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Two Nucleophilic Mutants of the *Micromonospora viridifaciens* Sialidase Operate with Retention of Configuration by Two Different Mechanisms

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Mutants of the Micromonospora viridifaciens *sialidase, Y370E and Y370F, are catalytically active retaining enzymes that operate by different mechanisms. Previous substitutions with smaller amino acids, including Y370D, yielded inverting sialidases. At least one water molecule can fit into the active-site cavity of this mutant and act as a nucleophile from the face opposite the leaving group (*Biochemistry ***2003***, 42, 12682). Thus, addition of a CH_2 unit (Asp versus Glu) changes the mechanism from inversion back to retention of configuration. Based on Brønsted β_{lg} values, it is proposed that the Y370E mutant reacts by a double-displacement mechanism (β_{lg} on k_{cat}/K_m -0.36 ± 0.04) with Glu370 acting as the nucleophile. However, the Y370F mutant (β_{lg} on k_{cat}/K_m -0.79 ± 0.12) reacts via a dissociative transition state. The crystal structure of the Y370F mutant complexed with 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid shows no significant active-site perturbation relative to the wild-type enzyme.*

Introduction

Glycosidase families 33 and 34 contain exosialidases (E.C. 3.2.1.18, neuraminidases),[1] which catalyze the hydrolysis of sialic acid from glycoconjugates with retention of the anomeric configuration.[2] These enzymes are involved in the pathogenesis of many human diseases, including influenza and cholera.[3,4] Sialidases are atypical retaining glycosidases in that the catalytic nucleophile is not a carboxylate residue, but rather a Tyr,[5–7] which becomes covalently linked in the sialosyl–enzyme intermediate (Scheme 1). These conclusions were based on the following observations: i) three tyrosine mutants of the *Micromonospora viridifaciens* sialidase (Y370A, Y370D and Y370G) were found to be catalytically active inverting enzymes,[5] and ii) an acid/base catalyst mutant (D59A) of the *trans*-sialidase from *Trypanosoma cruzi* is covalently modified on the active-site Tyr during the slow turnover of 3-fluoro-α-D-*N*-acetylneuraminosyl fluoride.[6,7]

Recently, it was shown that three mutants of the nucleophilic Tyr residue (Tyr370) in the *M. viridifaciens* sialidase are catalytically active inverting enzymes.[5] That is, replacement of this Tyr with smaller amino acids, such as Gly, Ala or Asp creates a "hole" that allows a bound water molecule to act as a nucleophile; this gives an inversion of configuration. To probe this phenomenon in greater detail, further mutagenic studies were performed.

Results and Discussion

The activities of five new nucleophile Tyr370 sialidase mutants were determined with respect to their hydrolysis of 2′-(4-methylumbelliferyl)-α-D-sialoside (MUαNeu5Ac; Table 1). Interestingly, the Y370H mutant is the least active of the five variants studied. A similar mutation is found naturally in *T. cruzi trans*-sialidases in which normal catalytic activity is destroyed upon substitution of an active-site Tyr with His (Y342H).[8] Mutants Y370N and Y370T were particularly good catalysts for the hydrolysis of MUαNeu5Ac, however, both enzymes lost activity upon storage at 0 °C and were therefore not fully characterized.

NMR studies were performed with the products in order to determine the stereochemical outcome of mutant-catalyzed hydrolysis of MUαNeu5Ac (Table 1). Given that the half-time for sialic acid mutarotation under the reaction conditions is approximately 80 min,[9,10] it is impossible to confirm whether the hydrolysis reaction of Y370H proceeds by inversion or retention of the anomeric configuration. Indeed, it is also impossible to determine the stereochemical outcome of the hydrolysis of substrates such as sialyl lactose that are turned-over slowly.

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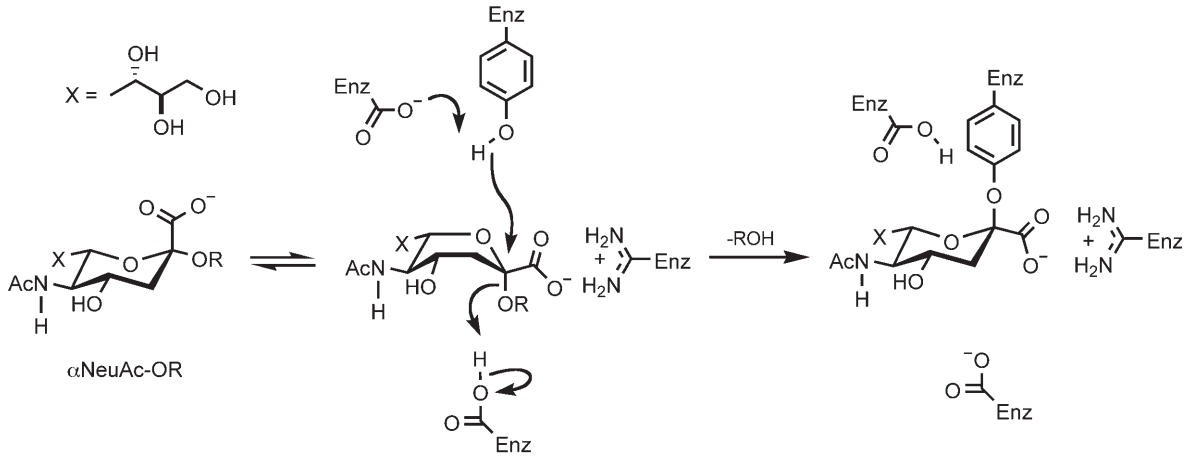


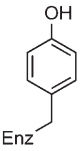
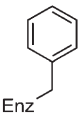
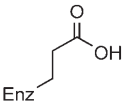
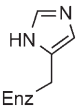
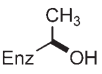
Scheme 1. The mechanism of the glycosylation step by sialidases.

However, we assumed that the stereochemical outcome of the hydrolysis of such substrates is the same as that for MUα Neu5Ac. In other words, the stereochemistry of the reaction is independent of the leaving group.

Time courses for the hydrolysis of MUαNeu5Ac are shown for two of these mutants, Y370E and Y370F (Figure 1). The data in Figure 1 show that α-sialic acid (αNeu5Ac) is the first reaction product. Thus, it can be concluded that, in contrast to Y370D,[5] Y370E, which possesses an additional CH_2 unit, and Y370F, which lacks an obvious nucleophile, operate with retention of configuration.

Kinetic parameters for the Y370E and Y370F retaining-mutant sialidases are given in Table 2. This comparison of the effect of aglycon structure on activity is revealed in the two Brønsted plots (Figure 2). The drop in activity for both mutants as the leaving group ability decreases is consistent with glycosidic C–O bond cleavage being rate-limiting—or partially so—for both k_{cat} and k_{cat}/K_m. This situation is in contrast to that of the wild-type sialidase, where a nonchemical step(s) is rate-limiting for both the k_{cat} and k_{cat}/K_m in the hydrolysis of aryl sialosides.[5] Thus, changes in the catalytic-rate constant from the wild-type enzyme to the various mutants (Table 1) do not yield any information concerning the free-energy differences between the corresponding transition states for C–O bond cleavage.

Table 1. Activity of sialidase mutants with MUαNeuAc at 37 °C, pH 5.25.

Sialidase	Side chain[a]	k_{cat} [s^{-1}]	k_{cat}/K_m [$M^{-1}s^{-1}$]	stereochemical outcome
wild-type[b]		51.5 ± 0.1	(7.23±0.04)×10^6	retaining
Y370D[b]		14.6 ± 0.6	(1.0±0.1)×10^5	inverting
Y370F		2.22 ± 0.13	(6.21±1.5)×10^4	retaining
Y370E		0.51 ± 0.01	(4.09±0.4)×10^3	retaining
Y370H		0.13 ± 0.01	(4.26±1.1)×10^2	n.d.[c]
Y370N		130[d]	n.d.	inverting
Y370T		11[d]	n.d.	inverting

[a] The protonation state of ionizable side-chains is not known. [b] Data taken from ref. [5] [c] At a protein concentration of 42 mg mL^{-1}, the rate of mutarotation was too fast for the determination of the anomeric configuration of the initially formed sialic-acid product. [d] These values are calculated from the maximal rates observed at high substrate concentration. Due to the rapid degradation of the mutant enzyme a complete Michaelis–Menten curve was not determined. That is, enzymatic activity dropped by greater than 50% over a period of 2 h even when the enzyme stock solution was stored on ice; n.d. = not determined.

Mechanism of action

As both Y370E and Y370F lack the customary nucleophilic oxygen atom, possible mechanisms for their action include: i) generation of an oxacarbenium ion intermediate with a lifetime that is sufficient to allow aglycon

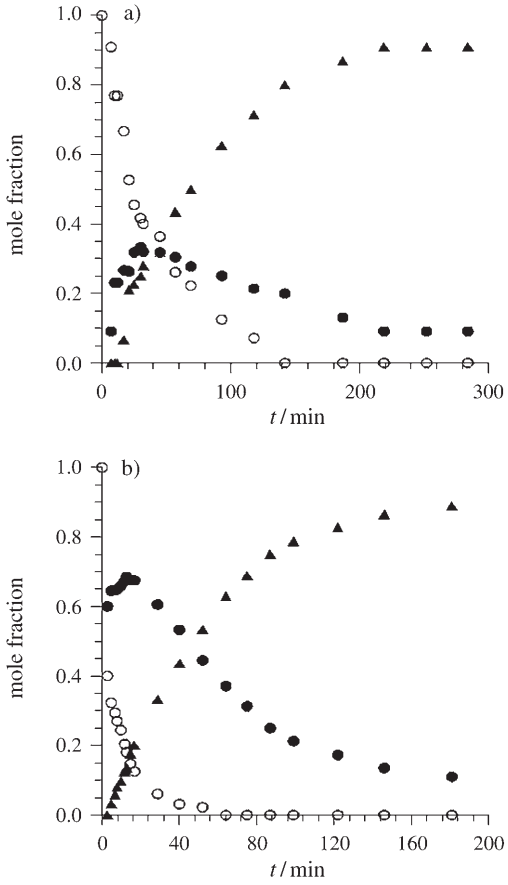


Figure 1. Mole fractions of MUαNeu5Ac (○), αNeu5Ac (●) and βNeu5Ac (▲) monitored by ^{1}H NMR spectrometry over time: a) Y370F-catalyzed hydrolysis and b) Y370E-catalyzed hydrolysis.

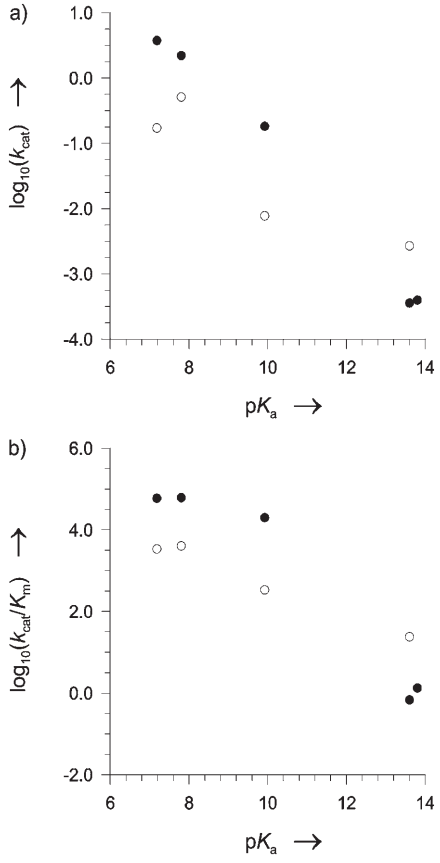


Figure 2. Effect of leaving-group ability on a) k_{cat} and b) k_{cat}/K_m for Y370E (○) and Y370F (●) at 37 °C, pH 5.25. Leaving-group ability represented as pK_a (BH^+) as follows: 4-nitrophenol (7.18); 4-methylumbelliferone (7.80); phenol (9.92); lactose (3-OH (13.6) and 6-OH (13.8)).

Table 2. The activity of Y370E and Y370F mutant sialidases with a panel of substrates at 37 °C, pH 5.25.

Leaving group	Y370E		Y370F	
	k_{cat} [s^{-1}]	k_{cat}/K_m [$M^{-1}s^{-1}$]	k_{cat} [s^{-1}]	k_{cat}/K_m [$M^{-1}s^{-1}$]
4-nitrophenol	0.170 ± 0.006	(3.4±0.4)×10^3	3.77 ± 0.25	(6.0±1.4)×10^4
4-methylumbelliferone	0.51 ± 0.01	(4.1±0.4)×10^3	2.22 ± 0.13	(6.2±1.5)×10^4
phenol	0.0078 ± 0.0004	(3.4±0.8)×10^2	0.18 ± 0.01	(2.01±0.13)×10^4
lactose (3′-OH)	0.0027[a]	24.1[a]	0.00036[a]	0.68[a]
lactose (6′-OH)	n.d.	n.d.	0.00040[a]	1.35[a]

[a] No error is associated with these values because they are estimates based on a single rate measurement at high concentration of substrate and enzyme; n.d. = not determined.

departure prior to being captured by water, that is, internal return (S_Ni); ii) generation of an oxacarbenium ion intermediate that is rapidly trapped by a nearby residue to give a sialosyl-enzyme intermediate that subsequently undergoes hydrolysis (S_N1); iii) direct displacement of the leaving group by an alternate nucleophile (S_N2) or iv) formation of an elimination product 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu5Ac-2en, DANA) followed by stereospecific hydration.

The fourth possibility can be ruled out by the following observations: i) it was noted that no signal for DANA was observed in the NMR spectra during the hydrolytic reactions catalyzed by the wild-type and three of the mutant sialidases (Y370D, Y370E and Y370F); ii) hydration was not observed during 18 h incubations of these four enzymes in the presence of DANA (Table 3); iii) DANA is a tight-binding reversible inhibitor of these enzymes (Table 3). This situation contrasts with that of other sialidases, including those

Table 3. Kinetic constants for the interaction of DANA with the wild-type, Y370D, Y370E and Y370F sialidases at pH 5.25.

	K_i [M]	Rate constant for hydration of DANA [s^{-1}][a]
wild-type	(1.4±0.7)×10^{-7}	< 0.1
Y370D	(4.3±1.3)×10^{-6}	< 4×10^{-2}
Y370E	(1.6±0.9)×10^{-6}	< 6×10^{-4}
Y370F	(5.5±2.2)×10^{-7}	< 5×10^{-4}

[a] Values were calculated based on the assumption that 10% hydration would have been detected.

from *Vibrio cholerae*[11] and influenza type B,[12] where it has been shown that DANA is formed during the corresponding hydrolytic reactions. It is difficult to rationalize differences in the catalytic ability of these enzymes to process DANA, given the similarity in their active-site architectures (Table 4). Although no evidence has been presented to date to suggest that sialidases react by an associative mechanism (S_N2-like), Yang et al. have reported that in an analogous *trans*-sialidase C–O bond cleavage occurs simultaneously with nucleophilic participation.[13]

Table 4. Interatomic distances between the pseudo-anomeric carbon of DANA and the closest side-chain carbon atom of four of the critical active-site residues from three different sialidases.

Residues	Influenza B [Å][a]	*V. cholera* [Å][b]	*M. viridifaciens* [Å][c]
Tyr(Cζ)–DANA	3.9	3.9	3.8
Glu(Cδ)–DANA	5.4	5.2	5.3
Asp(Cγ)–DANA	4.6	4.7	4.5
Arg(Cζ)[d]–DANA	5.5	5.6	5.5

[a] PDB ID: 1NSD.[12] [b] PDB ID: 1W0O.[11] [c] PDB ID: 1EUS.[24] [d] All distances are reported to the central Arg of the triad.

The smaller reactivity change for Y370E relative to that of Y370F (β_{lg} values on k_{cat}/K_m are -0.36 ± 0.04 and -0.79 ± 0.12, respectively) suggests that these mutants operate by different mechanisms. Thus, unless either a nonchemical step has unexpectedly become partially rate-limiting or general acid catalysis (proton donation to the leaving group) is somehow much more efficient for Y370E than for Y370F, it is likely that the hydrolysis reaction catalyzed by Y370E occurs by a concerted mechanism (S_N2-like); the likely nucleophile would then be the newly introduced Glu residue. The estimated lifetime for the sialosyl oxacarbenium ion in aqueous solution is about 3×10^{-11} s.[15] Therefore, in the case of Y370F, any such ion will be captured by a proximal enzymatic group rather than remain as a discrete intermediate long enough for the aglycon to diffuse from the active site. In a similar manner, Watson et al. proposed that the inverting Y370D mutant forms a transient oxacarbenium ion (S_N1) which is rapidly captured by a bound water molecule (β_{lg} value on $k_{cat}/K_m = -0.74 \pm 0.04$).[5] Further evidence for these conclusions could be provided by measurements of the anomeric ^{13}C-kinetic isotope effect for the various mutant-catalyzed hydrolysis reactions, as has been reported for a *trans*-sialidase.[13]

Nature of intermediates

Trapping experiments with 3-fluoro-α-D-sialosyl fluoride on the *trans*-sialidase from *T. cruzi* have been reported. These required the acid catalyst to be mutated before breakdown of the covalent intermediate, which was bound to the active-site Tyr, became partially rate-limiting. This condition was necessary for the observation of this intermediate by mass spectroscopy.[6] If Y370E produces a Glu-bound intermediate, due to the greater leaving ability of a carboxylate group relative to a phenolate group, the intermediate will be more labile than one in which the covalent bond is with a Tyr. Thus, a better trapping reagent will be required before the hypothesis of a glutamate-bound intermediate can be tested. Similarly, in order to probe which active-site residue could be captured by an oxacarbenium ion, a better trapping reagent will be needed with which to covalently modify Y370F. Possibilities for this role include either one of the oxygen atoms of Glu260 or the aromatic π-electrons to form an ester or a transient π-complex, respectively.

To examine any structural consequences of the Y370F mutation, crystals were grown in conditions previously reported for the D92G mutant.[14] Table 5 lists the data collection and refinement statistics. Atomic coordinates have been deposited at the Protein Databank with the accession code 1WCQ. Superimposition of the 1.8 Å wild-type *M. viridifaciens*–DANA complex (PDB ID: 1EUS) onto the three monomers of Y370F–DANA shows that the active site makes no significant changes after substitution of Tyr to Phe (Figure 3).

Table 5. Data collection and refinement statistics.

	Y370F + DANA
PBD ID	1WCQ
resolution range [Å]	124–2.1 (2.2–2.1)
unit cell dimensions [Å]	$a = b = 143.25$, $c = 160.25$
number of observations	799 157
number of unique measurements	110 930
completeness [%]	100 (100)
$I/\sigma(I)$	7.4 (1.5)
R_{merge}[a]	0.072 (0.47)
R factor [%]	17.5
R free [%]	23.8
rms deviation in bond length [Å]	0.021
rms deviation in bond angles [°]	1.835
esd of atom positions[b] [Å]	chain A: 0.198 ± 0.037
	chain B: 0.165 ± 0.026
	chain C: 0.181 ± 0.040
number of protein atoms	13 650
number of water molecules	1079
average B-factor for all atoms [Å^2]	chain A: 31.5
	chain B: 26.4
	chain C: 28.9
average B-factor for Neu5Ac2en [Å^2]	monomer A: 22.9
	monomer B: 27.9
	monomer C: 22.8

[a] $R_{merge} = \Sigma | I(k) - \langle I \rangle | / \Sigma I(k)$ where $I(k)$ is the kth measurement of the intensity of a reflection, $\langle I \rangle$ is the mean value of the intensity of that reflection and the summation is overall measurements. Values in parentheses relate to the highest resolution cell. [b] The standard radial uncertainty of an atom with the average B-factor was estimated by using the diffraction precision indicator method proposed by Cruickshank[25] as implemented in the program ESCET.[26]

The root mean square (rms) deviations for 357 C_α atoms of the WT structure (1EUS) compared to monomers A, B and C are 0.34, 0.36 and 0.31 Å, respectively. The closest distance of the Glu260 carboxylate group to the C2 of DANA, a neutral inhibitor, in the three monomers in Y370F was 4.37, 4.17 and 4.34 Å; this compares with 4.56 Å for the wild-type structure

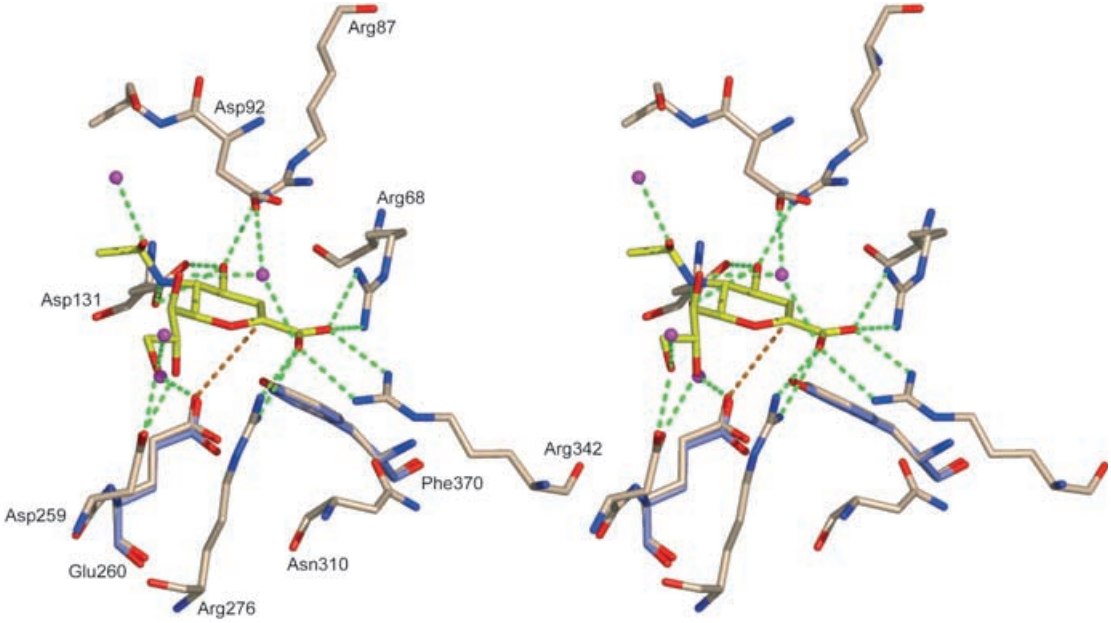


Figure 3. Superimposition of the active site (Tyr370 and Glu260) residues of the wild-type catalytic domain (PDB ID: 1EUS) and one of the three Y370F–DANA monomer complexes. Wild-type residues are shown in blue, and mutant Y370F is shown in wheat. The hydrogen bonding interactions are drawn as green dotted lines; the interaction between Glu260 and the C2 atom of DANA is shown in orange.

(Figure 3). A distance of this magnitude is too great for covalent bond formation. However, one must consider that there will be a significant electrostatic attraction between Glu260 and an oxacarbenium ion-like intermediate (or transition state). Therefore, in the context of a flexible active site, covalent bond formation during Y370F-catalyzed reactions cannot be ruled out.

In bacterial sialidases the mean distance between the carboxyl oxygens of the general-acid catalyst and the nucleophilic oxygen atom is in the range 6.9–7.6 Å (PDB IDs: 1EUU, 1KIT and 3SIL). Yet, the distance between the general-acid and nucleophile in retaining glycosidases that utilize two carboxyl groups is much shorter at 4.5–5.5 Å;[15,16] the corresponding distance in inverting glycosidases is around 9.0–9.5 Å.[15] Interestingly, the distance between the Glu introduced in Y370E and the general acid (Asp92) still permits hydrolysis to occur with retention of configuration. Yet, increasing this distance by the removal of one methylene unit (Y370D) permits a water molecule to be bound interstitially between the Asp and the bound substrate, and to act as a nucleophile during catalysis. Certainly, the catalytic machinery of *M. viridifaciens* sialidase is more tolerant to drastic alterations than is the case for most retaining glycosidases, in which a modest change to the nucleophile (E→D) results in a precipitous drop in activity even with activated substrates.[17,18]

In summary, three separate nucleophile mutants of the *M. viridifaciens* sialidase that possess significant catalytic activity react by three different mechanisms: Y370D by S_N1 inversion,[5] Y370E by S_N2 retention and Y370F by S_N1 retention. This finding has no precedent to date in the glycosidase literature.

Experimental Section

Materials: All restriction endonucleases and DNA modification enzymes were purchased from Gibco BRL or New England BioLabs (Beverly, MA, USA). All DNA manipulations were carried out according to standard procedures.[19] All chemicals were of analytical grade or better and were purchased from Sigma–Aldrich unless otherwise noted. MUαNeu5Ac was purchased from Rose Scientific (Edmonton, Canada). 3′SL was purchased from V-labs (Covington, LA, USA). DANA was prepared according to literature procedures,[20,21] except for the following minor modifications: after saponification of the methyl ester, the crude lithium salt of DANA was purified by anion-exchange chromatography by using Dowex 1X8 200–400 (acetate counter ion) as adsorbent (2 mL). The compound was eluted with water (20 mL) followed by an aqueous solution of pyridinium acetate (50 mL, 50 mM). After lyophilization, a mixture of DANA as the free acid and its corresponding pyridinium salt was obtained.

Expression vector: The leader sequence from *ompT* was inserted into the pET28a vector before the ligation of the sialidase gene. Plasmid pJW28Omp was made by inserting a 108 bp fragment from an *Xba*I/*Bam*HI digest of pET12a (Novagen) into the pET28a vector. In the wild-type sialidase gene,[5] the initiation codon was replaced with an *Eco*RI site (Eco*′ primer, 5′-CCGGAATTCACTGCGAATCCGTACCTCCGC-3′). The reverse primer incorporated a *Hind*III site. Thereby the natural stop codon was replaced to allow read-through for a C-terminal fusion construct that encoded His_6-tag (TerHin′ primer, 5′-CCCAAGCTTGCGCTGGCCTTCCACCTC-3′). The 2 kb PCR product was digested at the newly incorporated sites (*Eco*RI and *Hind*III), then ligated into digested and dephosphorylated pJW28Omp.

Mutagenesis: Site-directed mutagenesis was performed by using degenerate primers that contained NNK in place of the Tyr370 codon as reported for earlier mutants.[5] The forward mutagenic primer was Y370X-F′ (5′-GTCGNNKTCCACCCTGACCG-3′). The re-

verse mutagenic primer was Y370X-R′ (5′-GGTGGAMNNCGACATC-GAGCC-3′). The final mutant gene fragments were ligated into the JW28Omp vector. Plasmid DNA was isolated from single colonies and sequenced to identify the mutations.

Expression and purification: Expression was performed as reported previously.[5] At 40 h post-induction, culture supernatant (1 L) was incubated overnight at RT and 100 rpm with agarose Ni-NTA resin (25 mL, Qiagen) that was pre-equilibrated in binding buffer (50 mM phosphate, 5 mM imidazole, 100 mM NaCl, pH 8.0). The suspension was then packed in a 26 mm diameter column and run at 1 mL min^{-1}. The column was washed with wash buffer (150 mL; 50 mM phosphate, 10 mM imidazole, 100 mM NaCl, pH 8.0) until baseline was reached. Elution was achieved by increasing the imidazole concentration to 100 mM. Fractions containing sialidase were pooled and concentrated as before by using $(NH_4)_2SO_4$ precipitation followed by exchange by dialysis into storage buffer (10 mM Tris-HCl, 0.1 M NaCl, pH 7.2).[5] Purity of the sample was assessed by SDS-PAGE and silver staining. Aliquots of the pure enzyme were then stored at −80 °C. Total protein concentrations were determined with the Bradford assay by using bovine serum albumin as protein standard. The pure protein produced from the expression constructs were analyzed by N-terminal sequencing (NAPS Unit, University of British Columbia, Canada).

Enzyme kinetics and product studies: Measurement of the kinetic parameters for hydrolysis of the various activated and natural substrates, as well as NMR-based product studies, were carried out according to protocols described for the wild-type enzyme.[5] Product studies with Y370N and Y370T were performed at an enzyme concentration of 0.14 and 0.07 mg mL^{-1}, respectively. Due to the lower activity of mutants Y370E and Y370F, much more protein was required in the product studies (12 and 18 mg mL^{-1}, respectively) in order to achieve a hydrolysis rate significantly greater than the rate of mutarotation.[9,10] For Y370H mutarotation was faster than hydrolysis even at an enzyme concentration of 70 mg mL^{-1}.

In order to measure the binding affinity of DANA to the various sialidase mutants, the rate of MUαNeu5Ac hydrolysis at a concentration well below K_m was monitored with varying DANA concentrations. The data were then fitted to the competitive model for inhibition in order to obtain K_i by using the GraFit program. NMR experiments were performed with DANA (10 mM) in tartrate buffer (10 mM, pD 5.3) at RT to determine whether the enzymes could hydrate DANA. The concentration of enzyme in these reactions was one tenth of that used to determine the stereochemical outcome of MUαNeu5Ac hydrolysis. The DANA-containing reactions were monitored for at least 18 h. The maximal rate for hydration was calculated by assuming that 10% hydration would have been observed.

Crystallization and X-ray data collection: To examine the structural consequences of Y370F, crystals were grown in conditions previously reported for the D92G mutant.[14] Despite similar crystallization conditions, the Y370F protein crystallized in a different space-group, $P3_221$, with three monomers in the asymmetric unit. Crystals were soaked in DANA (10 mM) for 30 min at RT, and then transferred to cryoprotectant (20% glycerol in 16% PEG 3350, 0.2 M ammonium citrate) for 1 min prior to flash freezing. Data were collected at 100 K to 2.1 Å resolution on ESRF beamline ID14–2. Molecular replacement was used to locate the three monomers in the asymmetric unit by using the wild-type structure (PDB ID: 1EUU) and the program AMoRE.[22] The model was refined by using REFMAC,[23] with DANA and water molecules being fitted into maximum likelihood/σ_A-weighted $2F_o-F_c$ and F_o-F_c difference Fourier electron density maps.

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Keywords: enzyme catalysis · mutagenesis · neuraminidase · sialic acids · sialidase

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