Kinetic and Mechanistic Analysis of *Trypanosoma cruzi* Trans-Sialidase Reveals a Classical Ping-Pong Mechanism with Acid/Base Catalysis[†]

Iben Damager,[‡] Sabrina Buchini,[‡] Maria F. Amaya,[§] Alejandro Buschiazzo,[§] Pedro Alzari,[§] Alberto C. Frasch,[△] Andrew Watts,[‡] and Stephen G. Withers*[,‡]

Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, British Columbia, V6T1Z1 Canada, Unite de Biochimie Structurale, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris, France, and Instituto de Investigaciones Biotechnologicas, Universidad National de San Martin, CC 30, 1650 San Martin, Argentina

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ABSTRACT: The trans-sialidase from *Trypanosoma cruzi* catalyzes the transfer of a sialic acid moiety from sialylated donor substrates to the terminal galactose moiety of lactose and lactoside acceptors to yield α-(2,3)-sialyllactose or its derivatives with net retention of anomeric configuration. Through kinetic analyses in which the concentrations of two different donor aryl α-sialoside substrates and the acceptor substrate lactose were independently varied, we have demonstrated that this enzyme follows a ping-pong bi-bi kinetic mechanism. This is supported for both the native enzyme and a mutant (D59A) in which the putative acid/base catalyst has been replaced by the demonstration of the half-reaction in which a sialyl−enzyme intermediate is formed. Mass spectrometric analysis of the protein directly demonstrates the formation of a covalent intermediate, while the observation of release of a full equivalent of *p*-nitrophenol by the mutant in a pre-steady state burst provides further support. The active site nucleophile is confirmed to be Tyr342 by trapping of the sialyl−enzyme intermediate using the D59A mutant and sequencing of the purified peptic peptide. The role of D59 as the acid/base catalyst is confirmed by chemical rescue studies in which activity is restored to the D59A mutant by azide and a sialyl azide product is formed.

The widespread parasitic disease American trypanosomiasis, also known as Chagas disease, is a chronic, debilitating, multisystemic disorder that currently affects more than 20 million people in Central and South America. Annually, an estimated 50 thousand people die from the disease and 100 million people are at risk of infection in 21 Central and South American countries. The trans-sialidase from *Trypanosoma cruzi* (TcTS),[1] the protozoan agent of Chagas disease, is expressed on the parasite's surface and shed into the medium, thus constituting one of the few natural examples of surface glycosyltransferases in eukaryotes (*1*). Trypanosomes are unable to synthesize sialic acid and use the trans-sialidase to scavenge the monosaccharide from host glycoconjugates to sialylate mucin-like acceptor molecules present in the parasite plasma membrane. TcTS is a member of CAZY glycoside hydrolase family GH33 (http://www.cazy.org/) and has been classified as an exo-α-sialidase (EC 3.2.1.18). However, it behaves as a trans-sialidase in vivo and in vitro in the presence of suitable sugar acceptor substrates rather than catalyzing hydrolysis (*2, 3*). TcTS preferentially transfers α-(2,3)-linked sialic acid from the terminal galactose of the host glycoconjugates to a terminal galactose on the surface of the parasite with retention of anomeric configuration.

Since trypanosomes are incapable of sialic acid biosynthesis, *T. cruzi* trans-sialidase represents a promising target for the development of therapeutics to treat Chagas disease. To obtain such therapeutics, it is essential to understand the mechanism of the enzyme in detail. The kinetic mechanism of TcTS has been investigated earlier through studies in which rates were determined at a series of substrate concentrations using sialyllactose as the donor and either lactose or *N*-acetyllactosamine as the acceptor (*4, 5*). These experiments suggested a sequential mechanism in which sialyllactose binds first, followed by the acceptor (lactose or *N*-acetyllactosamine). This led to the assumption that there are two different lactose binding sites, which was difficult to rationalize with the three-dimensional structure of TcTS and with the observed similarities with sialidases. It was also unnecessarily complex, a ping-pong mechanism being more logical. Indeed, kinetic isotope effect studies had been interpreted as being consistent with a ping-pong mechanism, though they did not provide direct proof (*6*).

Structural and mechanistic studies on TcTS, involving trapping of the covalent intermediate and peptide mapping, have suggested that Tyr342 functions as the nucleophilic catalyst (*7, 8*). Further, the formation of a covalent inter-

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* To whom correspondence should be addressed. Phone: (604) 822-3402. Fax: (604) 822-2847. E-mail: withers@chem.ubc.ca.

[‡] University of British Columbia.

[§] Institut Pasteur.

[△] Universidad National de San Martin.

[1] Abbreviations: BSA, bovine serum albumin; CF_3MU-SA, (trifluoromethyl)umbelliferyl α-sialoside; PNP-SA, *p*-nitrophenyl α-sialoside; TcTS, trans-sialidase from *Trypanosoma cruzi*; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; wt, wild type.

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mediate involving Tyr342 with a 3-fluorosialoside has been directly demonstrated by X-ray crystallography (*7*). Additional crystallographic studies of stable enzyme–substrate and enzyme–product complexes obtained using inactive mutant enzyme forms revealed that the donor aglycone moiety in the Michaelis complex occupies the same site as the acceptor as well as revealing a conformational distortion of the bound substrate (*7, 9*). These results all point strongly toward a ping-pong mechanism, yet the published kinetic data were in contradiction. These structures also point to a role for Asp59 as the acid/base catalyst. However, this role has not been substantiated for TcTS by mechanistic analysis of mutants modified at that position.

In this paper, we report a kinetic characterization of wild-type TcTS with two different donor substrates, as well as of the mutant enzyme, D59A, in which the putative acid/base catalyst has been replaced. A ping-pong mechanism is clearly shown, the identity of the acid/base catalyst confirmed, and the formation of a sialyl−enzyme intermediate with an unmodified sialic acid proven.

MATERIALS AND METHODS

General. All buffer chemicals and other reagents were obtained from Sigma/Aldrich Co., unless otherwise stated. Recombinant TcTS was produced and purified as described previously (*9, 10*). The construct includes seven surface mutations to facilitate crystallization, but otherwise, the protein displays the same transglycosidase and hydrolase activities as wild-type recombinant TcTS. The single-point mutant $TcTS_{D59A}$ was produced using the Quick-Change kit (Stratagene) and purified as described before. Mass spectrometric analyses of proteins and peptides were performed on a PE-Sciex API-300 triple-quadrupole mass spectrometer. Protein samples (typically 50 μg) were injected into the mass spectrometer via an HPLC system equipped with a PLRP-S column (5 μm, 300 Å, 1 mm × 50 mm) and eluted using a 5 to 90% acetonitrile gradient system containing 0.1% trifluoroacetic acid over 3 min. MALDI-TOF spectra of sugar products were collected using a Voyager-DE-STR (Applied Biosystems) system in reflectron mode with an acceleration voltage of 20 kV using 2,5-dihydroxybenzoic acid as the matrix. The sample and matrix solution were mixed in a 1:10 ratio, and 1 μL of this sample was loaded onto the target plate and dried under vacuum.

Kinetic Analyses. All kinetic studies were performed at 25 °C by monitoring the hydrolyses of PNP-SA or CF_3MU-SA spectrophotometrically in a buffer containing 13 or 20 mM Tris-HCl, BSA (1 mg/mL), and 20 or 30 mM NaCl at pH 7.6 (200 μL final volume). Wavelengths and extinction coefficients were as follows: PNP, $\lambda = 400$ nm, $\epsilon = 11577$ cm^{-1} M^{-1}; CF_3MU, $\lambda = 385$ nm, $\epsilon = 6565$ M^{-1} cm^{-1}. A Varian CARY 4000 spectrophotometer attached to a temperature control unit was used. Kinetic parameters were determined by direct fit of the rate versus substrate concentration data to the Michaelis−Menten equation using GraFit (Leatherbarrow, R. J. *GraFit*, Erithacus Software Ltd., Staines, England).

The kinetic mechanism was explored by measuring rates at a series of concentrations of lactose (0–1 mM) at each of a series of concentrations of PNP-SA (0–10 mM) or CF_3MU-SA (0.1–0.6 mM). The following enzyme concentrations were employed: 0.01 mg/mL for the wild-type enzyme and 0.25 mg/mL for the D59A mutant. Reaction was started by addition of enzyme. Rescue of activity for the D59A mutant by azide was monitored kinetically by measuring rates at a fixed concentration of PNP-SA (1 mM) in the standard buffer while varying the concentration of sodium azide from 0 to 800 mM at a constant pH.

A pre-steady state burst of *p*-nitrophenol release was measured for the cleavage of PNP-SA (1 mM) by monitoring the absorbance at 400 nm after addition of the D59A mutant (1.7 μM).

Isolation and Analysis of the Azide Product. To the enzyme solution (20 μL, 1 mg/mL) in buffer were added *p*-nitrophenyl α-sialoside (1 mL, 0.44 mM) and azide (20 mM); the mixture was incubated for 30 s and then frozen in a dry ice/acetone bath and the reaction mixture freeze-dried. The residue was dissolved in methanol (20 μL), acetone (10 μL) added, and the solution cooled to 0 °C. (Trimethylsilyl)diazomethane (0.3 mL, 2.0 M in hexane) was added, and the reaction mixture was stirred for 15 min at room temperature and then frozen and freeze-dried. A ^{1}H NMR spectrum was recorded on a Bruker AV 300 spectrometer at 300 MHz in D_2O using dioxane as the internal reference [δ_H(dioxane) = 3.75], and the ^{1}H NMR data corresponded with the reported data in the literature for methyl (5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosyl)onate azide (*11*).

Monitoring of Azide Rescue of the D59A Mutant by ES/MS. D59A (20 μL, 2 mg/mL) was labeled by incubation with PNP-SA (5 μL, 44 mM) for 30 s, cooled in a dry ice/acetone bath, and then directly analyzed by mass spectrometry (control). For the rescue experiment, the sialyl−enzyme form of D59A was first formed by incubating the enzyme with PNP-SA as described for the control, and then sodium azide (5 μL, 0.02 M) was added and the mixture reacted 30 s before being cooled in a dry ice/acetone bath and then used directly for mass spectrometric analysis.

Labeling and Proteolytic Digestion. Labeling of the D59A mutant was achieved by incubating the enzyme (20 μL, 2 mg/mL) with PNP-SA (1 μL, 0.44 mM) for 30 s and then cooling it in a dry ice/acetone bath. Half of the reaction volume was used directly for mass spectrometric analysis. The other half was prepared for proteolytic digestion by adding H_3PO_4 until the pH reached 2 (60 μL). Pepsin (2 μL, 1 mg/mL in phosphate buffer, 180 mM, pH 2) was added, and the mixture was incubated for 15 h at room temperature. A control reaction was carried out by digestion of the unlabeled enzyme under identical conditions. The candidate labeled peptide from the peptic hydrolysate of the inactivated enzyme was isolated by HPLC using electrospray mass spectrometry (ES/MS) detection.

Electrospray Mass Spectrometry. Peptides were separated via a reverse-phase HPLC system interfaced with the mass spectrometer. For HPLC−MS experiments, proteolytic digests of labeled or unlabeled protein were loaded onto a C18 column (Nova-Pak, 3.9 mm × 150 mm; Waters, Milford, MA) and eluted with a gradient from 0 to 60% solvent B over the course of 60 min at a flow rate of 0.5 mL/min (solvent A is 0.05% trifluoroacetic acid and 2% acetonitrile in water, and solvent B is 0.05% trifluoroacetic acid and 80% acetonitrile in water). A postcolumn flow splitter was used to direct 90% of the sample into a fraction collector, while the remaining sample was sent to the mass spectrometer for

Table 1: Michaelis−Menten Parameters for PNP-SA[a]

	K_M^{app} (mM)	k_{cat}^{app} (s^{-1})	$(k_{cat}/K_M)^{app}$ (mM^{-1} s^{-1})
WT, hydrolysis, 0 mM lactose	1.09	1.57	1.44
WT, transfer, 2 mM lactose	5.80	6.77	1.17
D59A, hydrolysis, 0 mM lactose	0.030	0.031	1.03
D59A, transfer, 2 mM lactose	0.014	0.017	1.21

[a] Michaelis−Menten parameters (k_{cat} = 7.4 s^{-1}; K_M = 0.008 mM) were determined with lactose as the varied substrate in the presence of 6 mM PNP-SA. Average errors in kinetic parameters: ±7–10% for K_M and ±5–7% for k_{cat}.

analysis. The quadrupole mass analyzer was scanned over a mass-to-charge ratio range of 600–2400 amu, with a step size of 0.5 amu and a dwell time of 1 ms/step. In all ESI tandem MS (MS/MS) peptide sequencing experiments, the MS/MS product ion spectrum was obtained in the triple-quadrupole scan mode. Peptides previously fractionated by HPLC were introduced into the mass spectrometer either via a small column (Reliasil, 1 mm × 150 mm; Michrom BioResouces) with a flow rate of 0.05 mL/min or via a nanospray ion source (Protana). Following mass selection in the first quadrupole (Q1), the peptide of interest was fragmented by collision with nitrogen gas in the second quadrupole (Q2) and the fragment ions were analyzed in the third quadrupole (Q3). In a typical experiment, the following settings were used: Q3 scan range of *m/z* 600–2400 amu, step size of 0.5 amu, dwell time of 1 ms/step, orifice potential of 50 V, focusing ring voltage of 400 V, Q0 potential of −10 V, Q2 potential of −55 V (collision energy = Q0 − Q2 = 45 V), and source voltage of 5 kV. The collision gas thickness was varied to obtain optimal fragmentation of the parent peptide.

RESULTS

Determination of Kinetic Parameters for TcTS. Since TcTS is known to catalyze both hydrolysis and transglycosylation, kinetic parameters were first determined for the hydrolysis of PNP-SA in the absence of lactose. Hydrolysis kinetics followed Michaelis–Menten behavior, and as one can see in the first line of Table 1, a k_{cat} value of 1.6 s^{-1} and a K_M of 1.1 mM were determined. Kinetic parameters were then determined for PNP-SA at a single, fixed (2 mM) concentration of lactose (Table 1), revealing a 4-fold higher k_{cat} for the transglycosylation reaction than for hydrolysis. The K_M value is also approximately 5-fold higher, with the consequence that k_{cat}/K_M values are very similar. These findings suggest that the second step, deglycosylation, is rate-limiting since the rate is dependent upon the lactose concentration. Further, the approximately equal k_{cat}/K_M values found are consistent with a common first irreversible step, namely formation of the sialyl−enzyme intermediate.

Kinetic studies were then performed at a series of PNP-SA concentrations at each of a series of lactose concentrations. Since reaction progress was monitored via the increase in absorbance at 400 nm as a consequence of release of *p*-nitrophenol, our assay measures the total flux through the system, not just the transfer reaction which is the case for previous kinetic studies on this enzyme in which rates of transfer to radiolabeled acceptors were studied (*4, 5*). Michaelis–Menten behavior was again observed, and the Lineweaver–Burk plots obtained are shown in Figure 1A.

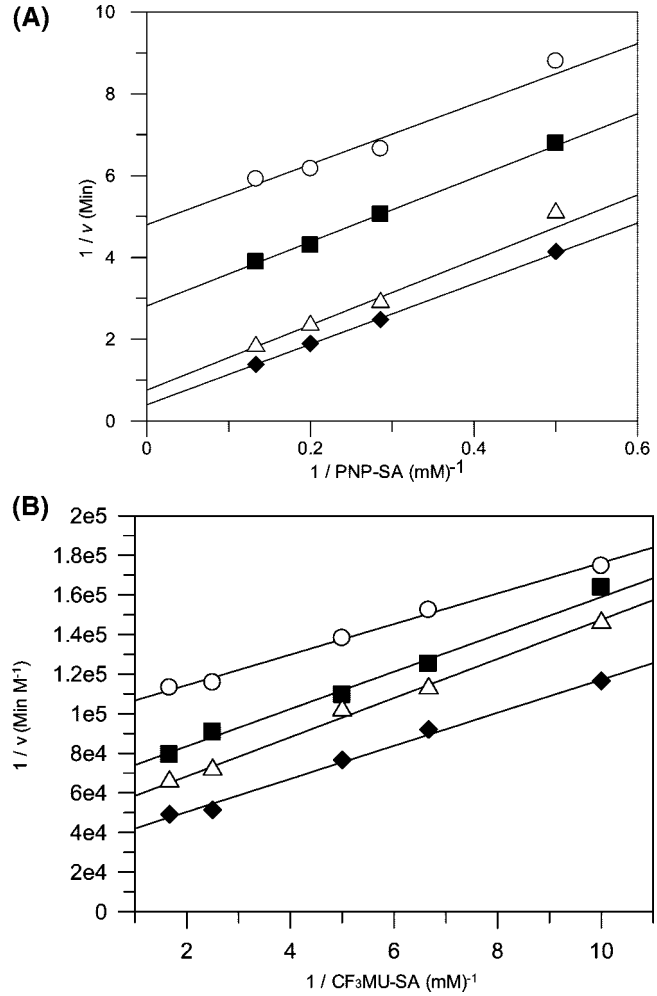

FIGURE 1: Lineweaver–Burk analysis of the TcTS kinetic mechanism. Double-reciprocal plot showing the activity of TcTS as a function of (A) PNP-SA concentration in the presence of 0.005 (○), 0.01 (■), 0.1 (△), and 1.0 mM lactose (◆) and (B) CF_3MU-SA in the presence of 0.01 (○), 0.05 (■), 0.1 (△), and 1.0 mM lactose (◆).

The lines observed in this case are clearly parallel, consistent with a ping-pong mechanism and not with the sequential mechanism proposed previously (*4, 5*). Replotting the data at a fixed (6 mM) PNP-SA concentration gives an apparent K_M for lactose of 8 μM and a k_{cat} of 7.4 s^{-1}. This low K_M value may well reflect the fact that the concentration of the lactosyl acceptors in vivo is quite low; thus, a low K_M is needed if transglycosylation is to dominate over hydrolysis.

To ensure that these results were not a consequence of the specific choice of substrate, we repeated the studies with a second aryl sialoside. In so doing, we decided to synthesize a substrate for which turnover could be monitored by both UV−vis and fluorescence. Previous experience had shown that methylumbelliferyl sialoside undergoes much slower spontaneous hydrolysis than the *p*-nitrophenyl glycoside, but that continuous monitoring of its cleavage by UV–visible spectroscopy is not convenient. Given the success of others with (trifluoromethyl)umbelliferyl glycoside substrates for other glycosidases, we synthesized (trifluoromethyl)umbelliferyl α-sialoside (CF_3MU-SA) according to a literature protocol (*12, 13*). Our expectation was that the lower pK_a of the (trifluoromethyl)umbelliferyl alcohol leaving group (pK_a = 7.26) would make this a more reactive substrate and

Table 2: Michaelis−Menten Parameters for CF_3MU-SA[a]

	K_M^{app} (mM)	k_{cat}^{app} (s^{-1})	$(k_{cat}/K_M)^{app}$ (mM^{-1} s^{-1})
WT, hydrolysis, 0 mM lactose	0.06	2.9	48.4
WT, transfer, 2 mM lactose	0.26	15.7	60.4

[a] Michaelis−Menten parameters (k_{cat} = 6.7 s^{-1}; K_M = 0.036 mM) were determined with lactose as the varied substrate in the presence of 1 mM CF_3MU-SA. Average errors in kinetic parameters: ±10–15% for K_M and ±10–13% for k_{cat}.

make the continuous monitoring of reaction more facile. CF_3MU-SA indeed turned out to be an ideal substrate, with low spontaneous hydrolysis rates, convenient spectrophotometric properties, and a significantly lower K_M. Kinetic parameters for hydrolysis of CF_3MU-SA by TcTS, as well as for transfer to 2 mM lactose, are presented in Table 2. Once again, k_{cat} and K_M are both 4–5-fold higher for transglycosylation than for hydrolysis, with the consequence of no effect on k_{cat}/K_M, again indicating that deglycosylation is rate-limiting. Interestingly, the K_M value for this substrate is much lower than that for PNP-SA. This indicates either that binding is inherently tighter, which is quite possible with a larger aglycone, or that the glycosylation step is faster; thus, more intermediate accumulates. Kinetic studies were then performed at a series of concentrations of both CF_3MU-SA and lactose, and the results are shown in Lineweaver–Burk format in Figure 1B. A series of parallel plots is indeed seen again, fully consistent with a ping-pong mechanism. This demonstrates that the parallel plots observed with PNP-SA are not an artifact of that specific substrate but are indeed a reflection of a ping-pong mechanism. Once again, a low K_M value for lactose was observed when the lactose concentration was varied and the CF_3MU-SA concentration kept constant at 1 mM, with the following Michaelis−Menten parameters: k_{cat} = 6.7 s^{-1}, and K_M = 0.036 mM.

Kinetic and Mechanistic Analysis of D59A TcTS. The kinetic parameters determined for hydrolysis of PNP-SA by the mutant of TcTS in which the putative acid/base catalyst, D59, has been replaced with alanine (D59A) are listed in Table 1. The results obtained are completely consistent with a role for D59 as the acid/base catalyst since the k_{cat} value drops substantially both for hydrolysis (50-fold) and for transglycosylation (400-fold), with the K_M value also dropping accordingly such that k_{cat}/K_M retains its approximate wild-type value. This behavior arises because the PNP leaving group does not require significant acid catalytic assistance for departure; thus, removal of the acid catalyst does not significantly slow the glycosylation step (as reflected in k_{cat}/K_M). However, the absence of the base catalyst does significantly slow the deglycosylation step (as reflected in k_{cat}), apparently more so for transglycosylation than for hydrolysis.

Direct support for this interpretation in the form of evidence for the accumulation of a sialyl−enzyme intermediate was achieved by the measurement of a pre-steady state "burst" of PNP release, as shown in Figure 2. Reaction of PNP-SA with a relatively large amount (1.7 μM) of D59A was continuously monitored spectrophotometrically, revealing a relatively rapid first phase involving formation of the sialyl−enzyme intermediate with release of PNP, followed by a slower second phase, which arises from steady state turnover of the substrate. By extrapolation of the linear phase back to time zero, a burst magnitude of 1.4 μM was obtained. This corresponds well with the amount of enzyme (1.7 μM), thus demonstrating the stoichiometric accumulation of a sialyl−enzyme intermediate.

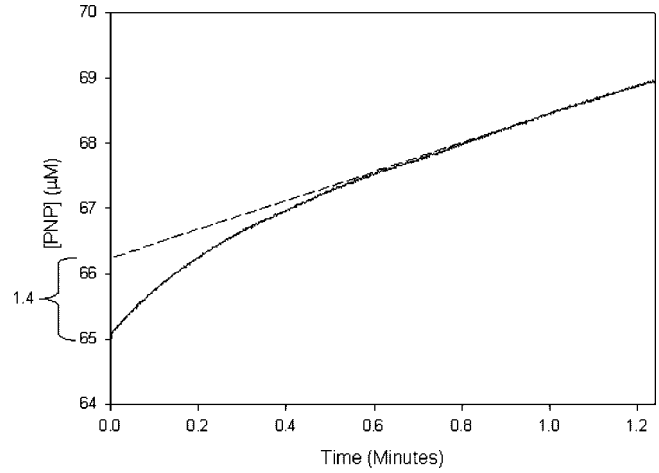

FIGURE 2: Burst phase kinetics observed upon reaction of PNP-SA with D59A TcTS. D59A TcTS (final concentration of 1.7 μM) was added to 1 mM PNP-SA in pH 7.6 buffer and the absorbance at 400 nm monitored. The substantial initial absorbance is due to PNP derived from earlier spontaneous hydrolysis.

This result was confirmed at the protein level by monitoring the reaction by electrospray mass spectrometry. Thus, D59A was incubated with PNP-SA and the sample loaded onto the ESI-MS apparatus via a short LC column (Figure 3A). In Figure 3, the solid line shows the mass spectrum of D59A TcTS to which no substrate had been added (71160), while the dashed line is that for D59A TcTS preincubated with PNP-SA (71450). The difference in masses (Δ = 290) is completely consistent with the expected mass increase due to covalent attachment of a sialyl residue (291 Da). Clearly, the sialyl−enzyme intermediate accumulates on the mutant enzyme. Further, as shown in Figure 3C, such an intermediate also accumulates, albeit to a lesser extent, on the wild-type enzyme upon incubation with PNP-SA. The small peak at 71493 corresponds well with the expected mass for the sialyl−enzyme form of wild-type TcTS.

Azide Rescue of D59A TcTS. A methodology has been developed for the assignment of acid/base catalysts in glycosidases via a search for azide "rescue" of enzyme activity in mutants modified at that position (*14–16*). This approach was also applied to the assignment of D59 as the acid/base catalyst in TcTS. Measurement of rates of cleavage of PNP-SA by D59A TcTS in the presence of a series of azide concentrations from 0 to 800 mM revealed a pronounced azide activation effect. The observed rate increased more than 20-fold from 0 to 100 mM azide and then decreased beyond that presumably due to a general inhibition by azide anion (Figure 4). The activation is due to azide reacting more rapidly with the covalent sialyl−enzyme intermediate than water does in the absence of the base catalyst. Since deglycosylation is rate-limiting in this case, the result is an increase in the observed steady state rate. This assertion requires, however, that the product obtained under these conditions indeed be the α-sialyl azide and not just sialic acid. To establish that this was the case, the reaction product was isolated and analyzed by MALDI-TOF MS. The observed ion at *m*/*z* 357.2 is exactly that of a sialyl

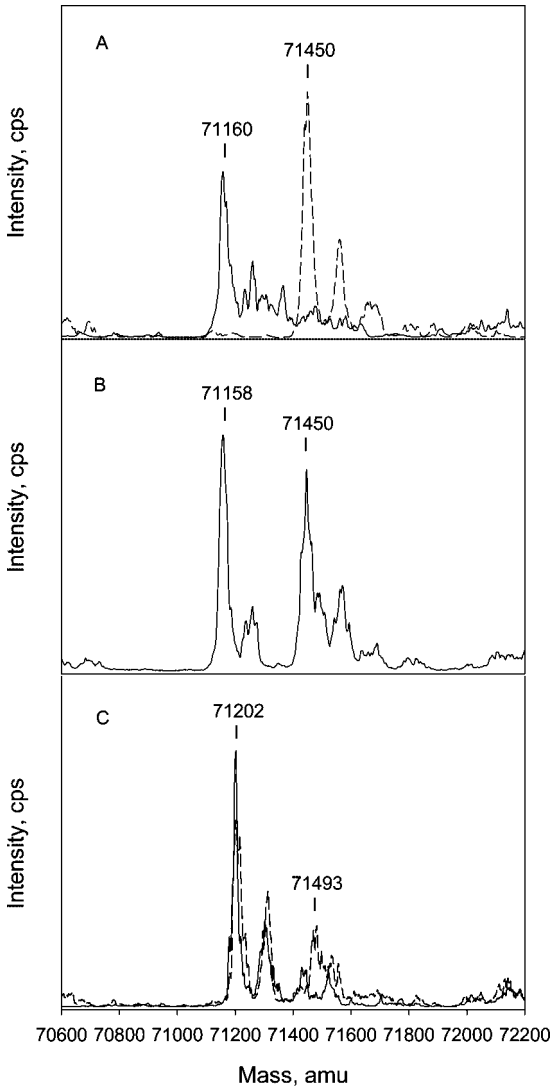

FIGURE 3: ESI-MS analysis of formation of an intermediate on D59A TcTS. (A) Electrospray ionization mass spectra of D59A TcTS and its sialyl−enzyme intermediate. The solid line is the spectrum of D59A TcTS with a mass of 71160 Da. The dashed line is the spectrum of D59A TcTS incubated with PNP-SA, yielding a mass of 71450 Da. The difference of 290 Da corresponds to one attached sialic acid moiety. (B) Turnover of the D59A TcTS sialyl−enzyme form by sodium azide. D59A sialyl-TcTS (71450 Da) was briefly incubated with sodium azide, partially releasing the free enzyme (71158 Da). (C) Electrospray ionization mass spectra of wild-type TcTS and its sialyl−enzyme form. The solid line is the spectrum of TcTS (71202 Da). The dashed line is the spectrum of TcTS incubated with PNP-SA (71493 Da).

azide product. To establish the anomeric stereochemistry of the product, the carboxylic acid was converted to its methyl ester and subjected to NMR characterization. Comparison of the ^{1}H NMR data with those in the literature identified the product as methyl (5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosyl)onate (*11*). Such formation of an azide with the same anomeric stereochemistry as the starting substrate is the expected result if Asp59 is indeed the acid/base catalyst.

Further confirmation of rescue with azide, at the protein level, was shown by ESI-MS analysis of a sample of the sialyl−enzyme intermediate before and after a brief incubation with sodium azide as shown in Figure 3B. Clearly, the sialyl−enzyme intermediate is being converted to free enzyme upon incubation with azide. Thiophenol was also

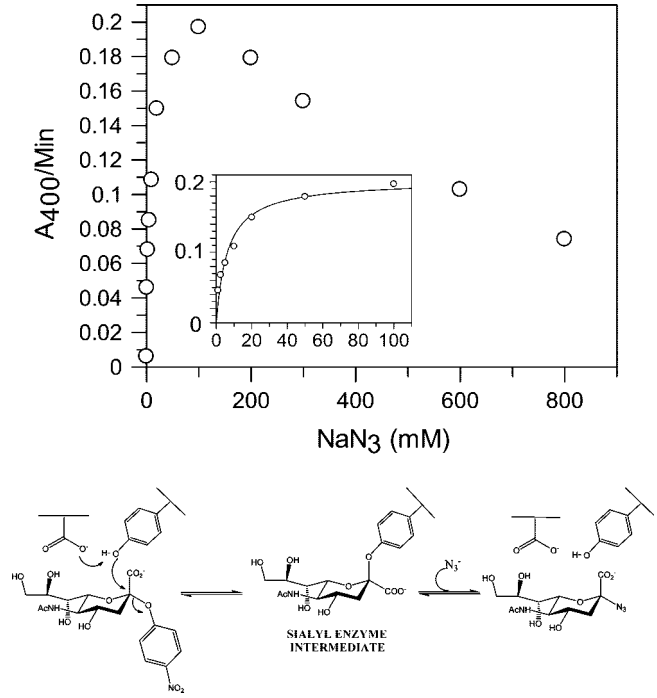

FIGURE 4: Rescue of D59A TcTS activity by increasing concentrations of sodium azide. The top panel shows kinetic data. The bottom panel shows the reaction in question. The inset shows an expansion of the plot from 0 to 100 mM azide.

shown to be a useful external nucleophile (data not shown), but neither sodium acetate nor potassium fluoride functioned in that role. Such differential behavior with different enzymes has been observed previously (*14–16*).

Verification of Tyr342 as the Catalytic Nucleophile. The ability to accumulate a sialyl−enzyme intermediate on the D59A mutant without the use of a fluorosugar analogue provided confirmation that Tyr342 acts as the catalytic nucleophile not only with fluorosugar substrates but also with unsubstituted sialosides, thereby sidestepping possible concerns that the use of fluorosugars may alter reaction pathways. Comparative HPLC−MS analysis of peptic digests of sialylated and free D59A identified a single pair of peptides at *m/z* 1084 and 1375 in the "free" and "bound" samples, respectively, that differ in mass by the expected 291 Da for an appended sialyl moiety. These masses correspond with those of the peptide identified previously using the fluorosugar approach (*8*). Indeed, further MS/MS analysis confirmed the sequence of DENSAYSSVL as obtained in earlier work.

DISCUSSION

The steady state kinetic data presented for both PNP-SA and CF_3MU-SA are entirely consistent with a ping-pong mechanism for TcTS. This interpretation is supported by the observation of the "half-reaction" of formation of the sialyl−enzyme intermediate upon reaction with the donor substrates in the absence of the acceptor lactose. This was observed at the protein level by ESI-MS with both the D59A mutant and the wild-type enzyme. It was also observed through burst kinetics with the PNP-SA substrate and the D59A mutant. Such a half-reaction is a requirement for a ping-pong mechanism but would not occur if the mechanism were ordered sequential as proposed previously.

A ping-pong mechanism is also fully consistent with the three-dimensional structure of TcTS as well as with structures of the trapped species along the reaction coordinate: the Michaelis complex, the covalent intermediate, and the enzyme−product complex. These structures reveal how the formation of the covalent sialyl−enzyme intermediate helps create the lactose binding site by, among other things, preorganizing the location of Tyr119 (*7*).

A ping-pong mechanism is also fully consistent with earlier kinetic isotope effect studies, which argued against an ordered sequential mechanism on the basis that similar β-^{2}H kinetic isotope effects were seen at low and high lactose concentrations (*6*). If the mechanism were ordered, then the isotope effect would have dropped to unity at the highest lactose concentrations due to trapping of the sialyllactose on the enzyme.

These results then call into question why such different kinetic behavior, involving converging rather than parallel Lineweaver–Burk plots, was seen previously. The most likely reason for the apparent difference in kinetic behavior is that, in the previous cases, rates were determined for the transfer reaction from sialyllactose onto isotopically labeled lactose and did not take hydrolysis into account. Since this reaction is accompanied by significant hydrolysis, to an extent that is dependent on the concentration of lactose present, then the rates measured were decreasingly affected by hydrolysis as lactose concentrations increased, leading to the observed convergence of lines in the Lineweaver–Burk plot. Apparent k_{cat}/K_M values, which are reflected in the slopes, cannot be the same in these cases. By contrast, our kinetic studies measure the total flux through the first step, and k_{cat}/K_M values (thus slopes) are therefore unaffected. This gave rise to the more reasonable parallel Lineweaver–Burk plots that are indicative of a ping-pong mechanism. Interestingly, an equivalent situation has been seen previously for several other group transfer enzymes that react through a covalent intermediate that can be hydrolyzed in a competitive pathway. These include a transglutaminase (*17*), a hexose phosphotransferase (*18*), and a nucleoside phosphotransferase (*19*). Indeed, in the latter case, the full set of kinetic equations was derived and data were fit to them in confirmation of the interpretation (*19*).

The clear demonstration of the half-reaction of formation of the sialyl−enzyme intermediate with release of *p*-nitrophenol directly supports the ping-pong mechanism via a covalent sialyl−enzyme intermediate. In addition, definitive evidence is also provided for Asp59 functioning as the acid/base catalyst in this specific enzyme by several means, including the use of azide rescue approaches. Studies on other GH33 sialidases had provided evidence for this in other systems, such as the neuraminidase from *Micromonospora viridifaciens* (*20*). However, this is the first definitive evidence in TcTS.

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