergents of α3-sialyltransferase II did not allow its purification, it was possible to use placenta membranes to characterize its linkage and acceptor specificity, and to use it in the enzyme assisted synthesis of biologically active oligosaccharides (Th. de Vries and D. H. Van den Eijnden, unpublished results). In the future the recombinant form of the enzyme might become available. Cloning of the gene of α3-sialyltransferase II might be possible by making use of PCR techniques involving primers based on the 'sialylmotif', that sialyltransferases may have in common [4].

Acknowledgements

The expert technical assistance of Wietske E. C. M. Schiphorst and Constan van de Broek is gratefully acknowledged. This work was supported in part by the Dutch Innovation Oriented Programme Research on Carbohydrates (IOP-k).

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