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N-Acetyl-4-deoxy-D-neuraminic Acid is Activated and Transferred on to Asialoglycoprotein

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The sialic acid analogue, *N*-acetyl-4-deoxy-neuraminic acid, is readily activated by CMP-sialic acid synthase from bovine brain. We also show that sialyl-transfer from CMP-*N*-acetyl-4-deoxy-neuraminic acid to asialo-α_1-acid glycoprotein is achieved at a high rate using Galβ1-4GlcNAc α(2-6)-sialyltransferase from rat liver.

In contrast to *Vibrio cholerae* sialidase, fowl plague virus sialidase liberates bound *N*-acetyl-4-deoxy-neuraminic acid from the glycoprotein. Thus, as opposed to the general view, the action of neither synthase nor transferase depends on the presence of the hydroxy group at C-4 of *N*-acetylneuraminic acid.

As terminal constituents in oligosaccharide chains of glycoproteins or glycolipids sialic acids play an important role in recognition phenomena involved, for example, in hepatic uptake of serum glycoproteins, virus-host cell interaction, intercellular communication, cell proliferation and tumour metastasis [1-5]. So far, only a few studies have been carried out on the substrate specificity of CMP-sialic acid synthase and sialyltransferase in respect to the sialic acid molecule itself.

As part of a program to investigate the relationship between chemical structure and biological function in the area of sialic acids we are studying the contribution of each part of the sialic acid molecule to enzyme-substrate interaction. In this way inhibitors of these key enzymes may also be found. In addition it might be possible to obtain glycoconjugates containing modified terminal sialic acid, thereby producing compounds with particular biochemical properties.

Abbrevations: BSA, bovine serum albumin; DTE, dithioerythritol; HPLC, high performance liquid chromatography; NeuAc, *N*-acetyl-D-neuraminic acid; 4-deoxy-NeuAc, *N*-acetyl-4-deoxy-D-neuraminic acid; 4-epi-NeuAc, 5-acetamido-3,5-dideoxy-D-glycero-D-talononulosonic acid; CMP-NeuAc, Cytidine-5'-monophospho-*N*-acetylneuraminic acid; CMP-4-deoxy-NeuAc, Cytidine-5'-monophospho-*N*-acetyl-4-deoxy-neuraminic acid; FPV-sialidase, Fowl plague virus sialidase; VCN, *Vibrio cholerae* neuraminidase.

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Activation and transfer of several sialic acid analogues substituted at position C-9 have recently been reported [6, 7]. In general, it is assumed that position C-4 in *N*-acetylneuraminic acid (NeuAc) is a crucial area for interaction of NeuAc with different enzymes involved in sialic acid metabolism. This is supported by studies on the substrate specificity of sialidases. Thus, 4-*O*-acetyl-NeuAc and 4-*O*-methyl-NeuAc are not cleaved by bacterial sialidases [8]. Relative to NeuAc, 4-*O*-methyl-NeuAc was only very slowly converted to the corresponding CMP-glycoside by CMP-sialic acid synthase [9]. 4-*O*-Acetyl-NeuAc and 4-epi-NeuAc were not activated at all [6, 10]. 4-*O*-Methyl-NeuAc was subsequently transferred from the CMP-glycoside to asialoglycoprotein, however, at a very low rate [11].

In order to address the question whether the action of synthase as well as of transferase indeed requires a hydroxy group at C-4, or at least a substituent of small size containing oxygen, we have synthesized a NeuAc analogue with the hydroxy group at C-4 replaced by hydrogen [12]. Independently, 4-deoxy-NeuAc was also prepared by Vasella *et al.* [13]. It turned out that 4-deoxy-NeuAc was a suitable substrate for CMP-sialic acid synthase from bovine brain [12]. Though CMP-4-deoxy-NeuAc was very labile, a high resialylation of asialo-α_1-acid glycoprotein was obtained using pure α(2-6)-sialyltransferase from rat liver.

Materials and Methods

Methods

All chemical buffer substances were of p.a. quality and came from E. Merck (Darmstadt, W. Germany) or Serva (Heidelberg, W. Germany).

Crystalline *N*-acetylneuraminic acid was prepared in this institute from edible bird's nests [14]. 4-Deoxy-NeuAc was synthesized chemically as described earlier [12]. Dithioerythritol (DTE) was purchased from Serva, cytidine-5'-triphosphate from Biomol (Ilvesheim, W. Germany), bovine serum albumin (BSA) and Triton CF-54 from Sigma (München, W. Germany). Both acetonitrile grade E and grade S came from Zinsser (Frankfurt, W. Germany). The aminopropyl column for preparative HPLC (0.7 cm × 12.5 cm, 5 μm particle size) was obtained from Serva, and *Vibrio cholerae* neuraminidase (EC 3.2.1.18) (1 U/ml) from Behring Werke (Marburg, W. Germany).

Fowl plague virus sialidase and α_1-acid glycoprotein were kindly supplied by Dr. R. Rott (Giessen) and Dr. K. Schmid (Boston), respectively. Pure Galβ1-4GlcNAc α(2-6)-sialyltransferase (EC 2.4.99.1) (2.95 U/ml) from rat liver was a generous gift from Dr. J.C. Paulson (Los Angeles). The enzyme was purified according to the published method [15].

Protein Determination

Protein content was measured by the method of Lowry *et al.* using bovine serum albumin as standard [16].

Desialylation of α_1-Acid Glycoprotein

α_1-Acid glycoprotein (9% NeuAc content) was desialylated with *Vibrio cholerae* neuraminidase (VCN). The enzyme was separated from asialoglycoprotein by affinity chromatography (unpublished results). Asialo-α_1-acid glycoprotein contained less than 0.1% bound NeuAc.

Determination of Galactose Acceptor Sites

Galactose content was determined after acid hydrolysis of the glycoprotein (1N HCl, 1h, 2h and 4h at 100°C) using galactose dehydrogenase as described previously [17].

Analytical HPLC

The HPLC system consisted of a Consta Metric III pump (0.1-10 ml/min, Latek), a Rheodyne injection valve, a Spectro monitor III with variable wavelength and a Latek one-channel recorder. CMP-NeuAc and CMP-4-deoxy-NeuAc were detected by chromatography on an aminopropyl-phase column (0.4 cm × 12.5 cm, Serva) which was run isocratically with a mixture of acetonitrile grade E/15 mM KH_2PO_4 in bi-distilled water, 1/1 by vol. CMP and CMP-glycosides were measured at 275 nm and calculated with respect to an external CMP-standard (100 μM).

NeuAc and 4-deoxy-NeuAc were detected by chromatography on an aminopropyl-phase column (0.4 cm × 25 cm, Serva), which was run isocratically with a mixture of acetonitrile grade S/15 mM KH_2PO_4 in bi-distilled water, 7/3 by vol. NeuAc and 4-deoxy-NeuAc were measured at 200 nm and calculated with respect to the corresponding external standards (0.5 mM). Retention time of 4-deoxy-NeuAc was 0.67-fold the respective value of NeuAc.

Preparation of CMP-Sialic Acid Synthase

CMP-sialiac acid synthase (EC 2.7.7.43) from bovine brain was partially purified as described elsewhere [6].

CMP-Sialic Acid Synthase Assay

The enzyme preparation used for kinetic studies exhibited a specific activity of about 200 mU/mg protein. One unit is defined as the amount of enzyme producing 1 μmol CMP-NeuAc per min. The enzyme assay (50 μl) contained 8 μmol Tris-HCl pH 9, 25 μg BSA, 500 nmol CTP, 50 nmol DTE, 2.5 mU CMP-sialic acid synthase and NeuAc or 4-deoxy-NeuAc. The reaction was initiated by CTP and terminated after an appropriate time at 37°C by addition of 60 μl cold acetone. Assay tubes were set on ice for 15 min, centrifuged at 9500 × g for 5 min and finally the supernatant was decanted.

Aliquots (25-40 μl) of this supernatant were analyzed for the amount of CMP-glycoside synthesized by HPLC as described above. No significant amount of CMP-NeuAc or CMP-4-deoxy-NeuAc was lost with the precipitate during this procedure. In contrast, CTP was precipitated almost completely.

Kinetic assays were performed in duplicate as described above using NeuAc or 4-deoxy-NeuAc at the following concentrations: 0.6, 1.0, 2.0, 3.0, 5.0 mM. Initial rates were determined after 6 min incubation time.

Preparative Synthesis of CMP-4-Deoxy-NeuAc

The preparative assay (2 ml) contained 320 μmol Tris-HCl pH 9, 80 μmol $MgCl_2$, 3 μmol DTE, 20 μmol CTP, about 1.7 U CMP-sialic acid synthase and 20 μmol 4-deoxy-NeuAc. After 45 min at 37°C, 500 μl of a solution containing 64 μmol Tris-HCl pH 9, 16 μmol $MgCl_2$, 0.6 μmol DTE, 6.0 μmol CTP and 0.65 U synthase were added. The mixture was incubated for another 45 min and the reaction was subsequently stopped by addition of 3.8 ml cold acetone. The preparative assay was processed further as described elsewhere [6, 7].

Purification of CMP-4-Deoxy-NeuAc

The CMP-4-deoxy-NeuAc synthesized was purified as described elsewhere [6, 7] with the following modifications: Semi-preparative HPLC was performed with an aminopropyl-phase column (0.7 cm × 12.5 cm). CMP-4-deoxy-NeuAc obtained from gel filtration chromatography on Bio-Gel P-2 was immediately lyophilized. Prior to use, the lyophilized powder was dissolved in bi-distilled water.

Characterization of CMP-4-Deoxy-NeuAc

Pure CMP-4-deoxy-NeuAc was characterized after acid hydrolysis as follows: 45 μl pure CMP-glycoside and 5 μl 12 N HCl were mixed. After 45 min at room temperature, the assay was neutralized with 50 μl 1 N NaOH and aliquots were subsequently analyzed. An increase in CMP and in 4-deoxy-NeuAc were calculated by HPLC as described above.

Contamination of CMP and 4-deoxy-NeuAc was determined by HPLC as above. The extinction coefficient of CMP-4-deoxy-NeuAc was measured via the amount of CMP liberated after acid hydrolysis as above. The content of inorganic phosphate in the purified CMP-glycoside was determined by the method of Ames [18]. Purified CMP-glycoside was checked for further impurities using the TLC system described by Higa and Paulson [10].

Transfer Assay

The transfer assay (200 μl) was composed essentially as described by Paulson and coworkers [10, 19] and contained 10 μmol sodium cacodylate buffer, pH 6.5, 200 μg Triton CF-54, 0..28 mg asialo-α_1-acid glycoprotein (155 nmol galactose acceptor sites), and 300 nmol CMP-NeuAc or 480 nmol CMP-4-deoxy-NeuAc, respectively. The CMP content in each CMP-glycoside was 3%. The reaction was started by addition of about 3 mU pure α(2-6)-sialyltransferase from rat liver, and assay tubes were incubated for 16 h at 37°C. Subsequently, glycoprotein was separated from CMP-glycoside and CMP by gel filtration chromatography on a column of Sephadex G-50 fine (0.8 cm × 15 cm) which was eluted with 10 mM NH_4HCO_3. The protein which eluted first was collected from the column effluent and lyophilized. Finally the lyophilized powder was dissolved in 260 μl bi-distilled water.

The following reference was performed for calculation of the glycoprotein recovery during the assay procedure described: An assay composed as above but lacking asialoglycoprotein, CMP-glycoside and sialyltransferase, and containing instead 0.28 mg α_1-acid glycoprotein (9% NeuAc content) was processed exactly as above. Finally the

amount of NeuAc released from the glycoprotein obtained was compared to the NeuAc content expected. In this manner a glycoprotein recovery of 80% was calculated.

Determination of Sialyl Transfer

The NeuAc or 4-deoxy-NeuAc transferred was released from the glycoprotein by Fowl plague virus sialidase. The assay (100 μl) contained 50 μmol sodium acetate pH5.5, 150 mU FPV-sialidase and about 15 μl resialylated glycoprotein obtained from the transfer assay. Assay tubes were incubated for appropriate times at 37°C, and aliquots (20-25 μl) were analyzed for NeuAc or 4-deoxy-NeuAc using HPLC as described above. Assays lacking sialidase in one case or lacking glycoprotein in the other case served as references and were processed as above.

Release of NeuAc or 4-deoxy-NeuAc transferred from the glycoprotein by VCN was investigated by an assay (100 μl) containing 50 μmol sodium acetate buffer pH 5.5, 0.9 μmol $CaCl_2$, 50 μmol NaCl, 5 mU VCN and about 10 μg resialylated glycoprotein. The assay was processed, and analyzed for NeuAc and 4-deoxy-NeuAc as above.

Activity of Fowl Plague Virus Sialidase

Sialidase activity was measured using the 4-methylumbelliferyl α-D-glycoside of *N*-acetylneuraminic acid as described [20].

Results and Discussion

Kinetic Data of the Activation Reaction

Activation of 4-deoxy-neuraminic acid to the corresponding CMP-glycoside was examined with CMP-sialic acid synthase from bovine brain. The partially purified enzyme preparation was free of contaminating enzyme activities which could interfere with the conversion of NeuAc or NeuAc analogues to the respective CMP-glycoside and was therefore suitable for kinetic studies and semi-preparative synthesis of CMP-glycoside [6, 7]. The reaction rate in kinetic assays was always measured at saturating concentrations of CTP (10 mM).

Enzyme activity was linear with protein concentration up to 19.5 μg per 50 μl (= 3.8 mU enzyme). The effect of the incubation time on the reaction rate was monitored with NeuAc and 4-deoxy-NeuAc at the highest and at the lowest substrate concentration used in the kinetic studies. Product formation in assays containing 2.5 mU enzyme and 0.6 mM of either substrate was linear with time for 10 min. Product formation in assays containing 2.5 mU enzyme and 5 mM of either substrate was linear with time for 30 min in the case of NeuAc, and for 20 min in the case of 4-deoxy-NeuAc. Thus, kinetic assays were performed with 2.5 mU synthase and the reaction was allowed to proceed for 6 min. Initial rates were determined from product formation measured by analytical HPLC at 275 nm as described in the Methods section.

Fig. 1 shows the activation kinetics of 4-deoxy-NeuAc and NeuAc plotted according to the method of Hanes [21]. The K_M value for NeuAc (1.55 mM) was similar to that determined in earlier studies (1.4 mM), using the identical synthase preparation from bovine

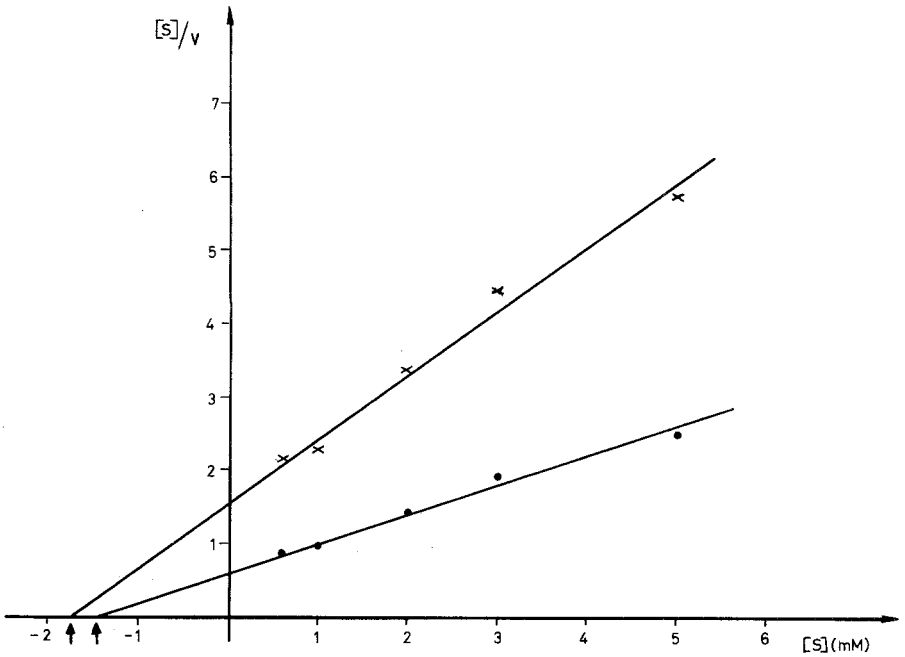

Figure 1. Activation kinetics of 4-deoxy-NeuAc (×) and NeuAc (●).

Substrate concentration (S) and initial reaction rate (v) are plotted according to the method of Hanes [21]. Initial rate was determined via product formation measured by analytical HPLC at 275 nm as described in the Methods section. Each point represents the average of duplicate assays. The K_M-values are indicated by arrows.

brain, but different assay conditions [6]. The K_M value measured for 4-deoxy-NeuAc (1.8 mM) was not significantly different from that of NeuAc (1.55 mM). In contrast, the V_{max}-value of 4-deoxy-NeuAc was only 0.45-fold the respective value of NeuAc. The kinetic data were obtained from Hanes plots [21] and are means of three kinetic studies.

Preparative Synthesis of CMP-4-Deoxy-NeuAc

Fig. 2 shows the time course for conversion of 4-deoxy-NeuAc and NeuAc to the corresponding CMP-glycosides. Though high synthase activity was used and the reaction was shifted towards product formation by further additions of enzyme and CTP, only 35-40% of 4-deoxy-NeuAc was activated. In contrast, about 70-75% of NeuAc was activated in an identical preparative assay.

The slow conversion of 4-deoxy-NeuAc correlates well with the low V_{max}-value and was additionally due to a marked degradation of CMP-4-deoxy-NeuAc during the incubation time. Further attempts to reach higher activation rates by several additions of synthase or by prolonged incubation time only reduced the amount of CMP-4-deoxy NeuAc finally synthesized. Thus, preparative assay was started with high synthase activity, and performed routinely for 1.5 h with one addition of enzyme and CTP during the time course as described in the Methods section.

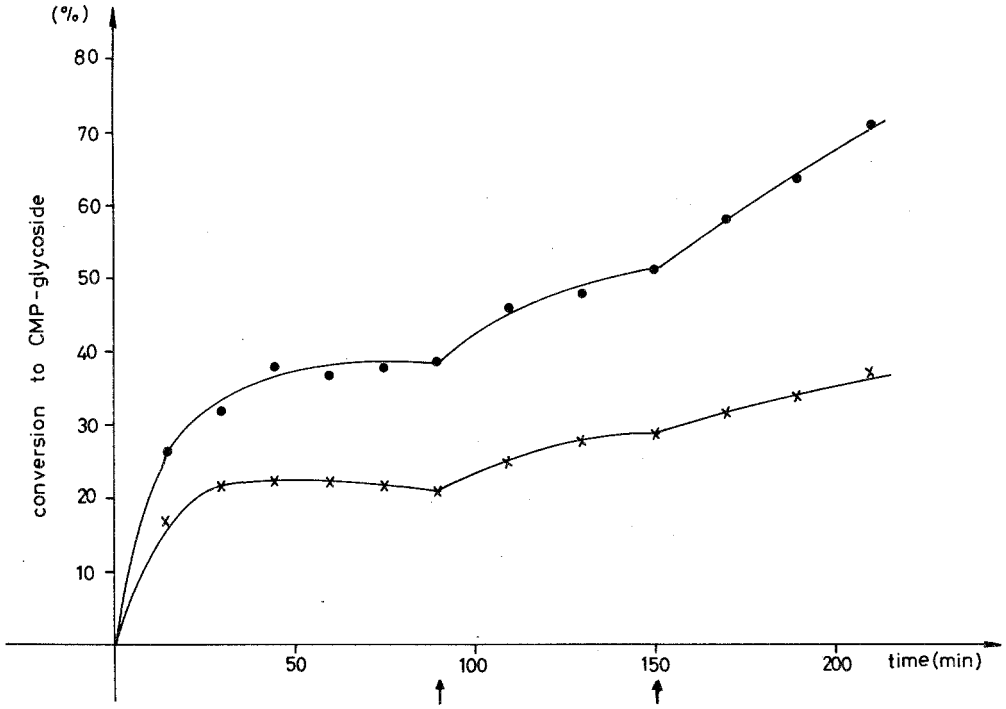

Figure 2. Time course for the preparative synthesis of CMP-4-deoxy-NeuAc (×) and CMP-NeuAc (●).

Preparative synthesis was performed essentially as described in the Methods section with 4-deoxy-NeuAc and NeuAc as substrate. CMP-sialic acid synthase activity used was modified as follows: Synthesis was started with 1 U enzyme. After 45 and 90 min, 0.4 and 0.6 U synthase, respectively, were added together with CTP, DTE and buffer as described in the Methods section. This is indicated by arrows. At various times 20 μl aliquots were withdrawn from the reaction mixture and the reaction stopped by addition of 25 μl cold acetone. The samples were put on ice for 15 min, centrifuged for 5 min at 9500 × g, and the supernatant was analyzed for the amount of CMP-glycoside synthesized by HPLC as described in the Methods section. Complete conversion of the substrate to a CMP-glycoside was taken as 100%.

The degradation of CMP-4-deoxy-NeuAc was monitored via CMP concentration by analytical HPLC as described in the Methods section. During preparative synthesis, CMP concentration increased constantly and reached 14% of the concentration of CMP-4-deoxy-NeuAc after 90 min. In contrast, concentration of CMP during synthesis of CMP-NeuAc was always less than 1.0% related to CMP-NeuAc.

Purification of CMP-4-Deoxy-NeuAc

Purification of CMP-4-deoxy-NeuAc was performed as described elsewhere for several CMP-activated NeuAc analogues [6, 7]. However, this procedure proved to be difficult due to the high lability of CMP-4-deoxy-NeuAc which was not observed for CMP-NeuAc or other CMP-NeuAc analogues purified in this laboratory. After purification which included semi-preparative HPLC, ethanol precipitation and gel filtration chromatography,

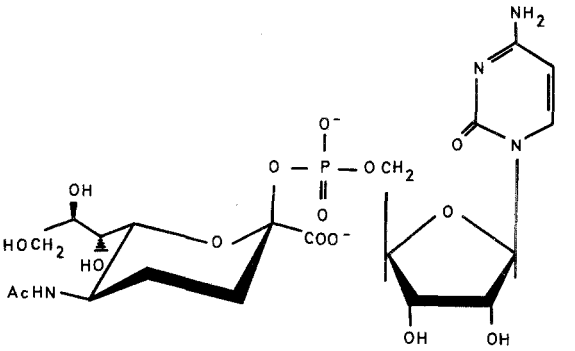

Figure 3. Structural formula of CMP-4-deoxy-NeuAc.

about 33% of CMP-4-deoxy-NeuAc synthesized in the preparative assay was obtained. The identical procedure gave about 55% of CMP-NeuAc. Considering in addition the low conversion rate during preparative synthesis, the overall yield of pure CMP-4-deoxy-NeuAc was 10-15% based on the amount of 4-deoxy-NeuAc used. In contrast, CMP-NeuAc was purified by the same procedure with 45-50% yield.

Characterization of CMP-4-Deoxy-NeuAc

Pure CMP-4-deoxy-NeuAc was characterized by analytical HPLC as described in the Methods section (for the structure formula, see Fig. 3). After acid hydrolysis a molar ratio of CMP/4-deoxy-NeuAc of 0.97/1.0 was calculated. Contamination with CMP or 4-deoxy-NeuAc amounted to 3-7%. During storage of CMP-4-deoxy-NeuAc in water at 4°C an approximately 2.5-fold increase in CMP was observed after 48 h. The extinction coefficients of CMP-4-deoxy-NeuAc and CMP-NeuAc were identical, as expected.

Sialyl Transfer

Transfer of 4-deoxy-NeuAc to asialoglycoprotein was examined with pure Galβ1-4GlcNAc α(2-6)-sialyltransferase from rat liver. The enzyme catalyses the transfer of sialic acids to non-reducing β-linked galactose in *N*-glycosidically bound oligosaccharide chains of glycoproteins [19].

The transfer assay described in the Methods section was aimed at favouring a high resialylation of asialo-α_1-acid glycoprotein. The amount of CMP-glycoside exceeded the potential galactose acceptor sites about 2-fold (nmol/nmol) in the case of CMP-NeuAc and about 3-fold (nmol/nmol) in the case of CMP-4-deoxy-NeuAc. The concentration of CMP-NeuAc (1.5 mM) and CMP-4-deoxy-NeuAc (2.4 mM) was 20-fold and 30-fold, respectively, the apparent K_M-value of Galβ1-4GlcNAc α(2-6)-sialyltransferase from rat liver for CMP-NeuAc. To enhance the stability of the CMP-glycosides, the pH was raised to 6.5 compared to the assay conditions described by Paulson and coworkers [10, 19]. Transfer of 4-deoxy-NeuAc could not be determined by the thiobarbituric acid method after acid hydrolysis of the glycoprotein (0.1 N HCl, 1 h at 80°C), since during acid hydrolysis (as above) 4-deoxy-NeuAc was degraded almost completely, and additionally the lack of the hydroxy group at C-4 blocks chromophore formation in the thiobarbituric acid reaction

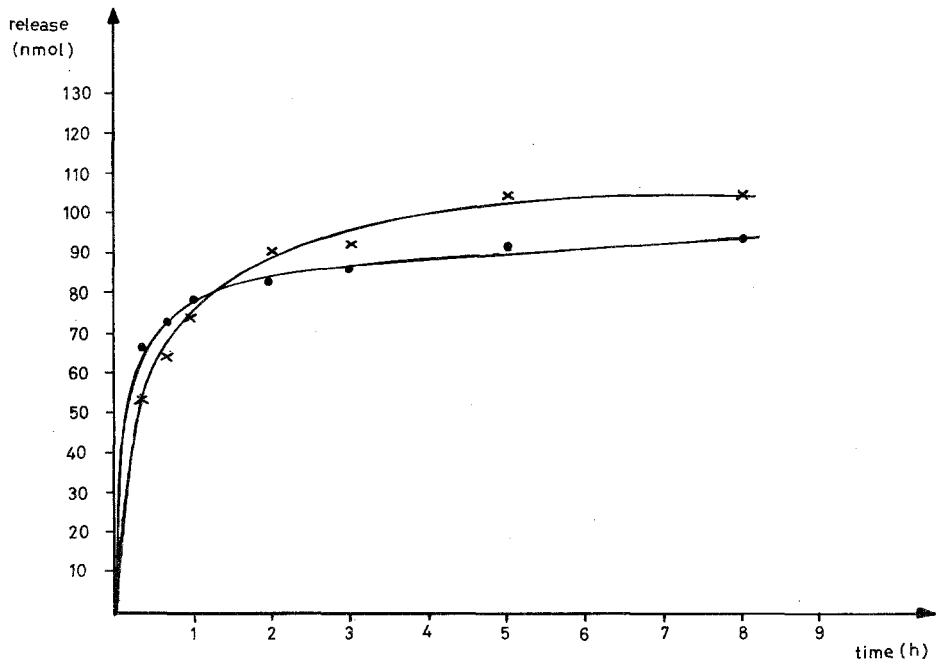

Figure 4. Time course for the release of transferred 4-deoxy-NeuAc (×) and NeuAc (●) by Fowl plague virus sialidase.

The transfer assay and sialidase assay were performed as described in the Methods section. 4-Deoxy-NeuAc or NeuAc released were calculated by analytical HPLC.

(unpublished results). Thus, transferred 4-deoxy-NeuAc and NeuAc were calculated by analytical HPLC after release from the glycoprotein by FPV-sialidase. This enzyme was chosen, because the methyl α-D-glycoside of 4-deoxy-NeuAc is resistant towards the action of several bacterial sialidases, but in contrast is readily cleaved by FPV-sialidase [12].

The time course for the release by FPV-sialidase of transferred 4-deoxy-NeuAc and NeuAc is shown in Fig. 4. A great excess of sialidase was used to ensure complete cleavage, because FPV-sialidase hydrolyzes the α(2-6)-glycosidic linkage formed by the sialyltransferase only very slowly [22, 23]. Cleavage was complete after 8 h in both cases, about 105 nmol 4-deoxy-NeuAc and about 95 nmol NeuAc being released from the glycoprotein, based on the galactose acceptor sites considering 80% glycoprotein recovery, as described in the Methods section. This means that high resialylation of 75% of the galactose acceptor sites was obtained with CMP-NeuAc and of 82% with 4-deoxy-NeuAc as donor substrate. α_1-Acid glycoprotein with a sialylation of 85% of the galactose acceptor sites represents the highest grade available (12% neuraminic acid content) [24].

In contrast, 4-deoxy-NeuAc was not released from the glycoprotein by VCN after 8 h using the procedure described in the Methods section. This was expected from the results obtained with the respective methyl α-glycoside [12]. The amount of NeuAc released from the glycoprotein by VCN after 8 h (108 nmol) was comparable to that obtained with FPV-sialidase.

The high transfer rate obtained with 4-deoxy-NeuAc was surprising. Though the pH in the transfer assay was raised to 6.5, CMP-4-deoxy-NeuAc was degraded very fast during the incubation, about 10-fold faster than CMP-neuraminic acid. Thus, it can be assumed that 4-deoxy-NeuAc is transferred at a higher rate than NeuAc considering the identical incorporation into asiolo-α_1-acid glycoprotein after 16 h.

In order to support this conclusion, a transfer assay was performed for 30 min essentially as described in the Methods section using CMP-neuraminic acid at 1.0 mM and CMP-4-deoxy-NeuAc at 1.0 mM or 2.0 mM. The transfer rate determined for 4-deoxy-NeuAc was 2- to 2.5-fold faster than that with NeuAc. This incorporation rate of 4-deoxy-NeuAc did not represent a true initial rate, because of the fast degradation of CMP-4-deoxy-NeuAc and the resulting increase in CMP, which is a competitive inhibitor of sialyltransferases.

Conclusions

In this report we have shown that *N*-acetyl-4-deoxy-neuraminic acid acts as a good substrate for CMP-sialic acid synthase from bovine brain. The K_M-value is similar to that of NeuAc, whereas the V_{max} differed giving only half the value obtained with NeuAc. Previously described modifications at C-4 influenced the activation reaction much stronger. Thus the V_{max} value determined for 4-*O*-methyl-NeuAc was decreased strongly to 20% of the V_{max} measured for NeuAc, whereas, 4-*O*-acetyl-NeuAc and 4-epi-NeuAc were not accepted at all as a substrate [6, 9, 10].

The results imply the following important conclusions. An axial substituent at C-4 as in 4-epi-NeuAc blocks activation. The size of the equatiorial sulbstituent at C-4 strongly in fluenres the activation reaction (methyl versus acetyl). On the other hand complete absence of an oxygen-containing substituent, in other words replacement of the hydroxy group by hydrogen, is well tolerated by the enzyme.

Surprisingly, CMP-4-deoxy-NeuAc decomposed at a much higher rate than CMP-NeuAc or the other CMP-glycosides prepared in this laboratory [6, 7]. The reason for the destabilizing effect of a second deoxy function (at C-4) is not clear. Despite this instability, 4-deoxy-NeuAc was readily transferred from the CMP-glycoside to asialo-α_1-acid glycoprotein by α(2-6)-sialyltransferase from rat liver. Though the CMP-glycoside decomposed rapidly during the transfer assay, the extensive resialylation obtained was apparently due to a very high initial rate.

The level of incorporation reached for 4-deoxy-NeuAc and NeuAc was in a comparable range. In contrast, 4-*O*-methyl-NeuAc gave only a very low resialylation of asialoglycoprotein [11]. However, it should be noted that in these studies a microsomal sialyltransferase preparation was used. The sialidases behave differently towards 4-deoxy-NeuAc, as the respective methyl α-glycoside was no substrate for the enzymes from *Vibrio cholerae, Clostridium perfringens* and *Arthrobacter ureafaciens* [12]. In sharp contrast, Fowl plague virus sialidase readily attacked this substrate [12]. Similarly, mammalian sialidases accept the methyl α-glycoside of 4-deoxy-NeuAc as a substrate, as demonstrated for bovine testis sialidase [12]. 4-Deoxy-NeuAc transferred to asialo-α_1-acid glycoprotein was completely released by FPV sialidase, but not by *Vibrio cholerae* sialidase, which is in accordance to the results obtained with the methyl α-glycoside.

In contrast to the generally adopted view, the results presented here indicate that a hydroxy group or an oxygen-containing substituent at C-4 is not required for enzymatic action of CMP-sialic acid synthase from bovine brain as well as for α(2-6)-sialyltransferase from rat liver. This means that hydrogen bonding involving C-4 of sialic acid is not a prerequisite for enzyme substrate interaction. However, replacement of the hydroxy group by hydrogen at this position apparently influences the catalytic rate of both enzymes in an opposite way. 4-Deoxy-NeuAc represents an additional synthetic analogue of sialic acid which can be used to modify glycoproteins enzymatically in the terminal position.

The fact that surprising differences in substrate specificity among enzymes involved in sialic acid metabolism can be recognized using synthetic NeuAc analogues is intriguing and stimulates further studies.

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