Substrate and Product Specificity of *Arthrobacter sialophilus* Neuraminidase*

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*Arthrobacter sialophilus* neuraminidase catalyzes the hydrolysis of N-acetylneuraminyl-α-oxygen, nitrogen, and azido glycosides. The most effective of these substrates examined was N-acetylneuraminyl-α-4-methylumbelliferylglycoside (AcNeu-α-4-MU; $K_a$ app. 0.6193 mM; $k_{cat}$ 136.4 sec$^{-1}$). The products resulting from the enzymic hydrolysis of N-acetylneuraminyl-α-azidoglycoside were N-acetylneuraminic acid and azide ion. N-acetylneuraminyl-α-2,3-thiogalactosylglycoside and N-acetylneuraminyl-α-2,6-thiogalactosylglycoside were competitive inhibitors of the enzyme having $K_i$ values of 1.52 mM and 1.70 mM, respectively. Dissociation constants for these thioglycosides were also determined by fluorescence enzyme titrations which gave values similar to those determined kinetically. N-Acetylneuraminic acid, but not its methyl ester, was a competitive inhibitor of neuraminidase. Its $K_i$ value, 0.18 mM, was also determined by both methods. 5-Acetamido-2,6-anhydro-3,5-dideoxy-α-glycero-β-talo-nonulosonic acid (2-deoxy-4-epi-AcNeu) was found to be a weak competitive inhibitor ($K_i$, 12.1 mM). *A. sialophilus* neuraminidase further catalyzes transglycosidation reactions with methanol as acceptor. Methanol had no effect on the release of 4-MU by enzymatic hydrolysis of AcNeu-α-4-MU, suggesting that the formation of the enzyme-glycone intermediate is the rate-determining step. The anomeric configuration of the product of this reaction, as shown by $^{13}$C-nmr spectroscopy, is N-acetylneuraminyl-α-methylglycoside. Neuraminidase, therefore, catalyzes its reactions with overall retention of configuration.

Studies involving the glycone specificity of exoglycosidases are of interest because they have the potential to delineate complimentary topographical limits for binding regions, as well as to provide additional information on mechanisms of action. Neuraminidases are glycohydrolases which catalyze the hydrolyses of sialyl-α-oxygen, nitrogen, and azido glycosides; whereas the α-sulfur glycosides are competitive inhibitors. In addition, we demonstrate that this enzyme can catalyze transglycosidation reactions, and establish that the configuration of its enzymic products are α- at their anomeric centers. This latter finding complements a recent report which used a different experimental method (16). These results are discussed in light of current general knowledge concerning the mechanisms of glycohydrolase activities.

**MATERIALS AND METHODS**

Neuraminidase from *A. sialophilus* was induced and purified as

The abbreviations used are: AcNeu, N-acetylneuraminic acid (I); AcNeu methyl ester, N-acetylneuraminic acid methyl ester (II); AcNeu-α-β-Me, N-acetylneuraminyl-β-methylglycoside (III); AcNeu-β-Me, N-acetylneuraminyl-β-methylglycoside (IV); AcNeu-α-4-MU, N-acetylneuraminyl-α-4-methylumbelliferylglycoside (V); AcNeu-α-N$_2$, N-acetylneuraminyl-α-nitrophenylglycoside (VI); AcNeu-α-N$_3$, N-acetylneuraminyl-α-nitrophenylglycoside (VII); AcNeu-α-S-2,3-Gal, N-acetylneuraminyl-α-2,3-thiogalactosylglycoside (VIII); AcNeu-α-S-2,6-Gal, N-acetylneuraminyl-α-2,6-thiogalactosylglycoside (IX); 2-deoxy-4-epi-AcNeu, 5-acetamido-2,6-anhydro-3,5-dideoxy-α-glycero-β-talo-nonulosonic acid (X); 2,3-dehydro-AcNeu, 5-acetamido-2,6-anhydro-3,5-dideoxy-α-glycero-β-talo-non-2-enonic acid; 2,3-dehydro-4-epi-AcNeu, 5-acetamido-2,6-anhydro-3,5-dideoxy-α-glycero-β-talo-non-2-enonic acid; 2,3-dehydro-4-keto-AcNeu, 5-acetamido-2,6-anhydro-3,5-dideoxy-α-glycero-β-talo-non-2-enonic acid; 2,3-dehydro-4-keto-AcNeu, 5-acetamido-2,6-anhydro-3,5-dideoxy-α-glycero-β-talo-non-2-enonic acid.

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previously described (4), and was judged to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. AcNeu (I) was prepared from "edible bird's nest" as previously described (17). AcNeu-methyl ester (II), AcNeu-a-Me (III), AcNeu-b-Me (IV), AcNeu-a-4-MU (V), and AcNeu-a-N-p-NP (VII) were prepared according to procedures as described in the literature (15, 18-20) (see Fig. 1). 2-Deoxy-4-epi-AcNeu (X), AcNeu-a-S-2,3-Gal (VIII), AcNeu-a-S-2,6-Gal (IX), and AcNeu-a-N (VI) (see Fig. 1) were prepared and purified by preparative high performance liquid chromatography and met 'H-nmr, 'C-nmr, and mass spectrometric criteria. These procedures will be published elsewhere. Methanol enriched with 13C was obtained from Merck Sharp and Dohne. Other chemicals and reagents were of the best commercially available grade.

Neuraminidase Assays—AcNeu-a-N3, AcNeu-a-Me, AcNeu-a-N-p-NP, and N-acetylneuraminic citost were assayed in 10 mM sodium phosphate, pH 6.0, at 37 °C as previously described (4). The kinetic constants for AcNeu-o-N-hydrolysis were determined by continuously monitoring the release of azide at 245 nm. A continuous fluorimetric assay with AcNeu-a-4-MU in 10 mM sodium phosphate, pH 6.0, at 37 °C (Perkin-Elmer 650-10S spectrofluorimeter) was used to determine the kinetic constants, essentially as described by Shulman et al. (21). Initial reaction rates were determined for all substrate concentrations. For the determination of kinetic constants, six substrate concentrations ranging from 0.30 to 2.0 mM the K M value were used. Lineweaver-Burk plots or the direct linear plot of Eisenhal-Cornish-Bowden (22) gave essentially identical values. The graphic procedures recommended by Segel (23) were used to determine K M values.

Direct Binding Assay—Spectrofluorimetric determinations of the dissociation constant for AcNeu-a-2,3-Gal, AcNeu-a-2,6-Gal, and AcNeu were performed by intrinsic protein fluorescence measurements (λ e, 280 nm, λ m, 297 nm) according to the procedure of Benesi and Hildebrand (24). Enzyme concentrations were always at least 100 times less than inhibitor concentrations.

Transglycosidation Reaction Conditions—N-Acetylneuraminic acid (0.080 mM) in 60% [13C]methanol (90% enrichment; v/v) was treated with A. sialophilus neuraminidase (0.5 pg) in 10 mM sodium phosphate buffer, pH 6.0, at room temperature, and the reaction was allowed to proceed until the concentration of AcNeu was 0.950 mM. The reaction mixture was heated at 100 °C for 20 min, washed thoroughly with methanol, freeze-dried and the product resolved in D2O. Fourier transform proton decoupled 13C-nmr spectra were measured at a frequency of 25.2 MHz with a Varian XL-100 spectrometer. Chemical shifts are expressed in ppm relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

RESULTS

Substrate Specificity Studies—A. sialophilus neuraminidase catalyzes the hydrolysis of oxygen, nitrogen, and azido glycosides; their K M and K cat values are given in Table I. With each substrate, the enzyme exhibited typical Michaelis-Menten kinetics. Catalytic hydrolysis of the AcNeu-a-N3 gave AcNeu (yield 98%) and azide ion (yield 100%) as products. From the K M/K cat ratios, the most effective neuraminidase substrate among those tested was AcNeu-a-4-MU, whereas AcNeu-a-N-p-PN was the least effective.

The two thioglycosides examined were competitive inhibitors with respect to AcNeu-a-4-MU, as shown in Fig. 2. At those concentrations of inhibitor tested, double reciprocal plots of v versus [S] were linear and the K M values were calculated as 1.52 mM and 1.70 mM for AcNeu-a-2,3-Gal and AcNeu-a-2,6-Gal, respectively. Control experiments with very large amounts of enzyme (40 units/assay) and prolonged incubation time (3 h) failed to indicate the release of free AcNeu.

A. sialophilus neuraminidase contains a single peak in its fluorescence emission spectrum at 327 nm. Addition of increasing amounts of either thioglycoside to neuraminidase resulted in the decrease of the intrinsic fluorescence of the enzyme, approaching maximal quenching of 10%, (see Fig. 3). Data points from the titration fit a theoretical curve drawn for 1:1 binding stoichiometry with the enzyme (Fig. 3, inset) from which dissociation constants of 1.57 mM and 1.31 mM for AcNeu-a-2,3-Gal and AcNeu-a-2,6-Gal, respectively, are obtained. These values are in excellent agreement with those determined kinetically.

Effect of Methanol on Glycoside Hydrolysis—The effect of methanol on the enzymatic hydrolysis of AcNeu-a-4-MU was examined, as shown in Fig. 4. Increasing concentrations of methanol do not significantly diminish the release of 4-MU, but substantially decrease the formation of AcNeu. The results of these kinetic studies support two conclusions: (a), that the enzyme can catalyze transglycosidation reactions and (b), that the formation of the enzyme-glycosyl intermediate is the rate-determining step.
Substrate and Product Specificity of Neuraminidase

The anomic configuration of the generated AcNeu-Me in this transglycosidation reaction was determined. The chemical shift in the $^{13}$C-nmr spectrum for the OCH$_3$ group (8 51.77 ppm) of the reaction product is almost identical to synthetic AcNeu-α-Me (δ 51.80 ppm (25, 26)). No AcNeu-β-Me (chemical shift for OCH$_3$, δ 51.00 ppm (25, 26)) could be detected. Reference spectra demonstrated that the resonances of the OCH$_3$ group of AcNeu-α-Me and AcNeu-β-Me can readily be distinguished in a mixture of these two glycosides. These results demonstrate that the A. sialophilus neuraminidase catalyzes the hydrolysis of its substrates with overall retention of configuration at the anomic carbon.

Product Binding—Neuraminidases obtained from a number of sources (27-29) have been reported to be inhibited by AcNeu. As seen in Fig. 4, double reciprocal plots indicate that AcNeu is a competitive inhibitor with respect to AcNeu-α-MU with a $K_I$ value calculated as 0.182 mM; determination of the $K_D$ (0.167 mM) spectrofluorometrically gave a similar value. 2-Deoxy-4-epi-AcNeu was also a competitive inhibitor, albeit of low effectiveness, with respect to glycoside hydrolysis ($K_{Ie}$, 12.1 mM; Fig. 5). Neither AcNeu methyl ester nor 2-deoxy-4-epi-AcNeu methyl ester inhibited the A. sialophilus neuraminidase at $10^{-4}$ M.

**DISCUSSION**

There is an extensive literature concerning the properties of neuraminidase as a result of its use as a macromolecular probe for the removal of sialic acids from cell surfaces (1-3) and because of its potential pathogenesis in several human diseases (2, 3, 30, 31). Little, however, is known about its mechanism of action. The preparation of substantial quantities of homogeneous neuraminidase from A. sialophilus (4) has now enabled us to initiate a detailed biochemical study of this enzyme.

We have demonstrated with A. sialophilus neuraminidase that variations in the structure of the aglycone residue of the AcNeu-α-glycosides affect both $K_{ma}$ and $K_{mc}$ values. However, the relationship between $K_{ma}$ and $K_{mc}$ was highly irregular with different aglycones (Table I). Similar variations in the $K_{ma}$ and $V_{max}$ values for other synthetic and natural substrates have been reported for neuraminidases obtained from V. cholerae and influenza viruses (1, 31-34). The most efficient substrate

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**Fig. 3.** Direct fluorimetric binding determination of the $K_D$ of AcNeu-α-S-2,3-Gal and AcNeu-α-S-2,6-Gal. Double reciprocal plots of the change in relative fluorescence (excitation 280 nm, emission 327 nm) versus the concentration of AcNeu-α-S-2,3-Gal (A) and AcNeu-α-S-2,6-Gal (B). Inset, replot of the measured per cent enzyme complexes versus the concentration of inhibitor. The solid lines represent the theoretical amount of enzyme complexes for the $K_D$ value calculated from the respective double reciprocal plot.

**Fig. 4.** Effect of methanol on the hydrolysis of AcNeu-α-4-MU by A. sialophilus neuraminidase. AcNeu-α-4-MU (0.02 mM) in 10 mM sodium phosphate, pH 6.0, was assayed at 37 °C in the presence of increasing concentrations of methanol. Methylumbelliferyl sugar was determined fluorimetrically as described in "Materials and Methods" (upper curve) and AcNeu was determined by the procedure described by Pappenberger and Hammond (35) (lower curve). Rates are expressed as % control (zero methanol).

**Fig. 5.** Inhibition of A. sialophilus neuraminidase by AcNeu and 2-deoxy-4-epi-AcNeu. The concentrations of AcNeu-α-4-MU are expressed in molarity and the velocities are expressed in units/mg of protein. Assays were run at pH 6.0 in 10 mM sodium phosphate buffer at 37 °C. A, AcNeu, (C) zero inhibitor; (D) 7.85 × 10⁻⁴ M inhibitor; (E) 2.05 × 10⁻⁴ M inhibitor; (F) 3.45 × 10⁻⁴ M inhibitor; (G) 5.75 × 10⁻⁴ M inhibitor. B, 2-deoxy-4-epi-AcNeu, (C) zero inhibitor; (D) 1.15 × 10⁻² M inhibitor; (E) 2.03 × 10⁻³ M inhibitor; (F) 3.20 × 10⁻³ M inhibitor. Insets, plots of $K_{Ie}$ versus inhibitor concentration.
for A. sialisphillus neuraminidase was the AcNeu-α-4-MU, as reflected by its $k_{cat}/K_m$ ratio (see Table I). This result may reflect the electron-withdrawing effect of this aglycone, in contrast to that shown by alkyl AcNeu derivatives.

Replacement of the anomeric oxygen or aglycone of AcNeu-α-glycosides has little effect on the apparent binding of these glycosides to the enzyme, but significantly alters the $k_{cat}$ values. This results in $k_{cat}/K_m$ ratios ranging from 1.79 × 10⁵ for the azido glycoside, to 1.52 × 10⁴ for the N-glycoside. The decrease effectiveness of AcNeu-α-N-p-NP as a substrate is clearly not due to binding, but to cleavage of its C–N bond (Table I). These findings conflict with an earlier report of Priwalowa et al. (15, 29) which claimed that N-glycosides are competitive inhibitors for V. cholerae neuraminidase. Aside from a species difference, one reasonable explanation for this discrepancy could be our use of the more sensitive fluorimetric assay for AcNeu (35).

As has been analogously observed with β-galactosidase (36), AcNeu-α-N₃ is an extremely effective substrate for Arthrobacter neuraminidase. Replacement of the oxygen atom by sulfur to yield alkyl thioglycosides leads to complete loss of catalytic activity. Determination of $K_i$ values, as shown here, confirms that these values are equilibrium dissociation constants. Control experiments with large amounts of enzyme, prolonged incubation time, and a more sensitive assay for AcNeu, ruled out the possibility that these thioglycosides were poor substrates. A comparable pattern of substrate specificity with galactosyl-β-glycosides containing different heteroatoms has also been observed for β-galactosidase (36–38).

That glycohydrolases (e.g. lysozyme (39–41) and β-galactosidase (37, 42, 43)) can catalyze transglycosidation reactions has been extensively documented. The utilization of the chromogenic substrate, AcNeu-α-4-MU, has allowed us to demonstrate that neuraminidase can also catalyze the transfer of N-acetylneuraminyl groups to methanol. The data in Fig. 3 indicate that the rate of free AcNeu production declines with increasing methanol, suggestive of partitioning of an intermediate between water and methanol with the generation of AcNeu-α-Me. The simplest kinetic scheme for this transglycosidation reaction is

$$E + S \xrightarrow{k_1} ES \xrightarrow{k_2} ES^* \xrightarrow{k_{(H_2)O}} E + P_1 \xrightarrow{k_{(MeOH)}} E + P_2$$

where $E$, $S$, and $ES$ stand for enzyme, substrate, Michaelis complex, and second intermediate complex, $P_1$ is the glycone part of the substrate, $P_2$ is AcNeu, and $P_3$ represents AcNeu-α-Me. Since methanol has no effect on the release of $P_1$, $k_2$ must be smaller than $k_1$ or $k_3$ and the release of umbelliferone is the rate-determining step for this substrate. Although the exact mechanism of the transglycosidation reaction cannot be deduced, it is clear that the enzyme-bound intermediate cannot spontaneously react with water but must have a sufficient lifetime to allow for the diffusion of the leaving group (aglycone) and the diffusion of methanol into the active site. That $P_1$ has the α-configuration was determined by 13C-nmr spectroscopy. From these results, it follows that A. sialisphillus neuraminidase catalyzes the hydrolysis of AcNeu-α-glycosides with overall retention of configuration. The latter finding is in agreement with a recent report that the α-anomer of AcNeu is released by V. cholerae neuraminidase (16).

By two independent methods, we have established that AcNeu is a competitive inhibitor of the enzyme with a $k_i$ value of 0.18 mm. Since neuraminidase releases strictly the α-anomer of AcNeu (16) and also synthesizes α-glycosides in transglycosidation reactions (see above), it appears reasonable to assume that the enzyme binds only the α-anomer. It has been previously determined that the β-anomer of AcNeu is thermodynamically more stable, and that only 5-8% of the sugar in solution has the α-configuration (16). Therefore, the minimal dissociation constant for AcNeu binding to neuraminidase is about $10^{-6}$ M.

Modification of the carboxyl moiety of sialic acid-α-keto-sides to either an ester, amide, or alcohol makes the resultant derivative neither a substrate nor inhibitor of neuraminidases (6–9). Since neither the methyl esters of AcNeu nor 2-deoxy-4-epi-AcNeu are enzyme inhibitors, this functionality is also obligatory for product binding.

Although a mechanistic interpretation of the $K_m$ values reported in Table I is not possible, it appears that the aglycone exerts a strong influence on substrate affinity. The active site of A. sialisphillus neuraminidase, therefore, can be visualized as having two binding subsites, one a specific site complementary to AcNeu which determines whether a given glycoside is a substrate, and a second, less specific subsite for the aglycone. The existence of such analogous subsites has been reported for chymotrypsin (44) and for other enzymes (45, 46). The nature of the hetroatom present in AcNeu-glycosides does not significantly affect binding to the enzyme but manifests itself in altering the $k_{cat}$ values. As also observed with β-galactosidase (38), replacement of the oxygen by sulfur in alkylthioglycosides makes the resultant compounds competitive inhibitors. The nature of the aglycone also influences the catalytic efficiency of neuraminidase, as reflected by changes in the $k_{cat}/K_m$ values; and, as recently reported for β-galactosidase (47), may also change the rate-determining step for the reaction.

The results reported in this paper begin to establish the active site of A. sialisphillus neuraminidase with respect to the role played by the aglycone in enzyme catalysis. Studies are currently in progress to completely characterize the binding site of this enzyme and to elucidate its mechanism of action. A detailed understanding of these properties may provide insight into the design of mechanism-based inhibitors.

REFERENCES

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