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Enzymatic transfer of sialic acids modified at C-5 employing four different sialyltransferases

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We present kinetic studies on the enzymatic transfer of several synthetic sialic acid analogues, modified at C-5, to distinct glycoprotein glycans by sialyltransferases differing in acceptor- and linkage-specificity. Biochemical properties of sialic acids were modified by introducing formyl-, trifluoroacetyl-, benzyloxycarbonyl-, and aminoacetyl-groups to the amino group at C-5 of neuraminic acid. The latter substitution renders the corresponding α-glyocoside resistant towards sialidases. The respective CMP-sialic acid analogues were prepared by CMP-sialic acid synthase with a yield of 13–55%.

The kinetic parameters of several sialyltransferases for the 5-substituted CMP-glycosides differed significantly. Relative to parent CMP-NeuAc, reaction rates of human- and rat liver Galβ1, 4GlcNAc α2,6-sialyltransferases ranged from 50 to 170%, of GalNAc α2,6-sialyltransferases from 40–140%, and of Galβ1,3GalNAc α2,3-sialyltransferase from 20–50%. Resialylation of asialo-α_1-acid glycoprotein by 5-*N*-formyl- and 5-*N*-aminoacetyl-neuraminic acid employing rat liver Galβ1,4GlcNAc α2,6-sialyltransferase proceeded to about 80% of galactose sites which is identical to the extent achieved with parent NeuAc.

According to our data, neosialoglycoconjugates which carry sialic acids modified at the *N*-acetyl group can be prepared for structure-function analysis, as this position seems crucial for recognition of adhesion proteins and influenza viruses.

Keywords: sialic acid analogues; CMP-glycosides; sialyltransferases, resialylation

Introduction

The carbohydrate component attached to soluble or membrane bound glycoproteins and glycolipids is of increasing biological interest. For example, specific sequences of cell surface glycoconjugates influence antigenic characteristics, and are involved in virus binding and cell-cell interactions [1]. The terminal constituents of glycan moieties are predominantly sialic acids that take part in many carbohydrate mediated recognition phenomena as a consequence of their exposed position and negative charge [2]. Sialyloligosaccharides enable influenza virus binding to host cells; interaction of plasmodium parasites with erythrocyte surface involves sialylated O-glycans, interaction of haematopoietic cells with vascular endothelium depends on the expression of sialyl-Lewis sequences, and α2,6-sialoglycans represent B-cell differentiation antigens [3–10]. Only recently the immunoglobulin adhesion proteins CD22, sialoadhesin and MAG were described as cell surface lectins recognizing certain sialoglycans [11–13]. Enzymatic transfer of synthetic sialic acids to soluble or cell surface glycoconjugates can specifically modify the biochemical properties of a glycoconjugate. We have previously demonstrated the feasibility of transferring sialic acid analogues modified at C-9 and C-4 employing sialyltransferases with different acceptor specificity [14–19]. This approach was successfully applied to constitute a synthetic influenza C virus receptor on host cells that resists cleavage by the receptor-destroying virus esterase and even prevents infection [20, 21], to study the specificity of the Golgi transporter for CMP-activated sialic acids and to remodel the cell surface of erythrocytes and the IM-9 B-cell line [22, 23].

The present study was performed to further investigate the substrate specificity of several sialyltransferases, specific for common N-linked or O-linked acceptor glycans, with regard to the *N*-acetyl group of sialic acid. In accordance with previous studies carried out with 9-substituted sialic acid analogues the results demonstrate that synthetic sialic acids modified at C-5 can be effici-

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ently incorporated in the terminal position of distinct glycan sequences according to the acceptor and linkage specificity of the sialyltransferase (ST) employed.

Materials and methods

MATERIALS

All chemicals used were of analytical grade and purchased from Merck (Darmstadt) or Serva (Heidelberg). Cytidine-5′-triphosphate (CTP) was obtained from Biomol (Hamburg), bovine serum albumin, Triton CF-54 and Triton X-100 from Sigma (München). Both acetonitrile grade E and grade S were from Zinsser (Frankfurt). CMP-NeuAc (NeuAc, 5-*N*-acetyl-β-D-neuraminic acid) was prepared enzymatically as described [15] and contained less than 4% CMP. α1-Acid glycoprotein and antifreeze glycoprotein (from serum of *Pagothenia borchgrevinki*) were prepared according to [18]. Fetuin was obtained from Serva (Heidelberg), and further purified on Sepharose 6B. All glycoproteins were desialylated by mild acidic hydrolysis [15].

Galβ-1,4GlcNAc α2,6-ST (rat liver; 2 U ml^{-1}, EC 2.4.99.1) and Galβ1,3GalNAc α2,3-ST (porcine liver; 0.2 U ml^{-1}, EC 2.4.99.4) were purchased from Boehringer (Mannheim), and Galβ1,4GlcNAc α2,6-ST from human liver (1.5 U mg^{-1}, EC 2.4.99.1) was isolated as described [24]; GalNAc α2,6-ST (1.9 U ml^{-1}, EC 2.4.99.3) from porcine submaxillary glands, purified according to the published method, was kindly supplied by Dr J.C. Paulson [25].

Protein was measured by the Bio-Rad protein assay and galactose sites were determined as outlined previously [15].

METHODS

*Synthesis of 5-*N*-acyl-neuraminic acid analogues*

5-*N*-Formyl-neuraminic acid [26] and 5-*N*-aminoacetyl-neuraminic acid [14] were prepared as described. 5-*N*-Trifluoroacetylation of neuraminic acid benzyl α-glycoside [14] afforded, after hydrogenolytic cleavage of the benzyl group, 5-*N*-trifluoroacetyl-neuraminic acid. 5-*N*-Benzyloxycarbonyl-neuraminic acid was originally synthesized by a chemical procedure [27] starting from *N*-benzyloxycarbonyl glucosamine.

Analytical HPLC-system [15, 18]

CMP and CMP-glycosides were measured at 275 nm with an aminopropyl phase column (0.4 cm × 12.5 cm, Serva, Heidelberg) [15, 18]. NeuAc, 5-*N*-formyl-Neu (Neu, neuraminic acid), 5-*N*-trifluoroacetyl-Neu, 5-*N*-benzyloxycarbonyl-Neu and 5-*N*-aminoacetyl-Neu were determined at 200 nm using a Spherisorb NH2 column (0.4 cm × 25 cm, Zinsser, Frankfurt), which was operated isocratically with a mixture of acetonitrile grade S/15 mM KH_2PO_4 (7/3 or 8/2, v/v) at 1–2 ml min^{-1}. The 5-*N*-acyl-neuraminic acids released from the CMP-glycosides or from the acceptor glycoproteins were identified according to their retention times and quantified with respect to corresponding external standards as outlined earlier for 9-substituted NeuAc analogues [15, 18].

Preparative synthesis and purification of CMP-glycosides

CMP-sialic acid synthase was partially purified from bovine brain as described previously (250 mU ml^{-1}, about 200 mU mg^{-1}) [15] and concentrated to about 3 U ml^{-1} by ultrafiltration.

Preparative enzymatic synthesis of CMP-NeuAc analogues modified at C-5 of the NeuAc moiety was performed at pH 9.0 as described [15, 18]. The final purification step on Bio-Gel P-2 was omitted in case of CMP-5-*N*-benzyloxycarbonyl-Neu.

Characterization of CMP-NeuAc analogues

The CMP-glycosides were characterized after acid hydrolysis as outlined earlier [15, 18]. CMP liberated was analysed by analytical HPLC at 275 nm, and the 5-substituted NeuAc analogues were quantified by analytical HPLC at 200 nm (see above). The content of CTP and inorganic phosphate were determined as described [15, 18].

Transfer assays

Sialyltransferase assays were performed as outlined [18, 14].

Assay 1 [18]. The reaction mixture (160 μl) for Galβ1,4GlcNAc α2,6-ST from rat liver (pH 6.0, 0.5% Triton CF-54; or pH 6.5, 0.1% Triton X-100) or human liver (pH 6.5, 0.1% Triton X-100) was composed as detailed previously and contained 1.7 mg ml^{-1} asialo-α1-acid glycoprotein (750 μM Gal sites) as acceptor; the assay at pH 8.5 was carried out accordingly.

Assay 2 [18]. The reaction mixture (100 μl) for GalNAc α2,6-ST from porcine submaxillary glands and Galβ1,3GalNAc α2,3-ST from porcine liver were composed as described (pH 6.5, 0.1% Triton X-100) and contained 1 mg ml^{-1} antifreeze glycoprotein (1400 μM Gal/GalNac sites) or 10 mg ml^{-1} asialofetuin (500 μM O-linked Gal/GalNAc sites) as acceptor.

Transfer reaction was started by addition of the respective sialyltransferase and terminated after appropriate times at 37 °C by addition of 1.3 ml cold 1% phosphotungstic acid in 0.5 N HCl. The assays were processed further as outlined earlier [15, 18].

Transferred NeuAc or NeuAc analogues were released from the glycoprotein by acid hydrolysis (0.1 N HCl, 1 h at 80 °C) and quantified by the thiobarbituric acid assay with respect to external standards as described [18, 28]. Further, transfer mediated by rat liver α2,6-ST was confirmed by analytical HPLC at 200 nm (see above). The extent of degradation during acid hydrolysis was as

follows: NeuAc, 10%; 5-*N*-trifluoroacetyl-Neu, 10%; 5-*N*-benzyloxycarbonyl-Neu, 15%; 5-*N*-aminoacetyl-Neu, 10% and 5-*N*-formyl-Neu, 50%. Release of the 5-substituted NeuAc analogues from acceptor glycoproteins by acid hydrolysis was virtually complete after 60 min.

Initial rates were determined in duplicate at 0.2, 0.5, or 1.0 mM concentration of the respective CMP-glycosides. Assay 1 was performed for 30 min with 0.5 mU rat liver α2,6-ST or 0.3 mU human liver α2,6-ST, assay 2 for 45 min with 2.0 mU GalNAc α2,6-ST or 0.3 mU α2,3-ST (respective activity was measured according to [18, 24]). Consumption of either substrate was always less than 15%.

For kinetic measurements initial rates were determined in duplicate at five concentrations of the respective CMP-glycoside near the corresponding K_m-value. Assay 1 was performed for 30 min with approximately 0.15 mU rat liver Galβ1,4GlcNAc α2,6-ST. To ensure linear transfer rates with incubation time, consumption of the donor substrates was limited to 20%, consumption of the acceptor substrate was less than 10%. Kinetic data were obtained from Hanes plots [18].

Resialylation assays

For the studies of maximal resialylation, assay 1 was modified to favour complete saturation of the acceptor substrate as described earlier [14, 15, 18]. Briefly, the reaction mixture (160 μl) contained 10 μmol Na cacodylate pH 6.7, 0.1 mg Triton CF-54, 1 mg BSA, 0.022 mg asialo-α1-glycoprotein (9.0 nmol galactose acceptor sites), and 1.5 mM of the respective CMP-glycoside. The reaction was started by addition of 3.5 mU rat liver α2,6-ST. Tubes were incubated for 17 h at 37 °C and assayed as indicated above.

Results

Preparative synthesis and purification of CMP-glycosides

5-*N*-Formyl-, 5-*N*-trifluoroacetyl-, 5-*N*-benzyloxycarbonyl- and 5-*N*-aminoacetyl-Neu (neuraminic acid) were converted to the corresponding CMP-glycosides (structures shown in Fig. 1) by CMP-sialic acid synthase from bovine brain on a preparative scale using the assay developed earlier [15, 18]. Final conversions of 65–80% were achieved for the NeuAc analogues modified at C-5 in accordance with the values obtained previously with several 9-substituted NeuAc analogues; conversion of parent NeuAc reached 72% [14, 15]. Only 5-*N*-benzyloxycarbonyl-Neu yielded a significantly lower conversion of 20% in accordance with the low V_{max} value determined for CMP-activation of this derivative (unpublished results). Purification of the CMP-glycosides synthesized was performed as described [14, 15] employing semi-preparative HPLC, ethanol precipitation and gel filtration. Overall yields of pure CMP-NeuAc analogues were 45–55% except for CMP-5-benzyloxycarbonyl-Neu, which yielded 13%. Pure CMP-glycosides could be stored at −20 °C for several months without significant decomposition.

Analytical data of pure CMP-glycosides

CMP-Activated NeuAc analogues modified at C-5 of the NeuAc moiety eluted at different retention times during HPLC chromatography and were distinguished from CMP-NeuAc or CMP. As observed with several CMP-NeuAc analogues, the interaction with the separating aminopropyl phase was strongly influenced by the substituent introduced at C-5; a benzyloxycarbonyl group reduced the elution time by 0.5-fold compared to parent CMP-NeuAc, whereas the formyl, trifluoroacetyl and aminoacetyl group only slightly influenced the retention time.

Purified CMP-glycosides were characterized by different methods [14, 15, 18]. An equivalent molar ratio of CMP to the respective 5-substituted NeuAc analogue was obtained after acid hydrolysis as determined by analytical HPLC (Table 1). Separation of the different 5-substituted NeuAc analogues and identification was achieved by applying the analytical HPLC system at 200 nm [14, 15, 18].

Contamination of CMP or NeuAc analogues accounted

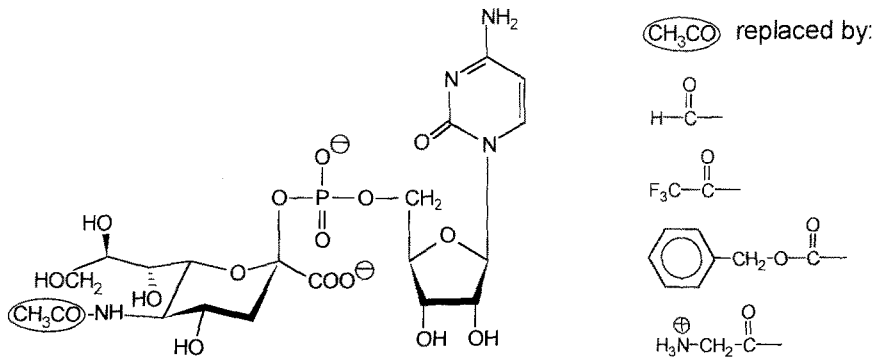

Figure 1. Structure formulae of NeuAc analogues modified at C-5. The acetyl group at C-5 was replaced by formyl-, trifluoroacetyl-, benzyloxycarbonyl-, and aminoacetyl groups.

Table 1. Characterization of purified CMP-NeuAc analogues

CMP-glycoside	*NeuAc analogues (mol per mol CMP)*	*CMP (%)*	*NeuAc analogue (%)*	*Inorg. phosphate (%)*	*A rel (275 nm)*
CMP-5-*N*-formyl-Neu	0.93	2.0	5.0	6.0	1.0
CMP-5-*N*-trifluoroacetyl-Neu	0.94	2.0	2.0	8.0	1.0
CMP-5-*N*-benzyloxycarbonyl-Neu	1.06	7.0	3.5	35.0	1.0
CMP-5-*N*-aminoacetyl-Neu	1.05	1.5	5.0	11.0	1.0

The molar ratio of CMP to 5-substituted NeuAc analogue was determined by analytical HPLC after acid hydrolysis (see Materials and methods). Contaminations of CMP, NeuAc analogue, and inorganic phosphate, relative to the amount of CMP-glycoside, were calculated as described in Materials and methods. The absorption coefficient relative to that of CMP-NeuAc (A rel) was measured by determining the amount of CMP after acid hydrolysis.

for less than 6% (Table 1). In each preparation the content of CTP, a potent sialyltransferase inhibitor, was below 0.2% related to the concentration of CMP-glycoside. Inorganic phosphate was negligible except for CMP-5-*N*-benzyloxycarbonyl-Neu where the final purification step was omitted (Table 1).

Initial rate assay

Incorporations of 5-*N*-formyl-, 5-*N*-trifluoroacetyl-, 5-*N*-benzyloxycarbonyl- and 5-*N*-aminoacetyl-Neu into glycoprotein glycans were compared to that of NeuAc employing four purified sialyltransferases with different linkage and acceptor specificity, Galβ1,4GlcNAc α2,6-ST (rat liver and human liver [24, 29]), GalNAc α2,6-ST (porcine submaxillary glands [25]), and Galβ1,3GalNAc α2,3-ST (porcine liver). The acceptor substrate for both the rat and human liver α2,6-ST was asialo-α_1-acid glycoprotein, for the porcine submaxillary gland α2,6-ST and porcine liver α2,3-ST antifreeze glycoprotein and/or asialofetuin. Increasing donor substrate concentrations were applied to estimate the saturating concentration of each CMP-NeuAc analogue, as this represents the important kinetic parameter for efficient preparation of neoglycoconjugates.

After release from the acceptor glycoproteins by acid hydrolysis transferred NeuAc analogues or parent NeuAc were measured colorimetrically by the thiobarbituric acid method with respect to a corresponding external standard [28]. In order to confirm these values, as an example sialyltransfer by rat liver α2,6-ST was also determined employing analytical HPLC at 200 nm, which allowed the identification of each NeuAc analogue according to the retention time and the quantification of amount liberated relative to a corresponding external standard [14, 15, 18]. Transfer values obtained by these two methods differed by less than 15%. Transfer rates of rat and human liver α2,6-ST measured with CMP-NeuAc analogues modified at C-5 were identical or even higher with respect to the value of parent CMP-NeuAc (Table 2). As an exception, 5-*N*-aminoacetyl-Neu was transferred at a rate about 50% lower by both α2,6-STs, and 5-*N*-trifluoroacetyl-Neu with a lower rate (50%) by the human enzyme. Raising the concentration of each CMP-glycoside from 0.5 mM to 1 mM, the reaction rate of rat liver α2,6-ST did not increase significantly (Table 2). This result indicates that saturation was reached at 0.5 mM, which accords to the K_m-values calculated (30–63 μM; Table 4). Saturation of the human liver enzyme had

Table 2. Initial transfer rates of rat liver and human liver Galβ1, 4GlcNAc α2,6-sialyltransferase for several CMP-NeuAc analogues

	Initial rates (%)						
	Rat liver α2,6-ST					*Human liver α2,6-ST*	
Donor substrate (mM)	0.2	0.5		1.0		0.2	0.5
pH value	6.5	6.0	6.5	6.0	6.5	6.5	6.5
CMP-NeuAc	83	94	100	100	100	99	100
CMP-5-*N*-formyl-Neu	81	90	114	90	115	101	113
CMP-5-*N*-trifluoroacetyl-Neu		84		93		50	49
CMP-5-*N*-benzyloxycarbonyl-Neu		170		170		ND	ND
CMP-5-*N*-aminoacetyl-Neu	40	30	48	38	51	71	65
pH 8.5		92		116			64

Assay 1 (pH 6.0, 6.5, or 8.5 with rat liver ST; pH 6.5 or 8.5 with human liver ST) was performed in duplicate with asialo-α_1-acid glycoprotein as acceptor and 0.2 mM, 0.5 mM and 1.0 mM CMP-glycoside as described in Materials and methods. Initial rates were determined on the basis of two to three independent experiments and are expressed as a percentage with respect to the value obtained at 1 mM CMP-NeuAc (rat liver 100% = 0.3 nmol min^{-1}; human liver 100% = 0.13 nmol min^{-1}). The values were obtained by the thiobarbituric acid method; maximum limit of error was ±10%. (ND, not determined).

already been achieved at 0.2 mM donor concentration (Table 2).

Table 2 further shows the influence of the pH value on the transfer of zwitterionic 5-*N*-aminoacetyl-Neu by rat liver α2,6-ST. The positive charge of the aliphatic amino group at C-5 (pK about 8.5) declined with increasing pH value. In agreement with the decrease in protonation, the transfer rate of this analogue increased by three-fold from pH 6.0 to 8.5, and at pH 8.5 even reached the value measured with parent NeuAc (Table 2). In contrast, the human liver enzyme was not markedly influenced by the pH-dependent protonation of this amino derivative (Table 2).

Employing porcine submaxillary glands GalNAc α2,6-ST, transfer rates obtained with CMP-NeuAc analogues at 1 mM were in the same range as that of parent CMP-NeuAc (80–140%, Table 3); only the reaction rate of 5-*N*-aminoacetyl-Neu was reduced by about 60%. The influence of the protein structure on the transfer mediated by GalNAc α2,6-ST was studied by comparing asialofetuin and antifreeze glycoprotein as acceptors. In contrast to the corresponding values measured with asialofetuin, significantly lower relative rates were determined for 5-*N*-trifluoroacetyl- and 5-*N*-aminoacetyl-Neu using antifreeze glycoprotein (Table 3). Initial rates obtained with porcine α2,6-ST at 1 mM of each CMP-NeuAc analogue were 1.2–1.6-fold higher than the values determined at 0.5 mM (Table 3). This kinetic property primarily reflects the low donor substrate affinity of this transferase represented by the high K_m value of 500 μM for the physiological donor CMP-NeuAc [18, 25]. Obviously, the substituent introduced at C-5 of the NeuAc moiety did not markedly influence the donor substrate affinity of the enzyme, as the transfer rate of parent CMP-NeuAc and of the CMP-NeuAc analogues increased to the same extent with increasing donor concentration (Table 3). This result was in accordance with the transfer kinetics obtained previously with several CMP-activated 9-substituted NeuAc analogues [18].

Employing α2,3-ST from porcine liver, for each 5-substituted NeuAc analogue, incorporation rates were found to range from 23 to 50% (Table 3). Initial rates were close to maximum values at 0.2 mM of each CMP-glycoside. In general, the reaction rate of this enzyme declines to 0.2–0.5-fold when the acetyl group at C-5 is modified.

Kinetic data

Accurate kinetic parameters of the rat liver α2,6-ST were determined for CMP-5-*N*-formyl-, CMP-5-*N*-benzyloxycarbonyl- and CMP-5-*N*-aminoacetyl-Neu. K_m-values obtained ranged from the 0.6–1.4-fold relative to the value of CMP-NeuAc (Table 4), with a K_m of 45 μM as determined previously [18]. Notably, the positive charge of 5-*N*-aminoacetyl-Neu did not significantly affect the transferase affinity. The V_{max}-values of the rat liver α2,6-ST measured with CMP-NeuAc and the CMP-NeuAc analogues were in the same range, 5-*N*-benzyloxycarbonyl-Neu, however, was incorporated significantly faster. Further, the data agreed with the initial rates listed in Table 2.

Resialylation

In order to investigate maximal incorporation of different NeuAc analogues, the transfer assay was modified as follows. To enhance stability of the CMP-glycosides the pH was raised to 6.7. To improve the resialylation extent

Table 3. Initial rates of porcine liver Galβ1,3GalNAc α2,3-ST and porcine submaxillary gland GalNAc α2,6-ST for several CMP-NeuAc analogues.

	Initial rates				
	Porcine α2,3-ST			*Porcine α2,6-ST*	
Donor substrate (mM)	0.2	0.5	0.5	1.0	
	AF	AF	AF	AF	AFG
CMP-NeuAc	93	100	65	100	100
CMP-5-*N*-formyl-Neu	21	23	56	81	95
CMP-5-*N*-trifluoroacetyl-Neu	40	50	49	82	26
CMP-5-*N*-benzyloxycarbonyl-Neu	ND	ND	88	140	ND
CMP-5-*N*-aminoacetyl-Neu	21	30	31	38	28

Assay 2 was performed with antifreeze glycoprotein (AFG) or asialofetuin (AF) as acceptor, and 0.2, 0.5, or 1 mM CMP-glycoside as described in Materials and methods. Transfer rates of α2,3-ST were determined on the basis of two independent experiments and are expressed as a percentage with respect to the value obtained at 0.5 mM CMP-NeuAc (100% = 0.02 nmol min^{-1}), of α2,6-ST as a percentage with respect to the value at 1 mM CMP-NeuAc (100% = 0.03 nmol min^{-1} with antifreeze glycoprotein; 100% = 0.06 nmol min^{-1} with asialofetuin). The values were obtained by the thiobarbituric acid method; maximum limit of error was ±10%.
ND, not determined.

Table 4. Apparent kinetic data of rat liver Galβ1,4GlcNAc α2,6-sialyltransferase for several CMP-NeuAc analogues.

Donor substrate	K_m (μM)	*Rel* V_{max}	V_{max}/K_m (1 mM^{-1})
CMP-NeuAc	45	1.0	22.2
CMP-5-*N*-formyl-Neu	63	0.95	15.1
CMP-5-*N*-benzyloxycarbonyl-Neu	30	1.7	56.7
CMP-5-*N*-aminoacetyl-Neu	46	0.66	14.3

Kinetic measurements were performed at five concentrations of CMP-glycoside using assay 1 as described in Materials and methods. Kinetic values were calculated on the basis of two to three independent kinetic experiments. V_{max}-values are expressed relative to that determined for CMP-NeuAc (= 1.0). Maximum limit of error was ±15%.

the amount of acceptor glycoprotein was reduced and of enzyme increased as described earlier [18, 14]. The results obtained with α2,6-ST from rat and human liver after 17 h incubation time are shown in Table 5. The values obtained previously [18] employing CMP-9-amino-NeuAc are included for comparison. The rat liver enzyme produced α1-acid glycoprotein resialylated by 80% with 5-*N*-formyl- and 5-*N*-aminoacetyl-Neu as well as with parent NeuAc (Table 5). The synthetic derivatives were about 30% less efficiently incorporated than parent NeuAc by the human liver α2,6-sialyltransferase (Table 5).

Discussion

The acetyl group at C-5 of the sialic acid molecule represents a crucial structural element for enzyme-substrate interactions of sialidase, CMP-sialic acid synthase and sialyltransferases. Kinetic data of sialyltransferases for the naturally occurring CMP-NeuAc and CMP-NeuGc are slightly different [30]. α-Glycosides of the synthetic 5-formyl-Neu were hardly attacked by bacterial sialidases [26]. Remarkably, a novel derivative, 5-*N*-aminoacetyl-Neu, was not cleaved at all by bacterial (*Vibrio cholerae, Arthrobacter ureafaciens* and *Clostridium perfringens*), viral (fowl plague virus), and mammalian (bovine testis) sialidases (unpublished results).

In this paper the preparation of the following CMP-activated sialic acid analogues modified at C-5 and the enzymatic transfer of the respective sialic acid analogues to different acceptor glycans, CMP-5-*N*-formyl-, CMP-5-*N*-trifluoroacetyl, CMP-5-*N*-benzyloxycarbonyl- and CMP-5-*N*-aminoacetyl-Neu is described (Fig. 1). 5-*N*-Trifluoroacetyl-Neu shows a reduced capacity for hydrogen bonding but sterically closely resembles the acetyl group. In 5-*N*-formyl-Neu the *N*-substituent is shortened by one carbon but still contains the basic amide structure. 5-*N*-Benzyloxycarbonyl-Neu carries a hydrophobic and space-filling substituent, which can be cleaved by catalytic hydrogenation. The sialidase resistant 5-*N*-aminoacetyl-Neu represents a zwitterionic compound which in the physiological milieu is positively charged.

The initial transfer rates of several sialyltransferases specific for a distinct glycosidic linkage and acceptor sequence were determined and compared to the values obtained with parent CMP-NeuAc. Considering these data, optimized conditions for transfer of the 5-substituted derivatives to the most common glycan acceptor structures could be deduced (Tables 2 and 3). The Galβ1,4GlcNAc α2,6-sialyltransferases purified from rat liver [29] and human liver [24] transferred the synthetic compounds with efficiency comparable to parent NeuAc (50–115%). GalNAc α2,6-sialyltransferase from porcine submaxillary glands was also not markedly affected by

Table 5. Maximum resialylation of asialo-α_1-acid glycoprotein by rat liver and human liver Galβ1,4GlcNAc α2,6sialyltransferases.

Donor substrate	*Resialylation (% Gal sites)*	
	Rat liver α2,6-ST	*Human liver α2,6-ST*
CMP-NeuAc	80	76
CMP-9-amino-NeuAc[a]	64	54
CMP-5-*N*-formyl-Neu	78	40
CMP-5-*N*-aminoacetyl-Neu	86	47

Resialylation assay (160 μl) was performed in duplicate at pH 6.7 and 1.5 mM donor substrate concentration, and incubated for 17 h at 37 °C as described in Materials and methods. Resialylation is given as a percentage of the total galactose acceptor sites and represents the average of three experiments. Maximum limit of error was ±8%.
[a]Values taken from [14, 18].

the substituents introduced at position C-5 (transfer rate 40–80% relative to NeuAc). As an exception, the benzyloxycarbonyl group increased the reaction rate of rat liver and porcine submaxillary gland sialyltransferase substantially (170% and 140%, respectively).

The catalytic rate of Galβ1,3GalNAc α2,3-sialyltransferase purified from porcine liver was slightly reduced as a consequence of the structural variation at C-5, but sialyl transfer still proceeded sufficiently (20–50% of the NeuAc rate). Although 5-*N*-aminoacetyl-Neu was incorporated at reduced rates (about 50%) compared with the other sialic acids modified at C-5 by each transferase, this sialidase resistant analogue nevertheless can be efficiently attached to N- and O-linked acceptor glycans. This result provides an important advantage compared with 9-amino-NeuAc which also resisted sialidase cleavage [31], but was only accepted as substrate by the α2,6-sialyltransferase from rat and human liver [14, 15, 18]. Despite the positively charged amino group, K_m-values of rat liver sialyltransferase for CMP-5-*N*-aminoacetyl-Neu and CMP-NeuAc were the same (Table 4). In contrast, the K_m value of this enzyme for CMP-9-amino-NeuAc was about 15-fold higher.

Both Galβ1,4GlcNAc α2,6-sialyltransferases resialylated galactose acceptor sites of asialo-α_1-acid glycoprotein with 5-*N*-formyl- and 5-*N*-aminoacetyl-Neu to a high extent (80–85% for the rat and 40–50% for the human enzyme) (Table 5). Resialylation mediated by the rat enzyme yielded a significantly higher substitution of galactose sites with 5-*N*-aminoacetyl-Neu compared with 9-amino-NeuAc, whereas the human enzyme resialylated to the same extent (Table 5). Since structural variations at C-5 of NeuAc had the lowest impact for sialyltransfer by the rat liver transferase, this enzyme represents an optimal tool for the synthesis of respective neosialoglycoproteins. Thus, in spite of the extensive sequence homology between the rat and the human liver α-2,6-sialyltransferase, donor substrate affinity and sensitivity toward structural modification at positions C-5 or C-9 of the NeuAc moiety turned out to be markedly different.

Based on the kinetic transfer data described here, a novel fluorescent CMP-glycoside, CMP-5-fluoresceinyl-Neu, was synthesized, which proved to be superior to the related fluorescent 9-substituted compounds [32]. Together with previous studies on the transfer of 9-substituted and 4-substituted sialic acid analogues [14, 15, 16] the kinetic data presented here allow the synthesis of a panel of neoglycoproteins or neoglycolipids, which are modified in important biochemical properties such as sialidase and esterase resistance or hydrophobicity by introduction of specifically tailored sialic acid analogues. Further, the investigations amplify the knowledge on the donor substrate specificity of sialyltransferases, which is an important prerequisite to develop strategies for the preparation of neoglycoconjugates containing novel biologically active sialic acid analogues. In a study currently under way the analogues presented in this paper proved to be useful for elucidating the structure-function relationship of sialic acid dependent recognition of adhesion proteins.

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