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Transfer of sialic acid in α2-6 linkage to mannose in Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4GlcNAc by the action of Galβ1-4GlcNAc α2-6-sialyltransferase

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The disaccharide Manβ1-4GlcNAc and the trisaccharide Manβ1-4GlcNAcβ1-4GlcNAc were each incubated with CMP-NeuAc and rat liver α2-6-sialyltransferase (CMP-NeuAc: Galβ1-4GlcNAc α2-6-*N*-acetylneuraminyl transferase). The resulting mixtures were fractionated by HPLC on Partisil 10 SAX, and the fractions obtained were investigated by TLC, GLC (monosaccharide analysis) and 500-MHz ^{1}H-NMR spectroscopy. The following products were identified: NeuAcα2-6Manβ1-4GlcNAc and NeuAcα2-6Manβ1-4GlcNAcβ1-4GlcNAc in yields of 4% and 27%, respectively. The sialylation of Manβ1-4GlcNAc-R by Galβ1-4GlcNAc α2-6-sialyltransferase contrasts the reported high specificity of this enzyme for the structural element Galβ1-4GlcNAc of *N*-linked carbohydrate chains of glycoproteins and related oligosaccharides.

Sialyltransferase, Galβ1-4GlcNAc α2-6; Sialyltransferase; Sialyloligosaccharide; Mannosidosis, β-

1. INTRODUCTION

The isolation and structural characterization of oligosaccharides and glycoamino acids from urine of patients with lysosomal storage diseases has contributed considerably to the knowledge about carbohydrate chains of glycoproteins. In general, the excreted compounds in oligosaccharidoses and glycoproteinoses can be recognized as parts of *N*-glycosidically linked carbohydrate chains, originating from incomplete lysosomal catabolism [1–3]. However, some of the identified urinary glycoconjugates in human aspartylglucosaminuria and caprine β-mannosidosis have not been described as normally occurring constituents of glycoproteins. For aspartylglucosaminuria, in addition to GlcNAc-Asn as the major compound, Galβ1-4GlcNAc-Asn, NeuAcα2-3/6Galβ1-4GlcNAc-Asn and larger glycoamino acids have been reported [4–7]. In caprine β-mannosidosis urine extensions of the primary storage compounds Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4GlcNAc with a Manβ1-4GlcNAcβ1-4 moiety have been found [8]. Recently, we have discovered NeuAcα2-6Manβ1-4GlcNAc in human β-mannosidosis urine (unpublished results). In all these cases, the structures of the major storage products are elements of the structures of the unusual compounds. Therefore, it has been proposed that the accumulated, abun-

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Abbreviations: NeuAc, *N*-acetylneuraminic acid; NeuAc-2-en, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid; CMP-NeuAc, cytidine 5′-monophospho-β-*N*-acetylneuraminic acid
Enzyme: Galβ1-4GlcNAc α2-6-sialyltransferase (CMP-NeuAc: β-D-Gal-(1-4)-β-D-GlcNAc α-(2-6)-*N*-acetylneuraminyl transferase) (EC 2.4.99.1)

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dantly occurring compounds can serve as substrates for glycosyltransferases. Our discovery of the presence of sialic acid in α2-6 linkage to the mannose residue of Manβ1-4GlcNAc prompted us to study the possibility of enzymatic synthesis of NeuAcα2-6Man linkages by rat liver Galβ1-4GlcNAc α2-6-sialyltransferase with Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4GlcNAc as substrates.

2. MATERIALS AND METHODS

2.1. *Materials*

Manβ1-4GlcNAc was isolated from pooled urines of two brothers with β-mannosidosis [9] by gel-permeation chromatography on Bio-Gel P-2 and repeated HPLC on Partisil 10 SAX. Manβ1-4GlcNAcβ1-4GlcNAc, isolated from the kidneys of β-mannosidosis goats [10], was a gift of Dr M.Z. Jones (Department of Pathology, Michigan State University, East Lansing, MI, USA). The purities of the di- and trisaccharide were thoroughly checked by HPLC and 500 MHz ^{1}H-NMR spectroscopy. Rat liver CMP-NeuAc: β-D-Gal-(1-4)-β-D-GlcNAc α-(2-6)-*N*-acetylneuraminyl transferase (EC 2.4.99.1) was obtained from Boehringer Mannheim (activity: 8 U/mg protein), and CMP-NeuAc (sodium salt) from Sigma.

2.2. *Incubations*

Manβ1-4GlcNAc (0.8 mg) and Manβ1-4GlcNAcβ1-4GlcNAc (1.0 mg) were each dissolved in 1.0 ml of 0.2 M Tris-maleate buffer, pH 6.7/glycerol (4:1, v/v), containing 1.0 mg bovine serum albumin, and 2.5 mg CMP-NeuAc was added. Each mixture was incubated with 50 mU (25 μl) α2-6-sialyltransferase at 37°C. To follow the course of reaction, aliquots (20 μl) of the Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4GlcNAc incubations were taken for HPLC-analysis at t = 0, 1, 2, 4, 6, 24 h and at t = 0, 1, 3, 6, 24 h, respectively. The incubations were stopped after 24 h by cooling to 0°C, and the various components were fractionated preparatively by HPLC. The HPLC-subfractions were desalted on a Bio-Gel P-2 column (50 × 1 cm, Bio-Rad) using water as eluent.

2.3. *Chromatographic procedures*

HPLC-analysis was performed isocratically on a Partisil 10 SAX column (250 × 4.6 mm, Whatman) using acetonitrile/30 mM KH_2PO_4, pH 4.9, as elution system (75:25, v/v, in case of Manβ1-4GlcNAc incubation and 70:30, v/v, in case of Manβ1-4GlcNAcβ1-4GlcNAc incubation) at a flow rate of 2.0 ml/min. The LKB 2150-HPLC system was connected to a diode array detector (Rapid Spectral Detector 2140, LKB) and an Olivetti M240 computer.

TLC was performed on Merck DC Alufolien and Plastikfolien Kieselgel 60 (0.2 mm, 10 × 10 cm) eluted two times with 1-propanol/acetic acid/water (85:1:15, v/v). The TLC plates were stained with orcinol/H_2SO_4 to detect hexose-

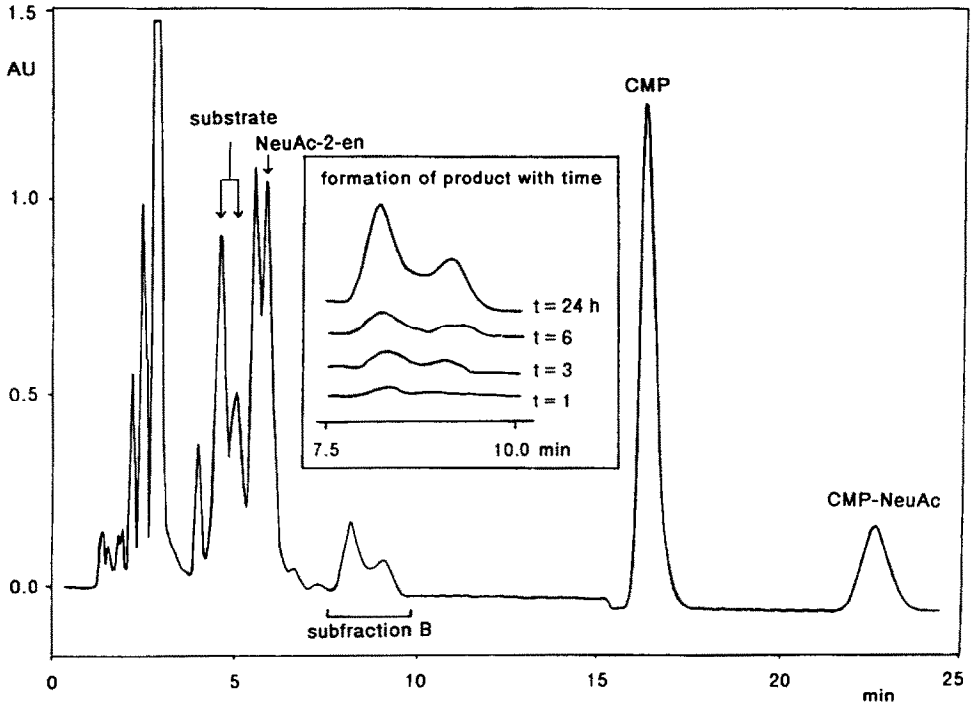

Fig.1. HPLC profiles of the Manβ1-4GlcNAcβ1-4GlcNAc incubation mixture on a Partisil 10 SAX column, eluted with acetonitrile/30 mM KH_2PO_4, pH 4.9 (70:30, v/v). The insert shows the formation of product with time (t = 1, 3, 6 and 24 h) and the complete profile represents the preparative HPLC profile obtained after 24 h. Subfraction **B** was collected as indicated. Due to anomerization, substrate as well as subfraction **B** occur as two peaks.

Table 1

Monosaccharide analysis data of the HPLC-subfractions **A** and **B**, representing the products of Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4GlcNAc incubations, respectively

Monosaccharide	A	B
Man[a]	1.0	1.0
GlcNAc	1.0	2.0
NeuAc	0.9	1.1
Amount (nmol)[b]	84	470
Yield (%)[c]	4	27

[a] The molar ratios are relative to Man = 1.00
[b] Amount indicates the total amount of Man in the subfractions
[c] Yield indicates the amount of Man present in the subfractions relative to the amounts of substrates used

containing compounds and with orcinol/$FeCl_3$/HCl to visualize sialic acid-containing compounds [11].

Monosaccharide analysis was performed by GLC. The trimethylsilylated methyl glycosides were analyzed on a capillary CP-Sil 5 WCOT fused silica column (25 × 0.32 mm, 0.11 μm film thickness, Chrompack) after methanolysis, *N*-reacetylation and trimethylsilylation [12].

2.4. *500 MHz ^{1}H-NMR spectroscopy*

500 MHz ^{1}H-NMR spectroscopy was carried out on a Bruker AM-500 spectrometer (Department of Chemistry, Utrecht University) at probe temperatures of 27, 32 and 37°C. Before recording ^{1}H-NMR spectra in ^2H_2O, the samples were repeatedly exchanged in ^2H_2O (99.96 atom% ^{2}H, Aldrich) with intermediate lyophilization [13]. Chemical shifts (δ) are given relative to internal acetone (δ = 2.225 ppm).

3. RESULTS

Incubation of Manβ1-4GlcNAc with CMP-NeuAc in the presence of rat liver α2-6-sialyltransferase gave rise to a small decrease of the amount of acceptor with time, as observed by HPLC. Due to the complexity of the HPLC elution profile, product formation was not directly deducible. On the other hand, for the Manβ1-4GlcNAcβ1-4GlcNAc incubation, the

Table 2

^{1}H-Chemical shift values of structural reporter group protons of the constituent monosaccharides of the HPLC-subfractions **A** and **B**, together with those of reference compounds Manβ1-4GlcNAc [15] and Manβ1-4GlcNAcβ1-4GlcNAc

Residue	Reporter group	Chemical shifts in[a]			
		Manβ1-4GlcNAc	**A**	Manβ1-4GlcNAc-β1-4GlcNAc	**B**
GlcNAc-**1**[b]	H-1α	–	–	5.189	5.195
	H-1β	–	–	4.696	4.696
	NAc	–	–	2.039	2.039/38[c]
GlcNAc-2	H-1α	5.211	5.207	–	–
	H-1β	4.724	4.736	4.610/01[c]	4.628/19[c]
	NAc	2.044	2.062	2.064	2.087/86[c]
Man	H-1	4.769	4.751	4.764	4.747
	H-2	4.071/61[d]	4.067/60[d]	4.060	4.059
	H-5	3.438	3.509	3.420	3.507
NeuAc	H-3a	–	1.724/20[d]	–	1.714
	H-3e	–	2.697	–	2.694
	NAc	–	2.033	–	2.033

[a] Chemical shifts (δ) are given for neutral solutions at 27°C (**B**), 32°C (Manβ1-4GlcNAcβ1-4GlcNAc) or 37°C (**A** and Manβ1-4GlcNAc), in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate in ^2H_2O acquired at 500 MHz, but were actually measured by reference to internal acetone (δ 2.225 ppm)
[b] GlcNAc-**1** is the GlcNAc residue at the reducing terminus of Manβ1-4GlcNAcβ1-4GlcNAc
[c] Two signals were observed due to anomerization of GlcNAc-**1** H-1
[d] Two signals were observed due to anomerization of GlcNAc-**2** H-1

HPLC profiles at t = 0, 1, 3, 6 and 24 h clearly showed the decrease of substrate and the formation of product with time (fig.1). In both incubations the amount of CMP-NeuAc gradually decreased until almost zero after 24 h, which is mainly caused by non-enzymatic cleavage of CMP-NeuAc into CMP and NeuAc-2-en [14]. A similar decrease of CMP-NeuAc was also observed in control experiments. After 24 h both incubation mixtures were separated by preparative HPLC into subfractions (fig.1), which were checked for the presence of NeuAc-containing oligosaccharides by monosaccharide analyses. Each incubation gave one NeuAc-positive HPLC-subfraction, denoted **A** and **B**, respectively. Monosaccharide analysis of **A** and **B** (table 1) revealed the presence of NeuAc, Man and GlcNAc, in molar ratios of 0.9:1.0:1.0 and 1.1:1.0:2.0, respectively. TLC of subfraction **A** showed a sialic acid/hexose-containing band, which migrated to the same position as NeuAcα2-6Manβ1-4GlcNAc, isolated from human β-mannosidosis urine. The compound pre-

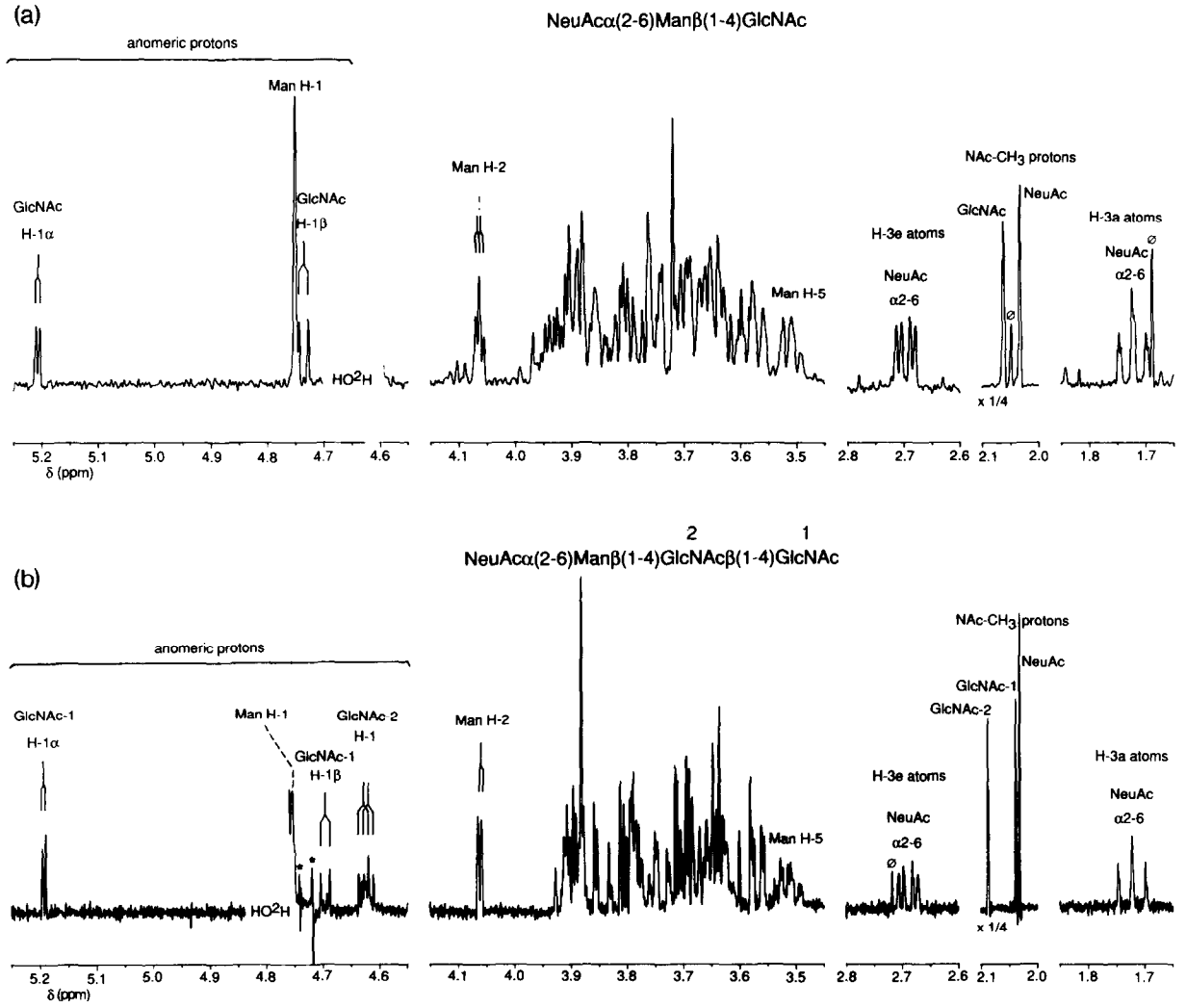

Fig.2. 500 MHz ^{1}H-NMR spectra of subfractions **A** (a) and **B** (b), obtained by incubation of Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4GlcNAc with Galβ1-4GlcNAc α2-6-sialyltransferase, recorded in ^2H_2O at 37°C and 27°C, respectively. The relative intensity scale of the *N*-acetyl regions differ from that of the other parts of the spectra as indicated. Spinning side bands are indicated by * and impurities by ∅.

sent in subfraction **B** showed a sialic acid/hexose-containing band below that of Manβ1-4GlcNAc-β1-4GlcNAc on TLC.

Subfractions **A** and **B** were subjected to 500-MHz ^{1}H-NMR spectroscopy. The chemical shift data of the structural-reporter groups are given in table 2, together with those of Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4GlcNAc. The NMR spectrum of **A** (fig.2a) was identical to that of NeuAcα2-6Manβ1-4GlcNAc, isolated from human β-mannosidosis urine. The α2-6-linked NeuAc residue in **A** is characterized by the acetamido, H-3a and H-3e signals at δ 2.033, 1.724/20 and 2.697, respectively. As compared to Manβ1-4GlcNAc [15], the attachment of NeuAc at C-6 of Man gives rise to significant chemical shift alterations for Man H-1 ($\Delta\delta$ −0.018) and Man H-5 ($\Delta\delta$ +0.071) and for the NAc signal of GlcNAc ($\Delta\delta$ +0.018). Based on the ^{1}H-NMR data, the monosaccharide analysis and the TLC behaviour, the structure of the compound in subfraction **A** was proved to be NeuAcα2-6Manβ1-4GlcNAc. This compound was synthesized in a yield of 4%.

The ^{1}H-NMR spectrum of subfraction **B** (fig.2b) revealed a similar set of NeuAc values as observed for NeuAc in subfraction **A** (acetamido, δ 2.033; H-3a, δ 1.714; H-3e, δ 2.694). Comparison of the ^{1}H-NMR data of **A** and **B** showed the same shift effects for Man H-1 ($\Delta\delta$ −0.017), Man H-5 ($\Delta\delta$ +0.087) and the acetamido group of GlcNAc-**2** ($\Delta\delta$ +0.023/0.022). The remaining structural reporter groups were hardly influenced, except GlcNAc-**2** H-1, which shifted downfield ($\Delta\delta$ +0.018). Based on the ^{1}H-NMR data and the monosaccharide analysis, the structure of the compound in subfraction **B** was proved to be NeuAcα2-6Manβ1-4GlcNAcβ1-4GlcNAc. The novel compound was formed in a yield of 27%.

4. DISCUSSION

This paper gives detailed information on the in vitro biosynthesis of NeuAcα2-6Manβ1-4GlcNAc and NeuAcα2-6Manβ1-4GlcNAcβ1-4GlcNAc from Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4GlcNAc, respectively, using CMP-NeuAc as glycosyl donor in the presence of rat liver α2-6-sialyltransferase. Up to now the latter enzyme has shown to be highly specific for the sequence Galβ1-4GlcNAc as part of *N*-linked carbohydrate chains of glycoproteins and related oligosaccharides, and adds NeuAc exclusively to the 6-position of galactose. Typical relative rates are: Galβ1-4GlcNAc = 1.00 (K_m = 1.62 mM) and Galβ1-4Glc = 0.01 (K_m = 129 mM), while Galβ1-6GlcNAc and Galβ1-3GlcNAc showed no reaction. The α2-6-sialyltransferase does not recognize Galβ1-6GalNAc, Galβ1-3GalNAc, GlcNAc-R (R = oligosaccharide), Galβ1-3GalNAc-Ser/Thr, GalNAc-Ser/Thr or other carbohydrates which are *O*-linked to glycoproteins, as substrates [16–18]. However, our experiments show that Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4GlcNAc can also serve as substrates, indicating a broader specificity for the enzyme, or less probable another sialyltransferase with this new substrate specificity is present in the commercial enzyme preparation.

This finding explains the accumulation of NeuAcα2-6Manβ1-4GlcNAc in addition to the major storage product Manβ1-4GlcNAc in human β-mannosidosis. The presence of NeuAc α2-6-linked to a mannose residue instead of a galactose residue suggests that the axial/equatorial position of the hydroxyl groups at C-2 and C-4 of the terminal sugar are of minor importance for the effectivity of the α2-6-sialyltransferase reaction. However, the presence of a -β1-4GlcNAc moiety seems to be preferable for α2-6-sialyltransferase action.

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