Mutagenesis of the Conserved Active-Site Tyrosine Changes a Retaining Sialidase into an Inverting Sialidase†

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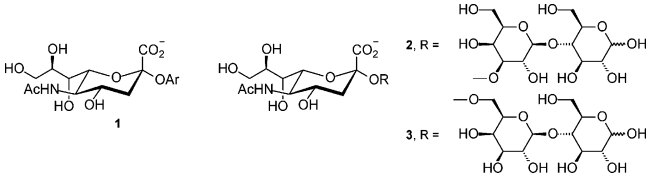
ABSTRACT: Mutagenesis of the conserved tyrosine (Y370) of the *Micromonospora viridifaciens* sialidase changes the mechanism of catalysis from retention of anomeric configuration to an unprecedented inverting mechanism in which water efficiently functions as the nucleophile. Three mutants, Y370A, Y370D, and Y370G, were produced recombinantly in *Escherichia coli*, and all are catalytically active against the activated substrate 4-methylumbelliferyl α-D-*N*-acetylneuraminide. The Y370D mutant was also shown to catalyze the hydrolysis of natural substrate analogues such as 3′-sialyllactose. A comparison of the pH-rate profiles for the wild-type and the Y370D mutant sialidase reveals no major differences, although with respect to the kinetic term k_{cat}/K_m, an ionized form of the aspartate-370 enzyme is catalytically compromised. For the wild-type enzyme, the value of the Brønsted parameter β_{lg} on k_{cat} is 0.02 ± 0.03, while for the Y370D mutant sialidase $\beta_{lg} = -0.55 \pm 0.03$ for the substrates with bad leaving groups. Thus, for the wild-type enzyme, a nonchemical step(s) is rate-limiting, but for the tyrosine mutant cleavage of the glycosidic C−O bond is rate-determining. The Brønsted slopes derived for the kinetic parameter k_{cat}/K_m display a similar trend (β_{lg} −0.30 ± 0.04 and −0.74 ± 0.04 for the wild-type and Y370D, respectively). These results reveal that the tyrosine residue lowers the activation free energy for cleavage of 6′-sialyllactose, a natural substrate analogue, by more than 24.9 kJ mol^{-1}. Evidence is presented that the mutant sialidases operate by a dissociative mechanism, and the wild-type enzyme operates by a concerted mechanism.

Sialidases (*N*-acetylneuraminosyl glycohydrolase, neuraminidase, EC 3.2.1.18) are exoglycosidases that catalyze the hydrolysis of sialic acid residues, linked to glycoconjugates (*1*). These enzymes are involved in the pathogenesis of many human diseases, including influenza and cholera (*2*, *3*). Within the sialidase superfamily, sequence homology (*4*) and X-ray crystal structure data (*5*) have revealed that the active site contains, in every case, a conserved tyrosine, a pair of acidic residues (glutamic and aspartic acid), and an arginine triad that interacts with the carboxylate group of the substrate (*6*). Furthermore, all known sialidases catalyze hydrolysis of the anomeric sialyl ketal linkage with overall retention of configuration (*7*−*10*).

Prior to initiation of this project, it was thought that the mechanism of these enzyme-catalyzed hydrolyses involved either (i) water directly attacking an oxacarbenium ion via an internal return mechanism (*11*), an idea initially advanced by von Itzstein and co-workers (*8*, *12*) or (ii) formation of a covalently bound sialyl-enzyme intermediate, a mechanism analogous to the standard double displacement reactions of retaining glycosidases that hydrolyze acetal linkages (*11*, *13*, *14*).

Recently, Withers and co-workers, using their fluorosugar methodology, have shown that during the slow turnover of their substrate analogue, the *trans*-sialidase from *Trypanosoma cruzi* becomes covalently modified on the active-site tyrosine residue (*15*). This significant finding suggests that the role of the tyrosine residue is as a nucleophile. However, a priori this observation does not allow one to differentiate between nucleophilic reactions that occur by means of a direct displacement (S_N2-like) or via an oxacarbenium ion intermediate (S_N1-like). Indeed, in a computational study, Thomas et al. concluded that an oxacarbenium ion is a potential intermediate during sialidase-catalyzed hydrolyses (*16*).

The present paper details a complementary series of kinetic and product studies that were performed on the wild-type and three tyrosine mutants of the sialidase from *Micromonospora viridifaciens* using a variety of substituted aryl α-D-*N*-acetylneuraminides (**1**) and two natural substrate analogues, 3′-sialyllactose **2** and 6′-sialyllactose **3**.



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MATERIALS AND METHODS

Materials. All restriction endonucleases and DNA modification enzymes were purchased from Gibco BRL or New England BioLabs (Beverly, MA). All DNA manipulations were carried out according to standard procedures (*17*). All chemicals were of analytical grade or better and were purchased from Sigma-Aldrich unless otherwise noted.

Cloning the nedA Gene. M. viridifaciens (ATCC 31146) was used as the source of the *nedA* gene since its sequence has been previously reported (*18*). A fresh colony was used to seed 500 mL of ATCC Medium172 (10 g of glucose; 20 g of soluble starch; 5 g of yeast extract; 5 g of *N*−*Z* amine type A; 1 g of $CaCO_3$ in 1 L). Cells were grown at 28 °C and 250 rpm for 4 days. Total DNA was isolated and purified using the method of Hopwood (*19*). The genomic DNA was digested with *Bcl* I at 50 °C overnight, and all fragments between 4 and 5 kb were excised from a 1% agarose gel and purified using the Qiaquick gel-extraction kit (Qiagen). Subsequent digestion of the 4−5 kb pool was performed with *Pst* I at 37 °C for 6 h, and fragments between 2 and 3 kb were gel-purified as above. This pool of fragments was ligated into *Bam* HI/*Pst* I-digested pUC19 vector (New England BioLabs Inc.) treated with alkaline phosphatase. Ligation mixtures were propagated in *Escherichia coli* DH5α, and 48 clones were amplified and purified using the Qiaspin plasmid purification kit (Qiagen). Restriction digests using *Eco* RI were used to screen the *Bcl/Pst* library for plasmids containing inserts of the expected size.

DNA Sequencing. Thirty-six plasmids from the *Bcl/Pst* library were sequenced to identify which clones contained the *nedA* gene insert. Nucleotide sequences were determined by the dideoxy-chain termination method (*20*) using the Sequenase II kit (Amersham). Reactions were performed on both strands using M13 forward or reverse primers and separated on a 6% polyacrylamide sequencing gel using a 373A Applied Biosystems automated sequencer.

Subcloning nedA Gene into Expression Vectors. The *nedA*-containing plasmid pJWBP13 was subjected to PCR amplification to add appropriate restriction sites for subcloning into various expression vectors. The initiation codon was replaced, and an *Nhe* I site was introduced at the front of the gene (Nhe*′ primer 5′-CGAGCTAGCACTGCGAATC-CGTACCTCCGC-3′). A *Hind* III site was incorporated following the natural stop codon from the *nedA* gene (SmaHin′ reverse primer 5′-CCCAAGCTTCTGGCCCCGG-GCCAC-3′). Amplification reactions were carried out in 10% DMSO using platinum Taq high fidelity DNA polymerase (Gibco BRL). The 2 kb PCR product was digested with *Nhe* I and *Hind* III, then ligated into the *Nhe/Hind*-digested and dephosphorylated pET28a vector (Novagen). The ligation mixture was transformed, propagated, and amplified as stated previously to obtain the expression plasmid construct pJWHS2, which encodes for the wild-type *M. viridifaciens* sialidase with an N-terminal His_6-tag. Plasmid construction was confirmed by restriction analysis and DNA sequencing using the T7-promoter and T7-terminator primers (Novagen).

Site-Directed Mutagenesis. The pJWHS2 plasmid was used as the template for the sialidase gene. Substitution of the Y370 codon to encode for aspartic acid was achieved by making the mutations in both strands in two separate PCR experiments. The front of the gene was amplified using the Nhe*′ primer with the reverse mutagenic primer Y370D-R′ (5′-GGTGGAGTCCGACATCGAGCC-3′) to give a 1.1 kb product (codon substitution is underlined). The back portion of the gene was amplified using the mutagenic primer Y370D-F′ (5′-GTCGGACTCCACCCTGACCG-3′) with the reverse primer SmaHin′ to produce a 0.9 kb fragment. These two purified fragments had an overlapping sequence at the site of mutation and were subsequently used to prime each other in an extension reaction of one cycle. The full 2 kb mutant gene was then amplified with the outer Nhe*′ and SmaHin′ primers. Following digestion with *Bam* HI and *Kpn* I, the purified 0.7 bp product was ligated into a similarly digested and dephosphorylated pJWHS2 vector and propagated as stated previously. Restriction and DNA sequence analysis was used to confirm the Y370D mutation in the pJWHS-YD3 plasmid, as well as to ensure that spurious mutations had not occurred during DNA manipulation. Additional sequencing primers included MV1846F′ (5′-AGGGCACCATCCGGATGTCC-3′) and MV1987R′ (5′-ATGCCGGTGCCCGGCTCG-3′). The Y370A and Y370G mutagenesis was performed using the same protocol as Y370D; however, the codon substitutions were GCC and GGC, respectively.

Recombinant Expression in E. coli. The recombinant pJWHS2 plasmid was transformed into *E. coli* strain BL21-(λDE3) (Novagen). A fresh single colony was used to inoculate 1 mL of TB broth (12 g of bactotryptone; 24 g of yeast extract; 4 mL of glycerol; 100 mL of K phosphate in 1 L) containing 50 μg/mL Kanamycin and incubated at 37 °C and 250 rpm for 1 h. This culture was used to seed a 30 mL culture that was grown for 2 h before scaling up to 1.5 L. When the culture had grown to an OD_{600} of approximately 1, cells were chilled down to room temperature and induced with 1.5 mM IPTG. Cells were incubated at room temperature until sialidase activity reached a plateau, typically 40−48 h post-induction.

Sialidase Purification. Culture broth (1500 mL) was centrifuged at 10 000*g* for 15 min. The supernatant was filtered (0.22 μm), mixed with $(NH_4)_2SO_4$ to 60% saturation, and stirred at 4 °C overnight. The precipitate was collected by centrifugation at 11 000*g* for 20 min, then resuspended in 80 mL of buffer A (50 mM Tris-HCl, pH 8.0). The solution was dialyzed at 4 °C overnight in 2 × 4 L of buffer A, then loaded onto an ion-exchange column equilibrated with buffer A (Source Q, Pharmacia). Proteins were separated using a linear gradient of 0.0−0.3 M NaCl over 1 L at a flow rate of 1 mL/min. Fractions containing sialidase activity were pooled and subjected again to $(NH_4)_2SO_4$ precipitation (as stated previously, resuspended in 10 mL) followed by exchange into buffer B (10 mM Tris-HCl, 0.1 M NaCl, pH 7.1). Ultrafiltration (30 kDa cutoff, Amicon) was used to concentrate the mixture to 1 mL. Concentrated samples were then loaded onto a Sephacryl-100-HR (Sigma Chemical Co.) gel-filtration column equilibrated with buffer B. The purity of the active fractions was assessed by SDS−PAGE and silver staining. Aliquots of the pure enzyme were then stored frozen at −80 °C for months without any detectable loss of activity.

Enzyme and Protein Assays. Throughout purification, sialidase activity was assayed based on the method of Potier et al. (*21*). The standard assay mixture contained 0.1 mM 4-methylumbelliferyl α-D-*N*-acetylneuraminide (MUαNeu5Ac,[1]

Rose Scientific Inc.) substrate in 50 mM NaOAc, pH 5.0. Samples were incubated at 37 °C for 10 min before the addition of stop solution. The release of free methylumbelliferone was measured at λ_{em} 365 and λ_{ex} 450 nm, using a Cary Eclipse fluorescence spectrophotometer. Total protein concentrations were determined by the Bradford assay (*22*) using bovine serum albumin as a protein standard.

N-Terminal Amino Acid Sequence. The purified wild-type sialidase was analyzed using 10% SDS−PAGE followed by electroblotting onto a Sequi-Blot PVDF membrane (BIO-RAD). The protein band was visualized with Coomassie brilliant blue R-250 and excised for sequencing. Edman degradation was performed at NAPS Unit, University of British Columbia.

Enzyme Kinetics. Michaelis−Menten parameters were measured under identical conditions for both wild-type and the Y370 mutants. Each 0.4 mL reaction was performed at 37 °C by equilibrating the buffer and substrate in the cell block for 3 min prior to the addition of enzyme (50 μL). Kinetic parameters were determined from a minimum of seven initial rate measurements within a substrate concentration range of at least $K_m/4$ to $4K_m$. The progress of the reactions was continuously monitored for 10 min using a Cary 3E spectrophotometer equipped with a Peltier temperature controller, and the derived initial velocities were corrected for background hydrolysis. Extinction coefficient differences were calculated by measuring the absorbance of the substrate and that of the product in each buffer and pH. The kinetic rate versus substrate data were fitted to the Michaelis−Menten equation using a standard nonlinear least-squares program (GraFit).

Thermostability. The effect of temperature on activity was determined by the sialidase assay method over a range of 10−65 °C. Using equivalent amounts of enzyme activity, duplicate reactions were carried out in 50 μL volumes containing 10 mM MU-αNeu5Ac in 100 mM NaOAc, pH 5.25. Following incubation for 12 min, 20 μL aliquots were removed from the reactions and added to 180 μL of stop solution. Samples were quantitated using a Cary Eclipse spectrofluorometer equipped with a 96-well plate reader accessory. After correcting for background hydrolysis at each temperature, the average relative rates were calculated.

pH-Rate Profile. To determine the effect of pH on catalysis, kinetic measurements were carried out over a pH range of 3.8−8.9. The buffers used were NaOAc-HOAc (pH range 3.8−5.7), 2-(*N*-morpholino)ethanesulfonic acid (MES-NaOH) (pH range 5.6−7.2), and tris-(hydroxymethyl)-aminomethane (Tris-HCl) (pH range 7.1−8.9), and ionic strength was maintained at 0.1 M (NaCl). The substrate for this experiment was MU-αNeu5Ac. Separate $\Delta\epsilon$ values were calculated for each of the 14 buffers (see Supporting Information, Table S1).

Brønsted Plots. The effect of leaving group ability on the kinetic parameters k_{cat} and k_{cat}/K_m was probed using a series of aryl *N*-acetylneuraminides. MU-αNeu5Ac, 4-nitrophenyl, 3-methoxyphenyl, and phenyl α-D-*N*-acetylneuraminide were purchased from Sigma-Aldrich, while 4-cyanophenyl, 3-nitrophenyl, and 3-chlorophenyl α-D-*N*-acetylneuraminides were synthesized according to literature procedures (*23*). All substrates (both commercial and synthesized) were estimated to be >95% pure by ^{1}H NMR spectroscopy. The reactions of aryl substrates were monitored as described previously at various wavelengths (see Supporting Information, Table S2). The hydrolyses of natural substrate analogues (α-(2 → 3) and α-(2 → 6) isomers of sialyllactose, purchased from V-labs) were monitored using a discontinuous assay for the liberated sialic acid product (Neu5Ac) according to standard procedures (*24*). The apparent binding constants of the two sialyllactose isomers with the Y370D mutant were measured by competitive binding assays using MU-αNeu5Ac as the substrate.

Product Studies. ^{1}H NMR spectroscopy (600 MHz Bruker AMX spectrometer) was employed to identify the products of the enzyme-catalyzed reactions (*7*). The reactions were carried out in 0.6 mL (25 °C, 10 mM tartrate buffer pD 5.1). The hydrolysis of 20 mM MUαNeu5Ac (sodium salt, Sigma-Aldrich) and 4-nitrophenyl α-D-*N*-acetylneuraminide (4NP-αNeu5Ac, Rose Scientific) in the presence of approximately 1 U wild-type or mutant enzyme was monitored until the reaction reached equilibrium. Spectral data were collected with 16 000 data points over a spectral width of 6024 Hz, with a relaxation delay of 2 s and 32 scans. An initial spectrum (referred to as time zero) containing substrate and buffer was acquired before the addition of enzyme.

RESULTS

Cloning and Sequencing. On the basis of the published sequence for the *M. viridifaciens nedA* gene (*18*), a simple strategy was designed that used sequential endonuclease digests of the genomic DNA to generate a restricted pool of *Bcl* I/*Pst* I fragments. Once inserted into pUC19, 36 individual plasmid inserts were sequenced to isolate a clone containing the desired gene. The *nedA* gene in pJWBP13 was sequenced in both directions to confirm the native sequence prior to modifying the gene by PCR for recombinant expression. Site-directed mutagenesis was confirmed by sequencing in both directions.

Expression and Purification. Several sialidase-fusion constructs were produced to achieve secreted expression in *E. coli*. The highest level of sialidase activity in culture supernatants was observed using an N-terminal His_6-tag, expressed in the BL21(λDE3) strain. The recombinant sialidase encoded in the pJWHS2 plasmid has a predicted molecular weight of 71 kDa. A protein of this approximate size with hydrolytic activity against the sialidase substrate MUαNeu5Ac was purified to homogeneity by ion-exchange and gel-filtration chromatography. Mutant enzymes were purified according to the same protocol. The average yield for the purified sialidases was 20 mg/L. Of note, the His_6-sialidase fusion protein expressed from the pJWHS2 plasmid did not bind to Ni−NTA agarose under a variety of binding conditions (data not shown). N-terminal amino acid sequence analysis revealed that the purified sialidase did not contain the encoded His_6-tag. Instead, the N-terminal region was similar to that observed when the unmodified gene was expressed in *Streptomyces lividans* by Sakurada et al. (*18*). Figure 1 shows the predicted N-terminal sequence deduced from the nucleotide sequence, along with the actual N-terminal region determined by Edman degradation. These

[1] Abbreviations: HOAc, acetic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MU-αNeu5Ac, 4-methylumbelliferyl α-D-*N*-acetylneuraminide; Neu5Ac, *N*-acetylneuraminic acid; PNP-αNeu5Ac, *p*-nitrophenyl α-D-*N*-acetylneuraminide; Tris, tris-(hydroxymethyl)-aminomethane.

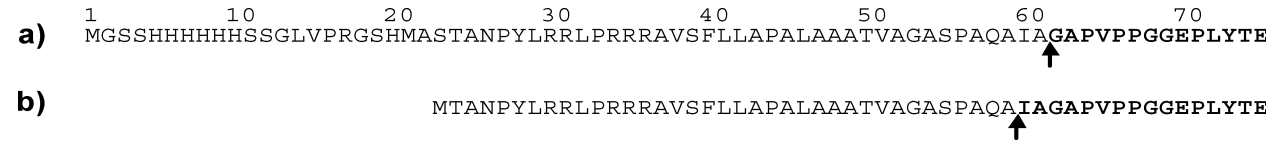


FIGURE 1: (a) Primary amino acid sequence of recombinant His_6-*NedA* gene in *E. coli* predicted from nucleotide sequence. (b) Predicted primary sequence of the unmodified *nedA* gene previously expressed in *S. lividans* (*18*). The amino acids in boldface represent the N-terminal amino acid sequence determined from the pure proteins; arrows indicate cleavage sites.

Table 1: Kinetic Parameters of Wild-Type Recombinant *M. viridifaciens* Sialidase and Y370 Variants using MU-αNeu5Ac at pH 5.25 and 37 °C

sialidase	relative k_{cat}[a]	relative k_{cat}/K_m[b]
wild-type	100 ± 5	100 ± 19
Y370A	2.9 ± 0.2	0.6 ± 0.16
Y370D	28 ± 2	1.4 ± 0.3
Y370G	86 ± 6	18 ± 4

[a] Absolute k_{cat} value for the wild-type is 51.5 ± 2.7 s^{-1}. [b] Absolute k_{cat}/K_M for wild-type is (7.2 ± 1.4) × 10^6 M^{-1} s^{-1}.

results suggest that perhaps the N-terminal region of the recombinant *nedA* gene operates as a signal sequence in *E. coli*. Given the fact that the purified sialidase is 61 amino acids shorter than expected, the theoretical MW is 65 kDa.

Enzyme Kinetics and Product Studies. An initial screen of sialidase activity at a series of temperatures using MU-αNeu5Ac as substrate revealed that the tyrosine mutants were catalytically active. Temperature−rate profiles for the recombinant wild-type and mutant enzymes were measured and showed that the mutant sialidases are slightly less stable than the wild-type at temperatures greater than 45 °C (Figure S1 in Supporting Information). Thus, all further enzyme-catalyzed rate constant measurements were performed at 37 °C. Given in Table 1 are the Michaelis−Menten kinetic parameters for the Y370 mutants and the wild-type recombinant *M. viridifaciens* sialidase using MUαNeu5Ac as the substrate. The kinetic properties of one mutant sialidase (Y370D) and the wild-type enzyme were characterized in greater detail. The variation on the kinetic parameters k_{cat} and k_{cat}/K_m as a function of pH are shown in Figure 2. Of note, Tris is not an inhibitor of these sialidase enzymes as the data for MES (pH = 7.2) and Tris (pH = 7.1) are similar (Figure 2).

Product studies were performed for the wild-type and all three Y370 variants. Figure 3 shows the time-course reaction for the wild-type-catalyzed hydrolysis of MU-αNeu5Ac monitored by ^{1}H NMR spectroscopy. The time-course reaction for Y370D-catalyzed hydrolysis of MU-αNeu5Ac is shown in Figure 4. Figure 5 is a graphical representation of the wild-type and Y370D time-course reactions; similar plots for the Y370A and Y370G mutant sialidases were obtained (Supporting Information, Figure S2). Equivalent observations were made when analogous reactions were performed with a second substrate, 4NP-αNeu5Ac (data not shown). Thus, the identity of the initial hydrolysis product was confirmed for both the wild-type sialidase and the Y370D mutant-catalyzed reactions.

A panel of aryl *N*-acetylneuraminides and two isomeric sialyllactose substrates were used to examine the Brønsted relationship for catalysis by both the wild-type and the Y370D mutant sialidases. As pK_a values have not been measured for the 3′- and 6′-hydroxyl of lactose, lower limits for these acidity constants were estimated in the following

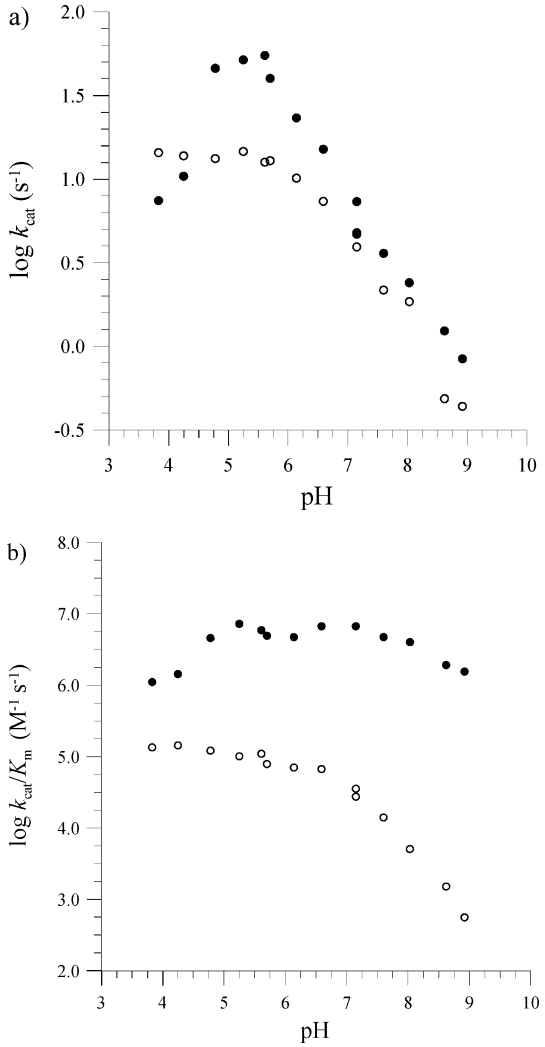


FIGURE 2: Effect of pH on kinetic parameters for wild-type (●) and mutant Y370D (○). (a) The pH dependence of k_{cat} and (b) the pH dependence of k_{cat}/K_M. All kinetic parameters were determined at 37 °C, using MU-αNeu5Ac as the substrate.

manner. The pK_a of the cyclic oligosaccharide β-cyclodextrin is 12.27 (*25*) and that for sucrose is 12.70 (*25*), and when these values are corrected for the number of hydroxyl groups in each molecule, the average pK_a value for a nonanomeric sugar hydroxyl group is approximately 13.6. Given that the 6′-OH group should be less acidic than the 3′-OH group, it has only one adjacent electron-withdrawing group; the values used in the Brønsted plots for the lactose derivatives **2** and **3** were 13.6 and 13.8, respectively. Also, because of the low hydrolytic activity of the Y370D mutant sialidase against both sialyl-lactose substrates, k_{cat} values were estimated using high enzyme concentrations and a single rate measurement at 10 mM substrate, and dissociation constants were obtained in a competitive binding assay. Figure 6 displays the so-derived Brønsted correlations, and the absolute values of the

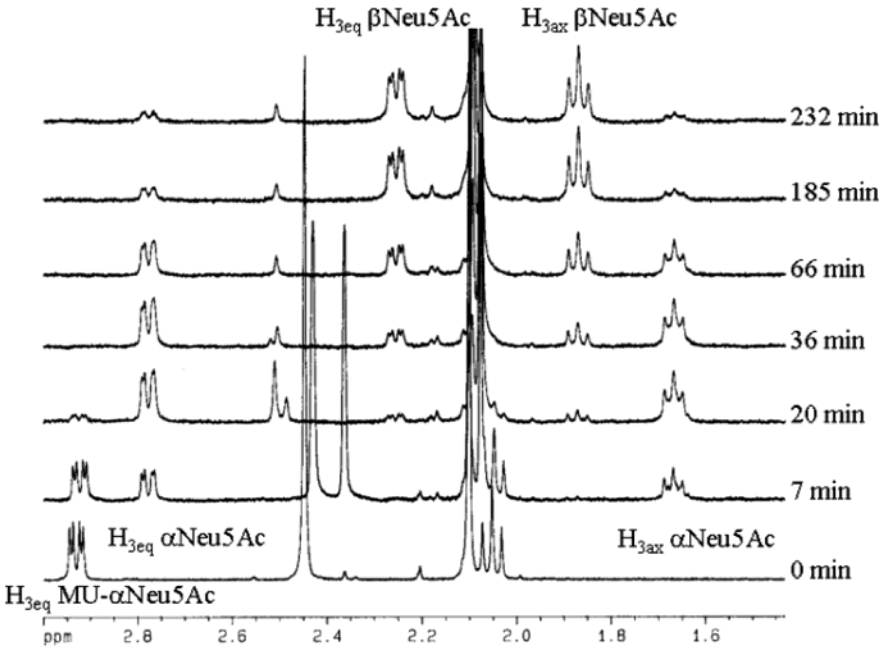


FIGURE 3: Time course of *M. viridifaciens* wild-type sialidase-catalyzed reaction monitored by 600 MHz ^{1}H NMR spectroscopy. Conditions: 20 mM MU-αNeu5Ac in 10 mM tartrate buffer, pD 5.2 at 25 °C; 1 U enzyme in 0.6 mL. Time zero spectrum was recorded before the addition of enzyme.

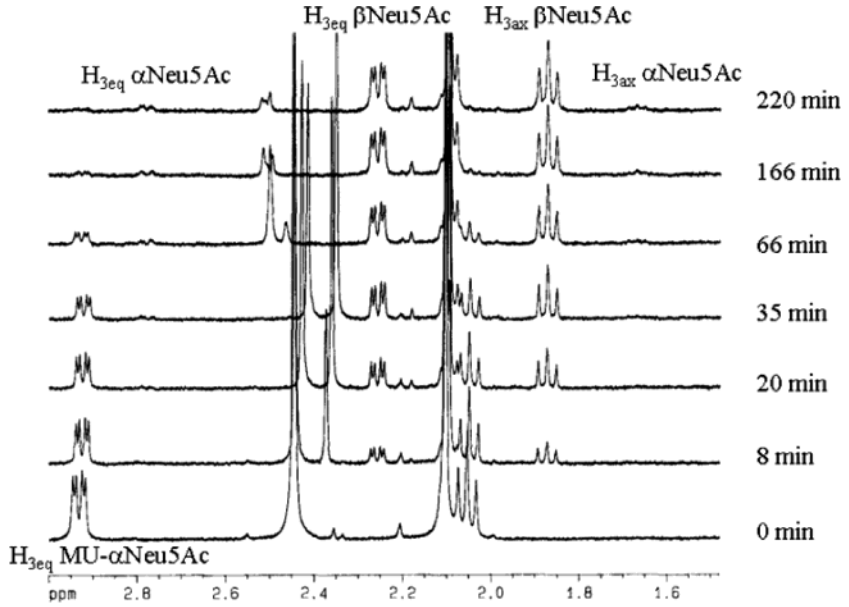


FIGURE 4: Time course of *M. viridifaciens* Y370D mutant sialidase reaction monitored by 600 MHz ^{1}H NMR spectroscopy. Conditions: 20 mM MU-αNeu5Ac in 10 mM tartrate buffer, pD 5.2 at 25 °C; 1 U enzyme in 0.6 mL. Time zero spectrum was recorded before the addition of enzyme.

kinetic parameters are tabulated in Tables S3 and S4 (Supporting Information).

DISCUSSION

The *nedA* gene that encodes for the *M. viridifaciens* sialidase has been cloned, sequenced, and recombinantly expressed in *S. lividans* (*18*). The 3-D crystal structure of this enzyme has also been solved (*26*). In the present work, the wild-type and Y370 mutants of this sialidase were produced recombinantly in *E. coli*. The recombinant form of the wild-type *M. viridifaciens* sialidase produced in the *E. coli* system has properties similar to those reported for the native form (*27*) as well as another recombinant form produced using *S. lividans* (*18*). For example, the stability of the recombinant enzyme has not been compromised, and the enzyme is most active between 50 and 55 °C (Figure S1, Supporting Information) (*18*, *27*). Also, the pH-rate profile, which is typical for this class of enzymes, is broad and bell-shaped, with a pH optimum that is similar to the reported value for the native enzyme (Figure 2) (*27*).

The *M. viridifaciens* sialidase mutants Y370A, Y370D, and Y370G were expressed successfully in *E. coli* at levels comparable to the wild-type enzyme. The observed pH-rate profiles for both the wild-type and the Y370D enzymes were roughly similar, although there were two distinct differences: (1) the Y370D mutant possessed a larger k_{cat} value

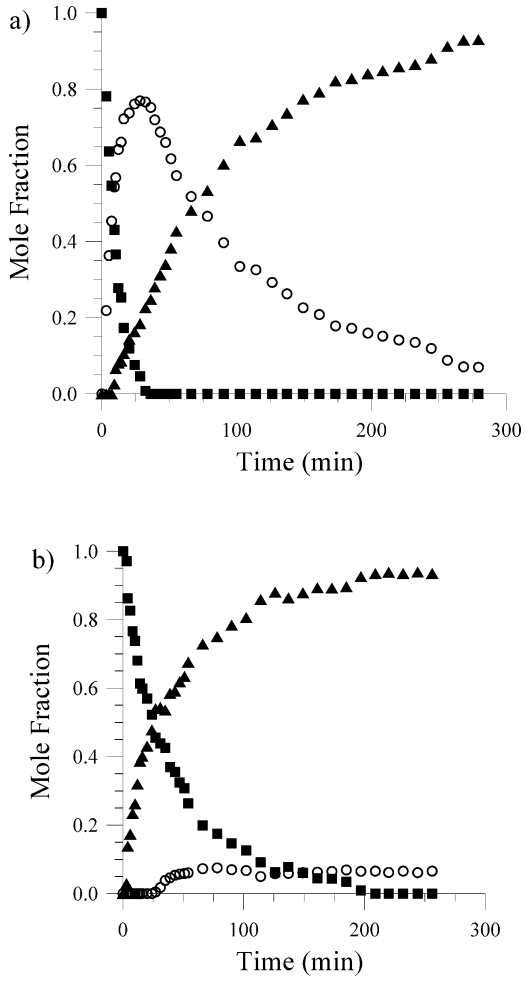


FIGURE 5: Mole fractions of substrate and products monitored over time. (a) wild-type-catalyzed hydrolysis and (b) Y370D-catalyzed hydrolysis. Values were calculated using the relative integrals of the H_{3eq} signals of MU-αNeu5Ac (■), αNeu5Ac (○), and βNeu5Ac (▲).

at low pH values; and (2) the value of k_{cat}/K_m for the Y370D mutant dropped at higher pH values, while the corresponding value for the wild-type sialidase hardly varied with pH. This decrease in activity for k_{cat}/K_m is consistent with the ionized form of the newly introduced aspartic acid residue being catalytically compromised.

Notably, all three tyrosine mutant sialidases showed significant catalytic activity (Table 1). This important finding can be contrasted to most other site-directed mutagenesis studies in which replacement of the conserved tyrosine residue of various sialyl processing enzymes resulted in either (i) incorrect folding or transport (Y406F influenza A/Tokyo/3/67 sialidase (*28*)) or (ii) a dramatic decrease in activity (Y409F influenza B/Lee/40 sialidase (*29*), Y347F *Clostridium perfringens* small sialidase (*30*), Y342F and Y342T *Trypanosoma cruzi trans*-sialidase (*31*), and Y370C human membrane-associated ganglioside sialidase (*32*)). Although no data was given, a single sentence in a report states that the Y347I mutant of the small sialidase from *C. perfringens* possesses wild-type activity (*33*).

Clearly, even with the natural substrate analogues **2** and **3**, the Y370D mutant sialidase shows a remarkable level of catalytic activity for an enzyme in which the catalytic nucleophile has been removed (Table S4, Supporting Information). The Y370D mutant sialidase shows a minor loss

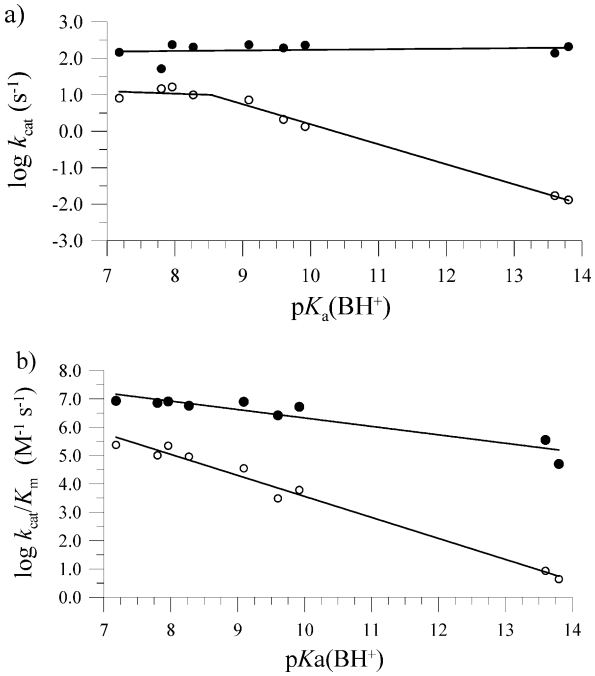


FIGURE 6: Brønsted plots. Effect of leaving group ability on (a) k_{cat} and (b) k_{cat}/K_m for the wild-type sialidase (●) and mutant Y370D (○). All experiments were performed at 37 °C and pH 5.25. Leaving group ability represented as pK_a (BH$^+$) as follows: 4-nitrophenol (7.18); 4-methylumbelliferone (7.80); 4-cyanophenol (7.96); 3-nitrophenol (8.27); 3-chlorophenol (9.09); 3-methoxyphenol (9.60); phenol (9.92); and lactose (3′-OH (∼13.6) and 6′-OH (∼13.8)).

Table 2: Relative Catalytic Activity of the Wild-Type and the Y370D Mutant Sialidase

leaving group	relative k_{cat}[a]	relative k_{cat}/K_m[b]
4-nitrophenol	18.1	35.4
3′-lactose	8.1×10^3	4.2×10^4
6′-lactose	1.6×10^4	1.2×10^4

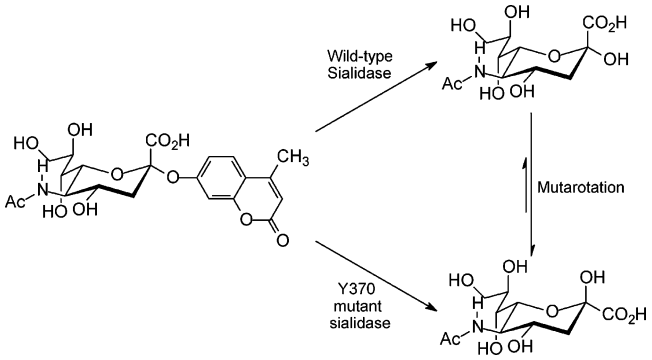
[a] Equals k_{cat}(wild-type)/k_{cat}(Y370D). [b] Equals k_{cat}/K_m(wild-type)/k_{cat}/K_m(Y370D).

of activity with the 4-nitrophenyl substrate and about a 10^4-fold reduction with the natural substrate analogues **2** and **3** (Table 2).

This change in activity can be compared to the *Agrobacterium* β-galactosidase nucleophilic mutant (Glu358Ala) that lost $>10^7$ of its catalytic power when the highly activated 2,4-dinitrophenyl β-D-galactoside was used as substrate (*34*). Thus, nucleophilic catalysis has a greater catalytic effect at secondary anomeric centers (aldopyranosides) than it does at the corresponding tertiary ketal centers.

Mechanism Utilized by Tyrosine Mutants. Product studies were performed to understand why these tyrosine mutants are catalytically active. These experiments reveal that the wild-type enzyme, like all other sialidases tested to date (*7*−*11*), operates via a retaining mechanism (Figures 3 and 5a), that is, the initial product of hydrolysis is αNeu5Ac, which undergoes mutarotation in solution to give an equilibrium mixture that favors the β-anomer. In contrast, the initial product of hydrolysis in the presence of the three Y370 mutants is βNeu5Ac (Figures 5b and S2). Thus, it can be concluded that simple replacement of the conserved tyrosine residue with a smaller amino acid changes the mechanism

Scheme 1



of the catalyzed reaction from one of retention to one of inversion of configuration (Scheme 1).

To the best of our knowledge, the observation that water can act as an efficient nucleophile for mutant retaining glycosidases to give an inverting glycosidase that exhibits significant (close to wild-type) activity with activated substrates is unprecedented. However, other workers have shown that mutants of acetal-hydrolyzing glycosidases missing the nucleophilic carboxylate residue can possess significant catalytic activity for inversion of configuration only when much stronger nucleophiles such as an azide (*34*) or formate (*35*) ion are added to the solution. On the basis of the results reported here, it is to be expected that the *C. perfringens* sialidase mutant Y347I reported by Chien et al. (*33*) also operates with inversion of configuration.

Contributions of the Tyrosine Residue to Catalysis. An important question that needs answering is that since water is capable of acting as a nucleophile in all three mutant sialidases studied, why does nature strictly conserve the tyrosine residue? The data listed in Table 2 show that a dramatic change in catalytic activity occurs between the wild-type and the Y370D mutant sialidases when the leaving group ability decreases.

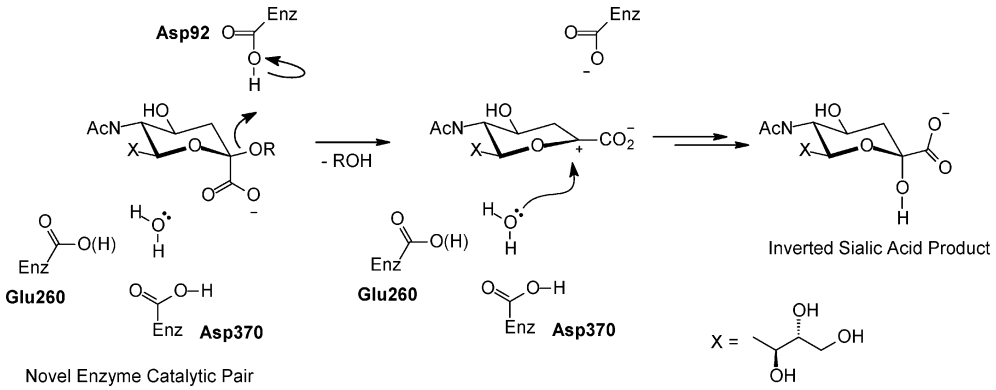
With respect to the kinetic term k_{cat}, cleavage of the glycosidic bond is not rate-determining for the wild-type sialidase ($\beta_{lg} = 0.02 \pm 0.03$, Figure 6a). The corresponding Brønsted plot for the Y370D mutant enzyme, which appears curved, indicates that for basic leaving groups (pK_a(ROH) $\geq$ 9.09), cleavage of the glycosidic bond is rate-limiting ($\beta_{lg} = -0.55 \pm 0.03$, Figure 6a). Consequently, the relative k_{cat} values listed in Table 2 for the natural substrates are lower limits for the catalysis of glycosidic bond cleavage afforded by the tyrosine residue. That is, the tyrosine residue lowers the activation free energy for cleavage of the glycosidic bond in 6′-sialyllactose (**3**) from the Michaelis complex by more than 24.9 kJ mol^{-1} at 37 °C.

Similarly, the Brønsted plot for the kinetic parameter k_{cat}/K_m showed a smaller dependence on leaving group ability for the wild-type enzyme ($\beta_{lg} = -0.30 \pm 0.04$, Figure 6b) than for the Y370D mutant ($\beta_{lg} = -0.74 \pm 0.04$, Figure 6b). For cleavage of the glycosidic bond at 37 °C, the activation free energy is raised by at least 24.2 kJ mol^{-1} on substitution of the tyrosine by an aspartic acid residue.

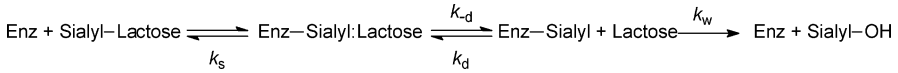
The picture that emerges from this study of the mechanism of action of the tyrosine mutants is shown in Scheme 2. For these enzymes, a new hole is generated, and water can now act as a nucleophile to give directly an inverted product. A noteworthy observation is that the Y370D mutant is more active than the wild-type enzyme at low pH values (Figure 2) where the presumed active-site general-base (E260) is likely to be protonated. Therefore, given the enforced proximity of this glutamate residue to the attacking nucleophile and that the rate of the mutant-catalyzed reaction is independent of the glutamate's protonation state, it can be concluded that the Y370D mutant reacts via a dissociative mechanism (S_N1) to give a transient oxacarbenium ion that is rapidly trapped by a water molecule located in the new active-site hole. In other words, varying the nucleophilicity of the attacking water molecule by changing what it is in intimate contact with (from a carboxylate group to a carboxylic acid) does not change the values for either of the two kinetic parameters (k_{cat} or k_{cat}/K_m). Also, the derived Brønsted β_{lg} values for the Y370D mutant enzyme are consistent with general-acid catalysis (presumably by Asp92) being required for departure of the leaving group.

This contrasts with the *trans*-sialidase from *T. cruzi* that Yang et al. have concluded, based on anomeric ^{13}C kinetic isotope effects, operates via an S_N2 mechanism (*36*). The data reported here also suggest that the wild-type sialidase from *M. viridifaciens* also reacts by a concerted mechanism

Scheme 2

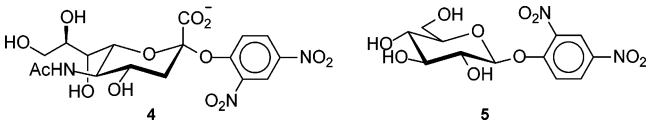


Scheme 3



because its rate of reaction does depend on the protonation state of the active-site general-base catalyst.

Why Does Nature Use a Tyrosine Nucleophile in Sialyl Transferring Enzymes? Withers and co-workers argued that because sialic acids have a carboxylate group attached to the anomeric center, it is electrostatically unfavorable for a carboxylate residue to displace the leaving group nucleophilically (*15*). However, the negative charge on the substrate's carboxylate group, upon binding to this family of enzymes, is more than counter-balanced by the three conserved positively charged arginine residues to which it binds (*5b*, *37*). As a consequence, the natural selection of a tyrosine rather than the usual carboxylate group (*11*, *14*) probably originates from differences in intrinsic reactivity. Specifically, sialyl-based ketal centers are inherently about 10^5 times more reactive than acetal-based glycosides. For example, using the reported β_{lg} value (60 °C) and the rate constant for spontaneous hydrolysis of 4NP-αNeu5Ac at 50 °C, the estimated rate constant for the spontaneous hydrolysis of 2,4-dinitrophenyl α-D-*N*-acetylneuraminide (**4**) at 50 °C is approximately 2.6 s^{-1} (*38*) while the corresponding reaction of 2,4-dinitrophenyl β-D-glucopyranoside (**5**) occurs with a rate constant of 3.4×10^{-5} s^{-1} (*39*).



Accordingly, it would be expected that the rate constant for reaction of a carboxylate-linked sialyl-enzyme intermediate with water would also be 10^5-fold faster than that for a typical glycosyl-enzyme intermediate (*11*), and this rate constant would be approximately 10^7-10^8 s^{-1} (k_w Scheme 3). Such a high intrinsic reactivity is counterproductive since any covalent enzyme intermediate must have a lifetime long enough to allow the aglycon (leaving group) to diffuse from the active site (k_{-d} Scheme 3) to avoid regenerating the starting material (k_s, Scheme 3).

Thus, a consequence of the greater inherent reactivity of ketal-based *N*-acetylneuraminides relative to acetal-based glycosides is that nature has selected to stabilize the sialyl-enzyme intermediate by making the leaving group worse (i.e., the intermediate is covalently linked to tyrosine rather than to the typical aspartate or glutamate).

CONCLUSIONS

In summary, we have successfully cloned, expressed, and purified a recombinant form of the *M. viridifaciens* sialidase for kinetic characterization. Results from these enzymatic studies have revealed that the replacement of the conserved tyrosine residue (Y370) with three smaller amino acids (alanine, aspartic acid, or glycine) changes the mechanism of action from one of retention of anomeric configuration to one of inversion.

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SUPPORTING INFORMATION AVAILABLE

Tables of wavelengths versus changes in extinction coefficients for kinetic assays. A plot of the effect of temperature on the rates of wild-type and mutant sialidase-catalyzed hydrolyses. Plots of the mole fractions of substrate and product for the Y370A-mutant and Y370G-mutant catalyzed hydrolyses of MUαNeu5Ac. Tables of k_{cat} and k_{cat}/K_m values for the hydrolyses catalyzed by the wild-type and Y370D mutant sialidases. This material is available free of charge via the Internet at http://pubs.acs.org.

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