Transfer of Synthetic Sialic Acid Analogues to N- and O-Linked Glycoprotein Glycans Using Four Different Mammalian Sialyltransferases†

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ABSTRACT: This paper presents kinetic properties of the transfer of several synthetic 9-substituted sialic acid analogues onto N- or O-linked glycoprotein glycans by four purified mammalian sialyltransferases: Galβ1,4GlcNAc α2,6sialyltransferase, Galβ1,4(3)GlcNAc α2,3sialyltransferase, Galβ1,3GalNAc α2,3sialyltransferase, and GalNAc α2,6sialyltransferase. The substituents at C-9 of the sialic acid analogues introduce special biochemical characteristics: 9-Amino-NeuAc represents, up to the present, the first derivative that is resistant toward bacterial, viral, and mammalian sialidases but is transferred by a sialyltransferase. 9-Acetamido-NeuAc, 9-benzamido-NeuAc, and 9-hexanoylamido-NeuAc differ in size and hydrophobic character from each other and from parent NeuAc. 9-Azido-NeuAc may be used to introduce a photoreactive label. The kinetic properties of the four sialyltransferases with regard to the donor CMP-glycosides differed distinctly depending on the structure of the substituent at C-9. CMP-9-amino-NeuAc was only accepted as donor substrate by Galβ1,4GlcNAc α2,6sialyltransferase (rat liver), but the K_m value was 14-fold higher than that of parent CMP-NeuAc. In contrast, 9-azido-NeuAc was readily transferred by each of these four enzymes. 9-Acetamido-NeuAc, which is a receptor analogue for influenza C virus, 9-benzamido-NeuAc, and 9-hexanoylamido-NeuAc were also accepted by each sialyltransferase, but incorporation values differed significantly depending on the enzyme used. For the first time, the resialylation of asialo-α_1-acid glycoprotein with 9-substituted sialic acid analogues by Galβ1,4GlcNAc α2,6sialyltransferase is demonstrated.

Sialyltransferases are responsible for the transfer of sialic acids from the respective CMP-glycoside onto glycoproteins or glycolipids (Corfield & Schauer, 1982). Up to the present four mammalian sialyltransferases with different acceptor and glycosidic linkage specificities have been purified from mammalian tissue (Paulson et al., 1977; Sadler et al., 1979a,b; Weinstein et al., 1982a; Miyagi & Tsuiki, 1982; Hesford et al., 1984; Joziasse et al., 1985; Sticher et al., 1988; Higa & Paulson, 1985).

Pure sialyltransferases have proven to be a useful tool for in vitro sialylation of soluble or membrane-bound glycoconjugates (Higa & Paulson, 1985; Paulson et al., 1978, 1984; Weinstein et al., 1982b; Kelm et al., 1986; Rogers et al., 1986; Powell et al., 1987; Markwell & Paulson, 1980; Paulson & Rogers, 1987), a method applied especially for studies to elucidate the structure of sialoglycoconjugate receptors. We have previously demonstrated the feasibility of the transfer of synthetic sialic acid analogues modified at C-9 and C-4 employing Galβ1,4GlcNAc α2,6sialyltransferase[1] from rat liver (Gross et al., 1987; Gross & Brossmer, 1987). In order to replace natural sialic acids by synthetic analogues with special biochemical properties, detailed kinetic data of sialyltransferases differing in acceptor specificity for the CMP-activated sialic acid analogues are a prerequisite. From these data optimal conditions for transfer of such derivatives onto various glycan acceptors can be deduced.

In this paper kinetic properties of four sialyltransferases for several CMP-activated sialic acid analogues modified at C-9 are compared: Galβ1,4GlcNAc α2,6sialyltransferase and Galβ1,4(3)GlcNAc α2,3sialyltransferase purified from rat liver (Weinstein et al., 1982a), which transfer sialic acid to terminal positions on N-linked glycans, and Galβ1,3GalNAc α2,3sialyltransferase and GalNAc α2,6sialyltransferase purified from porcine submaxillary glands (Sadler et al., 1979, 1979b), which transfer sialic acid to the core region of O-linked glycans.

All four sialyltransferases were capable of utilizing various CMP-sialic acid analogues modified at C-9 of the sialic acid molecule. Thus, within the specificity limitations of the individual sialyltransferases, the results demonstrate the general applicability of enzymatic synthesis in producing glycoconjugates containing synthetic NeuAc[2] analogues. It is, therefore, possible to introduce these analogues in a variety of carbohydrate sequences found in glycoproteins and glyco-

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[1] Enzymes: Galβ1,4GlcNAc α2,6sialyltransferase, EC 2.4.99.1; Galβ1,4(3)GlcNAc α2,3sialyltransferase, EC 2.4.99.5; GalNAc α2,6sialyltransferase, EC 2.4.99.3; Galβ1,3GalNAc α2,3sialyltransferase, EC 2.4.99.4.

[2] Abbreviations: HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; NeuAc, *N*-acetyl-β-D-neuraminic acid; 9-amino-NeuAc, 5-acetamido-9-amino-3,5,9-trideoxy-D-glycero-β-D-galacto-2-nonulosonic acid; 9-acetamido-NeuAc, 5,9-diacetamido-3,5,9-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulosonic acid; 9-benzamido-NeuAc, 5-acetamido-9-benzamido-3,5,9-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulosonic acid; 9-hexanoylamido-NeuAc, 5-acetamido-9-hexanoylamido-3,5,9-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulosonic acid; 9-azido-NeuAc, 5-acetamido-9-azido-3,5,9-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulosonic acid; CMP-NeuAc, cytidine 5′-(*N*-acetyl-β-D-neuraminic acid phosphate); ST, sialyltransferase.

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lipids according to the acceptor specificity of the transferases. However, some remarkable differences exist between the various enzymes concerning substitutions at C-9 of NeuAc, and not all derivatives are transferred equally by the enzymes studied.

Materials and Methods

Materials

All chemicals used were of analytical grade and purchased from Merck (Darmstadt, FRG) or Serva (Heidelberg, FRG). Crystalline *N*-acetylneuraminic acid was prepared in this laboratory according to Czarniecki and Thornton (1977). Cytidine 5′-monophosphate (CMP) was obtained from Boehringer (Mannheim), cytidine 5′-triphosphate was from Biomol (Ilvesheim, FRG), and bovine serum albumin, Triton CF-54, and Triton X-100 were from Sigma (München, FRG). Both grade E and grade S acetonitriles were from Zinsser (Frankfurt, FRG). Cytidine 5′-(*N*-acetylneuraminic acid phosphate) (CMP-NeuAc) and all other CMP-NeuAc analogues were prepared enzymatically as described previously (Gross et al., 1987) and contained less than 4% CMP, in the case of CMP-9-amino-NeuAc less than 1.5%. Galβ1,4GlcNAc α2,6-ST (2.95 units/mL) and Galβ1,4(3)-GlcNAc α2,3-ST (0.104 unit/mL) (both rat liver) and GalNAc α2,6-ST (4 units/mL) and Galβ1,3GalNAc α2,3-ST (57 milliunits/mL) (both porcine submaxillary glands) were purified according to published methods (Sadler et al., 1979a,b; Weinstein et al., 1982a).

α_1-Acid glycoprotein was prepared in Dr. K. Schmid's laboratory (Schmid, 1975) and antifreeze glycoprotein (fractions 3–5, from serum of *Pagothenia borchgrevinki*) in Dr. R. Feeney's laboratory (DeVries et al., 1970). Fetuin was obtained from Serva (Heidelberg, FRG) and further purified on Sepharose 6B. Isoelectric focusing was performed by U. Merkel and Dr. W. E. Merz in this institute (Merkel & Merz, 1984).

Methods

Protein Determination. The Bio-Rad protein assay was applied with bovine serum albumin as standard.

Desialylation of Sialoglycoproteins. After treatment with *Vibrio cholerae* sialidase as described (Gross & Brossmer, 1988a) asialo-α_1-acid glycoprotein or asialofetuin contained approximately 0.2% bound NeuAc.

Galactose Acceptor Sites. Sites were expressed in terms of galactose content of asialo-α_1-acid glycoprotein, antifreeze glycoprotein, and asialofetuin which was determined either after acid hydrolysis (1 N HCl, 100 °C, 2 and 4 h) with the galactose dehydrogenase assay (Wallenfels & Kurz, 1966) or after enzymatic release with β-galactosidase from bovine testis (100 milliunits, 20 h at 37 °C).

Analytical HPLC System (Gross et al., 1987). CMP and CMP-glycosides were measured at 275 nm on an aminopropyl phase column (0.4 cm × 12.5 cm, Serva) (Gross et al., 1987). NeuAc and 9-amino-NeuAc released from the glycoprotein were identified at 200 nm and quantified with respect to corresponding external standards (Gross et al., 1987) on a Spherisorb NH_2 column (0.4 cm × 25 cm, Zinsser).

Transfer Assays. Sialyltransferase assays were carried out essentially as described (Higa & Paulson, 1985; Gross et al., 1987).

Kinetic Assays. Assay 1. The reaction mixture (200 μL) for Galβ1,4GlcNAc α2,6-ST and Galβ1,4(3)GlcNAc α2,3-ST contained 12.5 μmol of sodium cacodylate buffer, pH 6.0, 200 μg of BSA, 1.0 mg of Triton CF-54, 0.31 mg of asialo-α_1-acid glycoprotein (130 nmol of galactose acceptor sites), and different concentrations of CMP-glycosides (30 μM to 5 mM). The concentration of asialo-α_1-acid glycoprotein in terms of galactose acceptor sites was 4-fold higher than the K_m value of both rat liver ST (Weinstein et al., 1982b).

Assay 2. The reaction mixture (100 μL) for GalNAc α2,6-ST and Galβ1,3GalNAc α2,3-ST contained 6.25 μmol of sodium cacodylate, pH 6.5, 200 μg of BSA, 0.1 mg of Triton X-100, 0.09 mg of antifreeze glycoprotein (105 nmol of galactose acceptor sites), and different concentrations of CMP-glycosides (200 μM to 5 mM). In the case of GalNAc α2,6-ST, assay 2 was also performed with 1.0 mg of asialofetuin (166 nmol of galactose sites) as acceptor. The concentration of antifreeze glycoprotein in terms of galactose acceptor sites represented the K_m value of porcine α2,6-ST or was 2-fold higher than the K_m value of porcine α2,3-ST (Sadler et al., 1979b; Rearick et al., 1979). The concentration of the O-glycosidically bound galactose acceptor sites of asialofetuin was 0.4-fold compared to the K_m value of GalNAc α2,6-ST for antifreeze glycoprotein. According to the published carbohydrate structure of antifreeze glycoprotein, an equal molar ratio of galactose/*N*-acetylgalactosamine was assumed (Feeney et al., 1986).

The transfer reaction was started by addition of the respective sialyltransferase and terminated after appropriate times at 37 °C by addition of 1.3 mL of cold 1% phosphotungstic acid in 0.5 N HCl. The assays were processed further as described earlier (Gross et al., 1987) except that for assay 2 the final extraction of the sediment with $CHCl_3$/MeOH was omitted. Finally, in each case the sediment was dissolved in 100 μL of 0.2 M NaCl.

Concomitantly, assay 1 containing in addition 0.3 mg of native α_1-acid glycoprotein, but no asialoglycoprotein, CMP-glycoside and sialyltransferase, was performed as described above to calculate glycoprotein recovery. Similarly, a modified assay 2 was carried out with 0.1 mg of antifreeze glycoprotein sialylated by GalNAc α2,6sialyltransferase (NeuAc content 48 nmol/mg). Finally, the amount of NeuAc released from the respective glycoproteins by acid hydrolysis (0.1 N HCl, 60 min, 80 °C) was compared to the theoretical NeuAc content. Glycoprotein recovery in transfer assays 1 and 2 was 95% and 83%, respectively.

Transferred NeuAc or NeuAc analogues were released from the glycoprotein by acid hydrolysis (see above) and quantitated by the thiobarbituric acid procedure (external standards included). The extent of degradation during acid hydrolysis was as follows: NeuAc, 10%; 9-amino-NeuAc, 10%; 9-azido-NeuAc, 10%; 9-benzamido-NeuAc, 15%; 9-hexanoylamido-NeuAc, 18%; 9-acetamido-NeuAc, 20%.

Initial Rate Assays. Initial rates were determined in duplicate at 1.0, 2.0, or 5.0 mM concentrations of the respective CMP-glycosides. Assay 1 was performed for 30 min with 0.35 milliunit of Galβ1,4GlcNAc α2,6-ST or for 45 min with 0.25 milliunit of Galβ1,4(3)GlcNAc α2,3-ST. Assay 2 was carried out for 30 min with 2.8 milliunits of GalNAc α2,6-ST or for 45 min with 0.25 milliunit of Galβ1,3GalNAc α2,3-ST. Consumption of either substrate was always less than 15%; in the case of CMP-9-amino-NeuAc it was only 3%.

For kinetic measurements initial rates were determined in duplicate at five concentrations of the respective CMP-glycoside near the respective K_m value. Assay 1 was performed for 45 min with approximately 0.07 milliunit of Galβ1,4GlcNAc α2,6-ST or up to 0.4 milliunit of Galβ1,4-(3)GlcNAc α2,3-ST; assays with CMP-9-amino-NeuAc contained about 0.3 milliunit of Galβ1,4GlcNAc α2,6-ST. To ensure that transfer rates were linear with incubation time,

Table I: Initial Transfer Rates of Rat Liver Galβ1,4GlcNAc α2,6Sialyltransferase and Galβ1,4(3)GlcNAc α2,3Sialyltransferase for Several CMP-NeuAc Analogues[a]

donor substrate	α2,6-ST initial rates (%)			α2,3-ST initial rates (%)	
	1 mM	2 mM	5 mM	1 mM	2 mM
CMP-NeuAc	100	99	100	100	100
CMP-9-amino-NeuAc	50	71	96	5	10
CMP-9-acetamido-NeuAc	100	112		53	53
CMP-9-benzamido-NeuAc	115	119		54	62
CMP-9-hexanoylamido-NeuAc	112	122		25	27
CMP-9-azido-NeuAc	139	148		133	147

[a] Assay 1 was performed in duplicate with asialo-α_1-acid glycoprotein as acceptor and 1, 2, or 5 mM CMP-glycoside as described under Materials and Methods. Initial transfer rates are expressed as a percentage with respect to the value obtained at 1 mM CMP-NeuAc (100% = 0.375 nmol/min for α2,6-ST; 100% = 0.11 nmol/min for α2,3-ST).

consumption of the donor substrates was restricted to be below 20%, whereas that of CMP-9-amino-NeuAc was kept below 7.5%. Further, the consumption of the acceptor substrate was always below 10%. Kinetic data were obtained from Hanes plots (Dixon & Webb, 1964) by linear regression analysis.

Resialylation Assays. For the studies of maximal resialylation, assay 1 was modified in order to favor complete saturation of the acceptor substrate: The reaction mixture (160 μL) contained 10 μmol of sodium cacodylate, pH 6.7, 0.1 mg of Triton CF-54, 1 mg of BSA, 0.022 mg of asialo-α_1-glycoprotein (9.5 nmol of galactose acceptor sites), and a 1.5 mM concentration of the respective CMP-glycoside. The reaction was started by addition of 3.5 milliunits of α2,6-ST (rat liver). Tubes were incubated for 17 h at 37 °C and assayed as described above.

Results

Initial Rates. Incorporation of 9-amino-, 9-acetamido-, 9-benzamido-, 9-hexanoylamido-, and 9-azido-NeuAc was compared to that of NeuAc by employing the following sialyltransferases with different acceptor specificity: Galβ1,4GlcNAc α2,6-ST and Galβ1,4(3)GlcNAc α2,3-ST (rat liver); GalNAc α2,6-ST and Galβ1,3GalNAc α2,3-ST (porcine submaxillary glands).

The acceptor substrate for each rat liver ST was asialo-α_1-acid glycoprotein with the terminal glycan sequence Galβ1,4GlcNAc, for each porcine submaxillary gland ST antifreeze glycoprotein with the glycan sequence Galβ1,3GalNAc (Sadler et al., 1979b; Higa & Paulson, 1985; Weinstein et al., 1982b; Rearick et al., 1979). Asialofetuin contains both of these terminal glycan structures.

Different donor substrate concentrations were used to estimate saturating concentrations for transfer of each NeuAc analogue. Initial rates of rat liver α2,6-ST for CMP-NeuAc analogues modified at C-9 showed comparable or even higher transfer as compared to that of parent CMP-NeuAc (Table I). In contrast, rat liver α2,3-ST yielded higher values, compared to CMP-NeuAc, only with CMP-9-azido-NeuAc and significantly lower rates with all other CMP-glycosides. There was no significant transfer with CMP-9-amino-NeuAc (Table I).

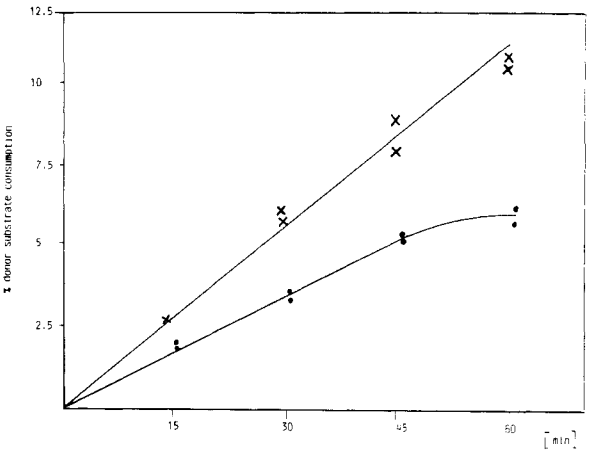

FIGURE 1: Time course for transfer of NeuAc (×) and 9-amino-NeuAc (●) onto asialo-α_1-acid glycoprotein by rat liver Galβ1,4GlcNAc α2,6sialyltransferase. Assay 1 was performed in duplicate at 1 mM CMP-glycoside concentration as described under Materials and Methods.

With porcine α2,3-ST, comparable transfer rates were obtained for CMP-NeuAc analogues and CMP-NeuAc (Table II). However, porcine α2,6-ST gave only low values for all 9-substituted CMP-NeuAc analogues except CMP-9-azido-NeuAc (Table II). Both porcine enzymes did not accept CMP-9-amino-NeuAc (Table II). The influence of the acceptor structure on the transfer of NeuAc analogues by GalNAc α2,6sialyltransferase was studied additionally with asialofetuin. Initial rates for the incorporation of all 9-N-acylated NeuAc analogues were about 5-fold higher (Table II), whereas 9-azido-NeuAc reached an identical transfer value as that obtained with antifreeze glycoprotein.

Increasing the concentration of each CMP-glycoside from 1 to 2 mM, initial rates determined with both rat liver ST were not significantly affected except in the case of CMP-9-amino-NeuAc (Table I).

GalNAc α2,6-ST did not require higher saturation concentration of the activated 9-substituted NeuAc analogues compared to CMP-NeuAc (Table II).

9-Amino-NeuAc is a sialidase-resistant NeuAc analogue (Gross & Brossmer, 1988a). Therefore, the time course for transfer of NeuAc and 9-amino-NeuAc by Galβ1,4GlcNAc α2,6-ST at 1 mM donor CMP-glycoside is compared in Figure

Table II: Initial Rates of Porcine Galβ1,3GalNAc α2,3Sialyltransferase and GalNAc α2,6Sialyltransferase for Several CMP-NeuAc Analogues[a]

donor substrate	α2,3-ST initial rates (%)		α2,6-ST initial rates (%)			
			1 mM		2 mM	
	1 mM	2 mM	AFG	AF	AFG	AF
CMP-NeuAc	100	88	80	80	100	100
CMP-9-amino-NeuAc	n	5	n	10	n	15
CMP-9-acetamido-NeuAc	78	101	10	40	15	50
CMP-9-benzamido-NeuAc	80	98	7	50	9	50
CMP-9-hexanoylamido-NeuAc	100	126	7	40	7	45
CMP-9-azido-NeuAc	84	109	150	145	165	160

[a] Assay 2 was performed with antifreeze glycoprotein (AFG) or asialofetuin (AF) as acceptor, and 1 or 2 mM CMP-glycoside as described under Materials and Methods. Transfer rates of α2,3-ST are expressed as a percentage with respect to the value obtained at 1 mM CMP-NeuAc (100% = 0.09 nmol/min) and transfer rates of α2,6-ST as a percentage with respect to the value at 2 mM CMP-NeuAc (100% = 0.33 nmol/min with antifreeze glycoprotein; 100% = 0.22 nmol/min with asialofetuin). n = negligible.

Table III: Apparent Kinetic Constants of Rat Liver Galβ1,4GlcNAc α2,6Sialyltransferase and Galβ1,4(3)GlcNAc α2,3Sialyltransferase for Several CMP-NeuAc Analogues[a]

donor substrate	α2,6-ST			α2,3-ST		
	K_m (μM)	rel V_{max}	K_m/V_{max} (1/mM)	K_m (μM)	rel V_{max}	K_m/V_{max} (1/mM)
CMP-NeuAc	50	1.0	20.00	70	1.0	14.30
CMP-9-amino-NeuAc	720	0.9	1.25			
CMP-9-acetamido-NeuAc	120	1.1	9.15	245	0.6	2.45
CMP-9-benzamido-NeuAc	30	1.1	36.65	220	0.6	2.75

[a] Kinetic measurements were performed at five concentrations of CMP-glycoside with assay 1 as described under Materials and Methods. V_{max} values are expressed relative to that determined for CMP-NeuAc (=1.0).

1. Incorporation of NeuAc was linear with time for at least 60 min; the corresponding donor substrate consumption was 11%. Incorporation of 9-amino-NeuAc was linear only up to 45 min, though the corresponding donor substrate consumption did not exceed 6%. This result was obviously due to an inhibition of 9-amino-NeuAc transfer by CMP, which is known as a sialyltransferase inhibitor (Beyer et al., 1981). To prove this assumption, the incorporation rate at 1 mM CMP-NeuAc or CMP-9-amino-NeuAc was determined in the presence and in the absence of 100 μM CMP: Whereas NeuAc transfer was not significantly influenced, incorporation of 9-amino-NeuAc was inhibited by 40%.

Kinetic Data. Kinetic parameters of the rat liver sialyltransferases were measured for three CMP-NeuAc analogues (Table III). Applying kinetic assay 1 described under Materials and Methods, incorporation of NeuAc by both enzymes was linear with time for 60 min at 40 μM CMP-NeuAc (donor substrate consumption up to 30%), which represented the lowest concentration used in the kinetic assays. Transfer by α2,6-ST at the lowest concentration of CMP-9-amino-NeuAc (300 μM) was linear with time for 45 min (donor substrate consumption below 7.5%).

K_m values of rat liver α2,6- and α2,3-ST determined for CMP-9-acetamido-NeuAc and CMP-9-benzamido-NeuAc ranged from 0.6-fold to 3.5-fold the value obtained with CMP-NeuAc (Table III). α2,6-ST was the only enzyme accepting CMP-9-amino-NeuAc as donor substrate, the K_m value being 14-fold higher compared to that of CMP-NeuAc (Table III). Thus, CMP arising from both enzymatic reaction and degradation competitively inhibits the transfer of 9-amino-NeuAc to a higher degree than that of NeuAc. V_{max} values of α2,6-ST for CMP-NeuAc and each CMP-NeuAc analogue were of comparable magnitude; V_{max} values of α2,3-ST for CMP-9-acetamido- and CMP-9-benzamido-NeuAc were about 50% compared to that of CMP-NeuAc (Table III).

Resialylation. In order to determine maximal incorporation of different NeuAc analogues, the transfer assay was modified as described under Materials and Methods. To enhance stability of the CMP-glycosides, the pH value was raised to 6.7 and the amount of acceptor glycoprotein reduced, yielding a 24-fold excess (nmol/nmol) of CMP-glycoside over the galactose acceptor sites. A high amount of enzyme was used compared to that in the initial rate assay, and the incubation time was extended. The results obtained with α2,6-ST (rat liver) are shown in Table IV. The saturation of galactose sites with NeuAc, 9-acetamido-NeuAc, and 9-hexanoylamido-NeuAc was identical (about 85%); 9-azido- and 9-benzamido-NeuAc were incorporated even to a higher extent, whereas 9-amino-NeuAc yielded an about 15% lower value compared to that of NeuAc. A higher excess of donor CMP-glycoside (48 nmol/nmol of Gal site) did not further improve resialylation (data not shown).

Resialylation of asialo-α_1-acid glycoprotein with NeuAc, 9-acetamido-NeuAc, or 9-amino-NeuAc (Table IV) was also studied by isoelectric focusing, which allows the qualitative sialylation pattern to be monitored (Figure 2). For comparison asialo-α_1-acid and native α_1-acid glycoproteins were included in the latter experiment. The sialylation pattern observed with NeuAc or 9-acetamido-NeuAc was very similar to that of the native glycoprotein with respect to distribution and relative intensity of the bands. As expected, transfer of 9-amino-NeuAc, being a zwitterion, yielded an band pattern identical with that of the asialo glycoprotein (Figure 2).

Table IV: Maximal Resialylation of Asialo-α_1-acid Glycoprotein by Rat Liver Galβ1,4GlcNAc α2,6Sialyltransferase[a]

donor substrate	resialylation (% Gal sites)
CMP-NeuAc	83
CMP-9-amino-NeuAc	66
CMP-9-acetamido-NeuAc	82
CMP-9-benzamido-NeuAc	95
CMP-9-hexanoylamido-NeuAc	84
CMP-9-azido-NeuAc	91

[a] Resialylation assay (160 μL) was performed in duplicate at pH 6.7 and at 1.5 mM donor substrate concentration and incubated for 17 h at 37 °C as described under Materials and Methods. The resialylation is given as a percentage of the total galactose acceptor sites and represents the average of three resialylation experiments.

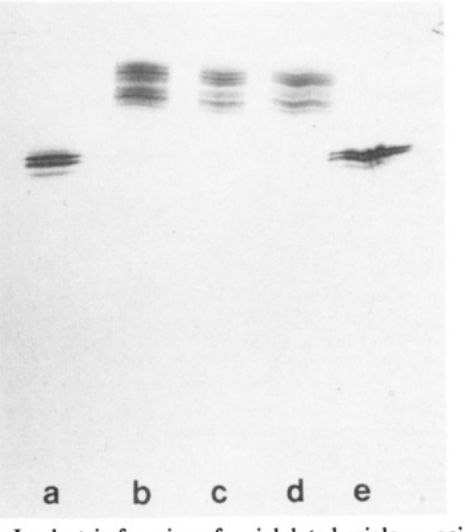

FIGURE 2: Isoelectric focusing of resialylated asialo-α_1-acid glycoprotein with NeuAc, 9-acetamido-NeuAc, and 9-amino-NeuAc by rat liver Galβ1,4GlcNAc α2,6sialyltransferase. After resialylation for 17 h at 37 °C, aliquots (1.5–2 μL) were withdrawn from the reaction mixture and directly applied to IEF. Transfer values determined by the thiobarbituric acid method are as shown in Table IV. (a) Asialo-α_1-acid glycoprotein; (b) native α_1-acid glycoprotein; (c) resialylation with NeuAc; (d) resialylation with 9-acetamido-NeuAc; (e) resialylation with 9-amino-NeuAc.

Discussion

To complete their biosynthesis, many soluble or membrane bound glycoconjugates require a final sialylation step, which is catalyzed by distinct sialyltransferases differing in specificity

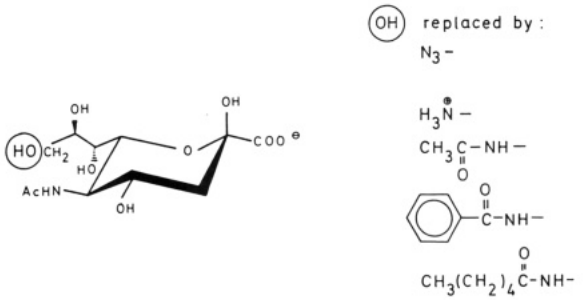

FIGURE 3: Structure formulas of NeuAc analogues modified at C-9. The hydroxy group at C-9 was replaced by amino, acetamido, benzamido, hexanoylamido, and azido groups.

with respect to the glycan acceptor structure and the linkage formed. Important biological functions of glycoconjugates depend on the degree of terminal sialylation (Reutter et al., 1982). In vitro transfer of naturally occurring sialic acids has been used, for example, to characterize the influenza virus receptor of several host cells and the receptor for macrophage binding to erythrocytes (Paulson et al., 1984; Kelm et al., 1986; Rogers et al., 1986). Replacement of naturally occurring sialic acids by synthetic analogues endowed with special properties can serve to influence certain biological functions of soluble or membrane-bound sialoglycoconjugates. In particular, those NeuAc analogues that deserve special interest are sialidase or *O*-acetyl-esterase resistant, are radiolabeled, fluorescently labeled, or photoreactively labeled, or differ in charge or hydrophobicity from the parent sialic acid.

Previous studies have demonstrated the feasibility of transferring synthetic sialic acid analogues onto asialoglycoprotein with Galβ1,4GlcNAc α2,6sialyltransferase from rat liver (Gross et al., 1987; Gross & Brossmer, 1987; Conradt et al., 1984; Beau & Schauer, 1980). In order to deduce optimal conditions for in vitro resialylation with sialic acid analogues, kinetic studies on the substrate properties of sialyltransferases specific for certain oligosaccharide structures are an essential prerequisite.

The surprising differences among four sialyltransferases toward substitution at C-9 of NeuAc, presented in this paper, demonstrate that synthetic sialic acid analogues are useful tools to study the donor substrate specificity of sialyltransferases. Data on the kinetic properties of each enzyme for differently substituted NeuAc analogues allow suitable derivatives to be chosen for a selective resialylation of N- or O-linked glycoconjugate glycans and additional analogues to be devised for special biological applications.

The chemical structures of the 9-substituted sialic acids which have been transferred by four sialyltransferases purified from rat liver and porcine submaxillary glands are shown in Figure 3. The initial transfer rates of each CMP-NeuAc analogue at saturating concentration are summarized in Figure 4. Owing to the limited availability of pure sialyltransferases, a complete kinetic study was restricted to the rat liver enzymes and three CMP-NeuAc analogues showing promising biological properties.

Transfer of 9-amino-NeuAc deserves special interest as it is up to now the only sialic acid analogue that resists cleavage by either bacterial, viral, or mammalian sialidases. The cleavage values determined for transferred 9-amino-NeuAc by application of high excess of *Vibrio cholerae*, fowl plague virus, *Clostridium perfringens*, and bovine testis sialidase were below 8% in each case, whereas transferred NeuAc was released by 80–95% under identical conditions (Gross & Brossmer, 1988a). The positive charge in the molecule (p*K* of the amino group 9.7) apparently causes specific changes

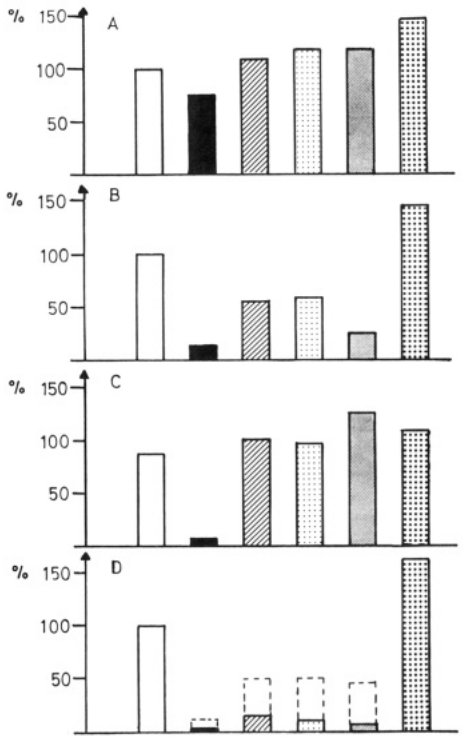

FIGURE 4: Initial rates of four sialyltransferases for CMP-NeuAc analogues modified at C-9 of the NeuAc moiety at 2 mM donor substrate concentration (as given in Tables I and II). (A) Galβ1,4GlcNAc α2,6-ST/asialo-α_1-acid glycoprotein; (B) Galβ1,4-(3)GlcNAc α2,3-ST/asialo-α_1-acid glycoprotein; (C) Galβ1,3GalNAc α2,3-ST/antifreeze glycoprotein; (D) GalNAc α2,6-ST/antifreeze glycoprotein (solid line) and GalNAc α2,6-ST/asialofetuin (hatched line). (Identical initial rates were obtained for CMP-9-azido-NeuAc with antifreeze glycoprotein and asialofetuin.) From left to right: NeuAc; 9-amino-NeuAc; 9-acetamido-NeuAc; 9-benzamido-NeuAc; 9-hexanoylamido-NeuAc; 9-azido-NeuAc.

in enzyme–substrate interaction. It is noteworthy that only one of the four sialyltransferases studied, rat liver Galβ1,4GlcNAc α2,6sialyltransferase, could transfer this zwitterionic analogue (Figure 4). At saturation concentration, the reaction rate obtained was as high as that of NeuAc (Table III).

In contrast to the sialidase resistance of asialo-α_1-acid glycoprotein resialylated with 9-amino-NeuAc, this asialoglycoprotein sialylated with 9-acetamido-, 9-azido-, and 9-benzamido-NeuAc was well susceptible to sialidase cleavage. Excess of *V. cholerae* sialidase released about 85% of transferred NeuAc analogues except 9-benzamido-NeuAc, which was liberated by 68% (unpublished experiments).

Transfer of 9-azido-NeuAc might be useful to obtain glycoconjugates containing a photoreactive label. It was therefore gratifying that all transferases readily accepted CMP-9-azido-NeuAc as donor substrate, yielding significantly higher transfer rates with both rat liver enzymes and porcine submaxillary gland α2,6sialyltransferase compared to the values obtained with CMP-NeuAc (Figure 4).

A different behavior of the four enzymes was also observed toward three 9-N-acylated NeuAc analogues (Figure 4). The structure of 9-acetamido-NeuAc is very similar to that of 9-*O*-acetyl-NeuAc, the major cell surface receptor determinant for influenza C virus; but in contrast to 9-*O*-acetyl-NeuAc, 9-acetamido-NeuAc resists cleavage by the receptor destroying *O*-acetyl-esterase (Herrler et al., 1985; Imhof et al., 1988).

Both 9-benzamido-NeuAc and 9-hexanoylamido-NeuAc contain a large, hydrophobic substituent at the amino group. With these N-acylated derivatives, rat liver α2,6- and porcine

submaxillary gland α2,3sialyltransferase yielded transfer values comparable to that of parent NeuAc (Figure 4). In contrast, the transfer value of the rat liver α2,3sialyltransferase was reduced by about 50% (Figure 4). Surprisingly, rat liver α2,6sialyltransferase showed a 4-fold lower K_m value for CMP-9-benzamido-NeuAc as compared to that of CMP-9-acetamido-NeuAc, although the acetamido group is a much smaller substituent (Table III). Further studies involving additional analogues may enable a correlation between the hydrophobic nature of the substituent at C-9 and a special hydrophobic region near to the active site of the enzyme to be unraveled.

In contrast, porcine GalNAc α2,6transferase gave only very slow incorporation rates into antifreeze glycoprotein with all N-acylated NeuAc analogues studied (Figure 4). In the case of 9-benzamido- and 9-hexanoylamido-NeuAc, sterical hindrance during transfer might explain this observation, because the acceptor GalNAc residue is directly linked to the native protein backbone which contains a large number of *O*-glycans. Compared to antifreeze glycoprotein, the initial transfer rate of all 9-N-acylated NeuAc analogues increased strongly when asialofetuin was used as acceptor (Figure 4).

Investigations with modified glycoproteins that lend themselves to the study of the serum half-life or receptor binding require a large amount of sialyltransferase to achieve masking of galactose sites (Reutter et al., 1982). For this purpose, rat liver Galβ1,4GlcNAc α2,6sialyltransferase was employed to study the resialylation of asialo-α_1-acid glycoprotein with sialic acid analogues. Use of 9-amino-NeuAc led to a sialylation degree of 66% of the galactose sites, a value which could not be improved by use of higher amounts of enzyme nor by a larger excess of donor substrate (Table IV). For comparison, under optimized conditions NeuAc transfer reached 83% sialylation (Table IV) in accordance to the previously published data (Weinstein et al., 1982b). The lower resialylation value obtained with 9-amino-NeuAc (66%, Table IV) was most likely a kinetic consequence of the low transferase affinity for CMP-9-amino-NeuAc, as a subsequent transfer led to an additional incorporation of either NeuAc or 9-amino-NeuAc to an extent of 30% (unpublished experiments). The results obtained demonstrate that even a sialic acid lacking the negative net charge, which is needed for interaction with sialidases or NeuAc aldolase, does not impede a high transfer.

Interestingly, in spite of their bulky hydrophobic substituent, 9-hexanoylamido- and 9-benzamido-NeuAc were incorporated to the same and a higher extent, respectively, than NeuAc itself (Table IV). On the basis of these findings it seems feasible to synthesize glycoproteins that contain an outer shell of hydrophobic groups in the oligosaccharide chains.

The results presented open the way for preparation of glycoproteins differently modified in the sialic acid moiety on a large scale by enzymatic synthesis. Further, the studied sialic acid analogues contribute to the knowledge of the donor substrate specificity of these enzymes, which is important for preparation of tailored analogues. The favorable enzyme–substrate interaction observed with large, space-filling substituents at C-9 (benzamido or hexanoylamido group) stimulated the synthesis of a sialic acid analogue carrying a fluoresceinyl residue at C-9. This fluorescent analogue could be activated and transferred onto glycoproteins by each of these sialyltransferases (Gross & Brossmer, 1988b).

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δ Opioid Receptor Selectivity Induced by Conformational Constraints in Linear Enkephalin-Related Peptides: ^{1}H 400-MHz NMR Study and Theoretical Calculations†

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ABSTRACT: Introduction into the structure of the linear hexapeptide DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr) or DTLET (Tyr-D-Thr-Gly-Phe-Leu-Thr) of *tert*-butyl groups as constraints different from cyclization leads to a large increase in the selectivity for δ opioid binding site in the case of DSTBULET [Tyr-D-Ser-(OtBu)-Gly-Phe-Leu-Thr] ($K_{i\delta}$ = 6.14 nM; $K_{i\mu}$ = 374 nM) and BUBU [Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr(OtBu)] ($K_{i\delta}$ = 4.68 nM; $K_{i\mu}$ = 475 nM) or a loss of affinity for DTTBULET [Tyr-D-Thr(OtBu)-Gly-Phe-Leu-Thr] ($K_{i\delta}$ = 866 nM; $K_{i\mu}$ = 4500 nM). This puzzling behavior is studied here by 400-MHz ^{1}H NMR spectroscopy in DMSO-d_6 solution and by theoretical calculations. When DSLET and DTLET are compared, the reduction in energetically accessible ϕ and ψ angles induced by the *tert*-butyl group in the D-Ser^2 residue decreases the degree of freedom in the N-terminal part of the peptides. For DSTBULET and BUBU, the rigidification of the backbone evidenced by the appearance of the large NOE's of Phe^4 NH–Gly^3 α and Gly^3 NH–α and by the loss of the C_7 folding around the D-Ser^2 residue found in DSLET could explain the drastic loss of affinity for μ opioid receptors. In DTTBULET, a large change in the spatial orientation around the D-Thr^2 (OtBu) residue forces the aromatic rings far from each other. Conformational analysis of these peptides by Metropolis calculations is in agreement with NMR analysis suggesting that the preferential g^- orientation of the Phe^4 side chain, yielding a short distance (≤10 Å) between the two aromatic rings, plays a crucial role in δ receptor selectivity. The most stable conformation of BUBU deduced from NMR is very similar to the conformation of δ-selective *cyclo*-[D-Pen^2,L-Pen^5]enkephalin and *cyclo*-[D-Pen^2,D-Pen^5]enkephalin, two severely constrained cyclic peptides. Moreover, the Tyr^1 and Phe^4 aromatic rings of BUBU can be easily superimposed on the corresponding rings in the rigid and selective δ-antagonist naltrindole.

Characterization of the biological and pharmacological responses associated with μ or δ opioid receptor stimulation requires the design of highly specific ligands for these binding sites (Hansen & Morgan, 1984; Roques, 1988).

The conformation of the enkephalins has been extensively studied by NMR spectroscopy [for reviews, see Schiller et al. (1984) and Khaled et al. (1985)], and these studies have shown that in solution the endogenous pentapeptides behave as highly flexible molecules able to assume various conformations of comparable low energy, allowing conformational adaptation to both μ and δ receptors. Sterically restricted linear or cyclic peptides, such as morphiceptin and Tyr-*c*-(N-γ-D-A_2Bu-Gly-Phe-Leu), respectively, which behave as μ-agonists (Chang et al., 1981; Schiller & DiMaio, 1982), represent convenient models to probe the bioactive conformation at the μ opioid receptor. Conformational studies by energy calculations have shown that these compounds present a few structures stabilized mainly by intramolecular H-bonds involved in β-turns eventually reinforced by a C_7 turn between the carbonyl of Tyr^1 and the NH of Gly^3 (Loew et al., 1986; Maigret et al., 1986). The occurrence of folded structures in μ-selective peptides is in agreement with the proposed bioactive conformation at the μ opioid receptor (Roques et al., 1976; Clarke et al., 1978; Loew et al., 1978; DiMaio & Schiller, 1980; Fournié-Zaluski et al., 1981; Castiglione et al., 1987; Schiller et al., 1987; Keys et al., 1988).

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