Glycoconjugate J (1988) 5: 411-417

Asialo-α_1-acid Glycoprotein Resialylated with 9-Amino-5-*N*-acetyl-D-neuraminic Acid is Resistant towards Bacterial, Viral and Mammalian Sialidases

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Received August 25, 1988.

Keywords: 9-amino-NeuAc, sialidase resistance, sialidases, sialic acid analogue, resialylation

We demonstrate that 9-amino-NeuAc transferred to asialo-α_1 acid glycoprotein resists cleavage by bacterial, viral and mammalian sialidases. This is the first synthetic sialic acid analogue, which can be activated and transferred to glycoprotein, but is not a sialidase (EC 3.2.1.18) substrate.

Sialic acid analogues, if synthesized with structure/activity correlation as an incentive, will become important tools. Substitution of sialic acid in glycoproteins and glycolipids may influence their biological function such as receptor binding or serum half life. In addition such analogues may block or influence the metabolism of sialic acid at the cellular level causing changes in cell surface glycan structures.
In order to be activated and transferred, the analogues should resemble the parent sialic acid as closely as possible. In this context substitution at C-9 of NeuAc was shown previously to be suitable, as these modifications did not impede action of CMP-sialic acid synthase and sialyltransferase [1-4].

An analogue replacing sialic acid on soluble and cell surface bound glycoconjugates and being resistant towards catabolic sialidases possesses considerable interest. Among the

Abbreviations: HPLC, high performance liquid chromatography; BSA, bovine serum albumin; NeuAc, *N*-acetyl-D-neuraminic acid, 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid; 9-Amino-NeuAc, 9-amino-5-*N*-acetyl-D-neuraminic acid, 5-acetamido-9-amino-3,5,9-trideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid; CMP-NeuAc, cytidine-5′-monophospho-*N*-acetyl-D-neuraminic acid; CMP-9-amino-NeuAc, cytidine-5′-monophospho-9-amino-5-*N*-acetyl-D-neuraminic acid; 9-azido-NeuAc, 5-acetamido-9-azido-3,5,9-trideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid.
Enzymes: sialidase, acylneuraminylhydrolase (EC 3.2.1.18); Galß1-4GlcNAc a(2-6)-sialyltransferase (EC 2.4.99.1)

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naturally occurring sialic acids only 4-*O*-acetyl-NeuAc was resistant towards sialidases, and cleavage of 9-*O*-acetyl-NeuAc proceeded at a reduced rate [5]. Here we demonstrate that 9-amino-NeuAc, which was earlier reported to be activated to the corresponding CMP-glycoside and transferred to asialoglycoprotein [3], is the first synthetic sialic acid analogue not released from a glycoprotein by sialidases from bacterial, viral and mammalian origin.

Materials and Methods

Materials

All chemicals used were of analytical grade and purchased from Merck (Darmstadt, W. Germany) or Serva (Heidelberg, W. Germany). Crystalline *N*-acetylneuraminic acid [6] and 4-methylumbelliferyl α-NeuAc were prepared in this Institute. Cytidine 5'-monophosphate (CMP) was obtained from Boehringer (Mannheim, W. Germany), bovine serum albumin and Triton CF-54 from Sigma (München, W. Germany). CMP-NeuAc (<4% free CMP) and CMP-9-amino-NeuAc (<1.5% free CMP) were prepared as described elsewhere [3]. Galβ1-4GlcNAc α-(2-6)-sialyltransferase (2 U/ml; 2 U/mg, EC 2.4.99.1.) was purified in this laboratory according to the published method [7]. *Vibrio cholerae* sialidase (1 U/ml) came from Behring Werke (Marburg, W. Germany) and *Clostridium perfringens* sialidase (5 U/mg) from Boehringer (Mannheim, W. Germany). Bovine testis sialidase was partially purified in this laboratory. α_1-Acid-glycoprotein and fowl plague virus were kindly supplied by Dr. K. Schmid (Boston, U.S.A.) and Dr. R. Rott (Giessen, W. Germany), respectively.

Synthesis of 9-Amino-NeuAc

9-Azido-NeuAc was prepared from the respective 9-*O*-tosylate as described elsewhere [1]. Catalytic hydrogenation (palladium oxide) then afforded 9-amino-NeuAc which was purified by chromatography on DEAE-Sephadex A 25. 9-Amino-NeuAc was characterized by elemental analysis and ^{1}H-NMR spectroscopy (300 MHz). It was pure as judged by TLC (silica gel; *n*-propanol/water, 5/3 by vol) and ^{1}H-NMR spectrum.

Desialylation of α_1-Acid Glycoprotein

The reaction mixture containing 50 mM sodium acetate pH 5.5, 7 mM $CaCl_2$, 0.01% sodium azide, 50 mg of α_1-acid glycoprotein and *Vibrio cholerae* sialidase (1 U) was dialyzed at 37°C for 24 h against 50 mM sodium acetate pH 5.5, 7 mM $CaCl_2$, 0.01% sodium azide (two buffer changes) to remove the NeuAc released. After chromatography (column 6 x 0.4 cm) on NeuAc-Sepharose-4B (2 μmol NeuAc/ml gel coupled by the divinylsulfone method, unpublished results), run with the buffer used for dialysis (5 ml/h), the effluent containing asialo-α_1-acid glycoprotein was dialyzed against twice-distilled water and lyophilized. This procedure allowed quantitative removal of sialidase as controlled by fluorimetry using 4-methylumbelliferyl α-NeuAc as substrate. The asialo-α_1-acid glycoprotein preparation contained less than 0.2% bound NeuAc.

Galactose Acceptor Sites

These sites were measured in terms of galactose content of the asialo-α_1-acid glycoprotein, which was determined after acid hydrolysis of the glycoprotein (1 N HCl,1 h, 2 h, and 4 h at 100°C) using the galactose dehydrogenase assay [8].

Resialylation Assay

The assay was performed according to the previous studies [9, 10]. Briefly, the reaction mixture (1 ml) contained 50 µmol sodium cacodylate pH 6.0, 1 mg BSA, 5 mg Triton CF 54, 1.5 mg asialo-α_1-acid glycoprotein (830 nmol galactose sites), and 0.7 µmol CMP-NeuAc or 6 µmol CMP-9-amino-NeuAc. Transfer was initiated by addition of 19.5 mU Galβ1-4GlcNAc α(2-6)-sialyltransferase (rat liver). After 17 h at 37°C the resialylated glycoprotein was obtained by gel filtration on Sephadex G-50 fine (column 0.8 x 15 cm, 10 mM NH_4HCO_3), lyophilized and dissolved in twice-distilled water.

Transferred NeuAc or 9-amino-NeuAc was quantified after acid hydrolysis (0.1 N HCl, 60 min, 80°C) by the thiobarbituric acid assay [11] and by analytical HPLC at 200 nm with respect to appropriate external standards [3].

Sialidase Assay

Cleavage of asialo-α_1 acid glycoprotein resialylated with NeuAc or 9-amino-NeuAc by α(2-6)-sialyltransferase as described above was performed as follows. The reaction mixture (100 µl) contained 7.5 µmol sodium acetate pH 5.5, in the case of *Vibrio cholerae* sialidase additionally 0.8 µmol $CaCl_2$ and 13 µmol NaCl, and about 20 µg resialylated glycoprotein (6.4-8.4 nmol transferred NeuAc or 9-amino-NeuAc). The reaction was started by addition of 10 mU *Clostridium perfringens*, or *Vibrio cholerae* sialidase, or 20 mU fowl plague virus sialidase. After appropriate times at 37°C, NeuAc or 9-amino-NeuAc liberated was quantified by the thiobarbituric acid assay with respect to external standards [11]. Corresponding controls were lacking sialidase.
Cleavage by bovine **testis** sialidase was performed as above with the following modifications. Assay (60 µl) contained 6 µmol sodium acetate, pH 4.4, 0.02% sodium azide, 60 µg BSA, about 14 µg resialylated asialo-α_1-acid glycoprotein (5 nmol NeuAc or 9-amino-NeuAc transferred) and 9 mU enzyme. Activity of bovine testis sialidase was determined by fluorimetry at pH 4.4 as described [12] using 0.625 mM 4-methylumbelliferyl α-NeuAc as substrate.

Results

Asialo-α_1-acid glycoprotein was prepared by treatment with *Vibrio cholerae* sialidase to preserve the native protein structure. Resialylation of asialo-α_1-acid glycoprotein by Galβ1-4GlcNAc α(2-6)-sialyltransferase (rat liver) was performed considering the kinetic data described (K_M-values for CMP-NeuAc and CMP-9-amino-NeuAc, 45 and 750 µM, respectively [4]). A high incorporation of NeuAc and 9-amino-NeuAc was achieved after 17 h

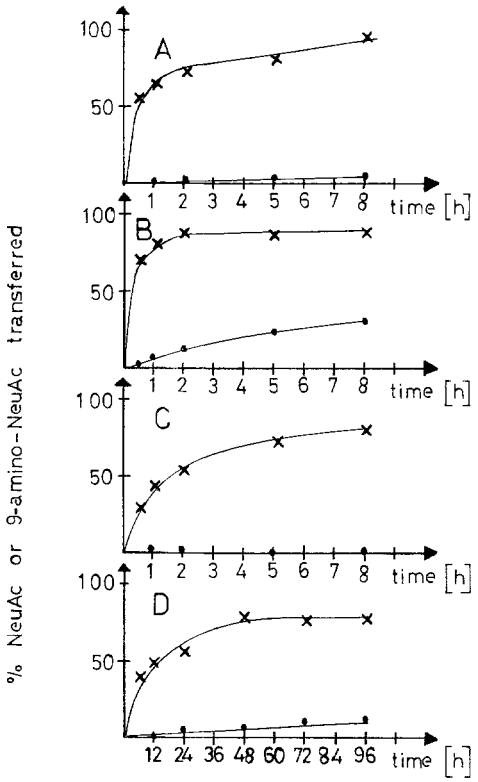

Figure 1. Time course for cleavage of transferred NeuAc (x-x) or 9-amino-NeuAc (-▲-) by several sialidases. Panel A, *Vibrio cholerae* sialidase (10 mU); panel B, *Clostridium perfringens* sialidase (10 mU); panel C, fowl plague virus sialidase (20 mU); panel D, bovine testis sialidase (9 mU). NeuAc or 9-amino-NeuAc was transferred to asialo-α_1-acid glycoprotein by Galß1-4GlcNAc α(2-6)-sialyltransferase from rat liver as described in the Materials and Methods section. Cleavage of the resialylated glycoprotein by sialidase was performed as described in Materials and Methods, and is expressed as a percentage with respect to the amount of transferred NeuAc or 9-amino-NeuAc present in the incubation mixture.

incubation at saturating concentrations of donor substrates (8-15 fold the K_M value). Final incorporation of 9-amino-NeuAc and NeuAc reached 65% and 75%, respectively, of the galactose acceptor sites. The glycoprotein products were isolated by gel filtration as described [13].

Fig. 1 shows time courses for the release of NeuAc and 9-amino-NeuAc by sialidases from different origin. Despite a high enzyme excess, 9-amino-NeuAc was not released by both *Vibrio cholerae* sialidase (below 7% after 8 h, Fig. 1A) and fowl plague virus sialidase (below 3% after 8 h, Fig. 1C). In contrast, NeuAc was cleaved after 8 h by 97% with the former and by 80% with the latter enzyme. A great excess of *Clostridium perfringens* sialidase completely liberated transferred NeuAc after 1 h; release of 9-amino-NeuAc, however,

proceeded only very slowly (8% after 1 h; 32% after 8 h; Fig. 1B). The lower release of NeuAc by fowl plague virus sialidase compared to both bacterial sialidases (80% versus >90% after 8 h) can be explained by the linkage specificity of the viral enzyme, which acts on α(2-3)-linked sialic acids at a 20-50 fold higher rate [14-16].

A mammalian sialidase has been purified from bovine testis (500 fold, 500 mU/mg) yielding a preparation free of proteolytic activity [17]. The enzyme strongly prefers cleavage of α(2-3)-linked NeuAc, whereas α(2-6)-linked NeuAc is only very slowly released [17]. Thus prolonged incubation times (48 h) were needed to release about 80% of NeuAc transferred by Galβ1-4GlcNAc α(2-6)-sialyltransferase (Fig. 1D). In contrast, after 48 h about 6%, and even after 96 h only about 12% of 9-amino-NeuAc were liberated (Fig. 1B). To compare with the bacterial and viral sialidases the values for 8 h incubation were 35% of NeuAc and below 3% of 9-amino-NeuAc released. Liberation of NeuAc by the mammalian enzyme did not exceed 80% even after 96 h because of the slow cleavage rate for α(2-6)-linked NeuAc and the decreasing enzyme activity in the incubation mixture. Activity was about 85% after 48 h incubation due to the stabilizing effect of BSA; extended incubation leading to a continous decrease of activity (about 70% after 72 h and about 55% after 96 h).

Discussion

A substitution of sialic acid as constituent of soluble glycoproteins and cell surfaces by a synthetic analogue would be of particular interest, if it is stable against turnover *in vivo*. For this purpose, a suitable synthetic derivative should be a substrate for CMP-sialic acid synthase and sialyltransferase, but once transferred it should resist the action of sialidases.

Several sialic acid analogues modified at C-9 and C-4 have previously been activated to the corresponding CMP-glycoside and subsequently transferred to asialoglycoproteins [2-4, 13, 18]. We show in this paper that 9-amino-NeuAc transferred onto asialo-α_1-acid glycoprotein by Galβ1-4GlcNAc α(2-6)-sialyltransferase (rat liver) is not cleaved by bacterial, viral and mammalian sialidases. Though we have applied an unphysiologically high excess of each enzyme (1.3 - 3.1 mU/nmol transferred analogue), 9-amino-NeuAc was not released significantly (below 8%) during incubation times sufficient to liberate 80-90% of transferred NeuAc. During a prolonged incubation (8 h) only the enzyme from *Clostridium perfringens* produced a slow release of the analogue. This observation shows again that substrate specificity of various sialidases significantly differs, even comparing only enzymes of bacterial origin [19].

In previous studies with the methyl α-glycoside of 9-amino-NeuAc we had found that sialidases of various origin did not accept this compound (unpublished results). As affinity of sialidases for serum glycoproteins is 100-1000 fold higher compared to the values for low molecular weight substrates [5], in this paper sialidase resistance was demonstrated applying a glycoprotein resialylated with 9-amino-NeuAc.

Contrary to 9-amino-NeuAc, other analogues substituted at C-9 (9-fluoro-, 9-acetamido-, 9-hexanoylamido-, 9-benzamido- and 9-azido-NeuAc), which were transferred to asialo-α_1-acid glycoprotein by Galβ1-4GlcNAc α(2-6)-sialyltransferase [2, 3], were readily released

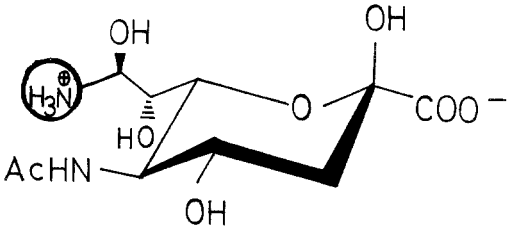

Figure 2. Structural formula of 9-amino-NeuAc. The hydroxy group at C-9 is replaced by an amino group.

either by bacterial, viral or mammalian sialidases, yet with different velocity and K_M-values (unpublished results). 4-*O*-Methyl- and 4-deoxy-NeuAc, both of which transferred to asialoglycoprotein, were not attacked by bacterial sialidases, but cleaved by a viral enzyme [13, 18].

Although 9-amino-NeuAc contains the entire carbon chain of parent NeuAc (Fig. 2), its properties differ considerably due to the zwitterionic character induced by the additional positive charge (pK = 9.7). At pH values below 7.7 the amino group is protonated, thus leaving 9-amino-NeuAc without net charge. In view of the fact that the amide of NeuAc is not converted to the CMP-glycoside [20], activation of 9-amino-NeuAc by CMP-sialic acid synthase at both pH 9 [12] and even pH 7.5 (unpublished result) with favourable kinetic data is remarkable. In contrast to the synthase, action of sialidases is strongly affected by a positive charge at C-9, the extent of inhibition being dependent on the origin of the enzyme.

From the results presented, 9-amino-NeuAc transferred to soluble or membrane bound glycoproteins is expected to resist sialidase action also *in vivo* and thus will become a useful probe for studying biological functions of sialic acid.

Acknowledgements

We thank Mr. Jean Michel Krause for expert technical assistance and are greatly indebted to Dr. K. Schmid, Boston University, for donating α_1-acid glycoprotein and Dr. R. Rott, University of Giessen, for supplying purified fowl plague virus. The studies were supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 136) and the Fonds der Chemischen Industrie.

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