Evidence of Ternary Complex Formation in Trypanosoma cruzi trans-Sialidase Catalysis*

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Trypanosoma cruzi trans-sialidase (TcTS) is a key target protein for Chagas disease chemotherapy. In this study, we investigated the implications of active site flexibility on the biochemical mechanism of TcTS. Molecular dynamics studies revealed remarkable plasticity in the TcTS catalytic site, demonstrating, for the first time, how donor substrate engagement with the enzyme induces an acceptor binding site in the catalytic pocket that was not previously captured in crystal structures. Furthermore, NMR data showed cooperative binding between donor and acceptor substrates, supporting theoretical results. In summary, our data put forward a coherent dynamic framework to understand how a glycosidase evolved its highly efficient trans-glycosidase activity.

Nearly 1% of the genome of any organism encodes glycoside hydrolases (GHSs) (1). Many of these genes have been classified and grouped into families according to their sequence similarities. Proteins from the same GH family can share the biochemical mode of action and even key residues for catalysis. Although it has been established that almost all of the classical mechanisms undergo acid/base catalysis with carboxyl-containing residues as the most frequent catalytic nucleophiles, three of the 130 GH families use a tyrosine for this role instead. This differential aspect was first described for the Trypanosoma cruzi trans-sialidase (TcTS) (2). TcTS is a retaining GH (3) member of family number 33 (GH33) (4) that preferentially transfers sialic acid units to terminal β-galactopyranoside (β-Galp)–containing molecules and synthesizes α2,3-linked rather than α2,6 linkages. TcTS is a potential target for Chagas disease chemotherapy. In this study, we investigated the cooperative binding of both substrates to TcTS is required for transfer reaction.

Background: Trypanosoma cruzi trans-sialidase (TcTS) (2). TcTS is a retaining GH (3) member of family number 33 (GH33) (4) that preferentially transfers sialic acid units to terminal β-galactopyranoside (β-Galp)–containing molecules and synthesizes α2,3-linked rather than acts as a hydrolase (5). TcTS activity has been involved in a vast myriad of functions in the biology of T. cruzi (6–9) and in the pathology of Chagas disease, a life-threatening illness that affects 10 million people in 21 endemic countries (10). In an effort to decipher the mechanism of TcTS catalysis, Watts et al. (11) demonstrated that fluor-based sialyl inactivators formed a covalent intermediate with the enzyme. The occurrence of such an intermediate was further observed in several enzymes of the GH33 family (12, 13). Based on structural (11) and kinetic studies (14), Tyr-342 is considered to be the catalytic nucleophile residue of TcTS (11), whereas Asp-59 is proposed to act as a general acid/base catalyst in a double displacement reaction (14) that follows a classical ping-pong mechanism (15) in which the sialosyl-aglycone may leave the active site to allow entry of an acceptor substrate. According to this hypothesis, however, a water molecule could attack the sialosyl-enzyme intermediate before the acceptor substrate reaches the binding site, which would result in hydrolysis rather than in an efficient sugar transfer. Thus, a mechanism involving a ternary complex would support the high rates for the transfer reaction wherein the acceptor moiety is located in the acceptor binding site before the sialic acid transfer, which would displace water molecules from the catalytic pocket. Such a model would require additional conformational rearrangements in TcTS because the crystal structure of this enzyme shows a notably narrow acceptor binding site for accommodating both the sialoside donor and β-Galp acceptor substrates simultaneously (16).

Numerous works presenting evidence of plasticity in the catalytic cleft of TcTS have arisen. Molecular dynamics (MD) simulations have shown that two hydrophobic residues, Tyr-119 and Trp-312, play important roles in TcTS flexibility by controlling the entry of substrates into the catalytic pocket (17, 18). Classical (19) and hybrid quantum mechanics/MD (20) simulations with mutagenesis studies (19, 21) have identified other key residues that probably contribute to the plasticity of the binding site. Moreover, experimental evidence for such...
plasticity arose from the observation that the non-functional variant of the trans-sialidase (TS\text{Y342H}) (22) undergoes conformational changes upon sialoside binding, suggesting the opening of a second binding site that accommodates a \(\beta\)-Galp moiety (6). Active site rearrangement following the binding of sialoside was further proposed for the active TcTS (23). Results of TS\text{Y342H} incubated with \(\alpha\)2,6-sialyllactose in the presence of lacto-N-tetraose show that incorrect fitting of sialoside into the binding site of TS\text{Y342H} does not trigger \(\beta\)-Galp binding, which corroborates this hypothesis (6). Additionally, surface plasmon resonance results show that lactose (Lac) binds to the inactive mutant TS\text{Y59N} in the presence of \(\alpha\)2,3-sialylactose (3-SL) (24). The sequential entry of both substrates, leading to the formation of a ternary complex, would support the catalytic efficiency of the sialic acid transfer reaction but would contradict the crystallographic data (24).

Herein, we investigated the implications of TcTS active site flexibility on its biochemical mechanism by analyzing the protein binding dynamics of both donor and acceptor substrates on three TcTS variants, the enzymatically active form (wild type TcTS (TS)) and two inactive mutants, TS\text{Y342H} and TS\text{D247A}, using MD simulations and NMR spectroscopy studies. We demonstrated for the first time that upon 3-SL binding TS and TS\text{Y342H}, but not TS\text{D247A}, undergo conformational changes, opening a second cleft in the catalytic site and allowing the acceptor substrate to bind. The simulations carried out with the Lac molecule bound to the second site of TS and TS\text{Y342H} showed a stable ternary complex with the acceptor substrate in position for a subsequent transfer reaction, which sheds light on the catalytic mechanism. Our results show remarkable structural plasticity upon engagement of acceptor substrate in the TcTS active site, a property that must be considered in future studies on the catalytic mechanism of the enzyme and in the design of new selective inhibitors.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of His-tagged Fusion Proteins**—Expression and purification of TS, TS\text{Y342H}, and TS\text{D247A} proteins were carried out according to Carvalho et al. (19). Briefly, a freshly transformed bacterial colony with the His-tagged protein insert was selected and inoculated in 100 ml of Luria Bertani medium. Twenty milliliters from this preinoculum was transferred to 4 liters of terrific broth medium and grown at 37 °C until an optical density at 600 nm of 0.8 was reached. Protein expression was induced by addition of 0.12 mM isopropyl \(\beta\)-D-thiogalactoside, and the cells were incubated overnight at 28 °C. The bacterial cells were harvested by centrifugation and washed with saline solution. The pellet was lysed with 20 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, 2% Triton X-100, 1 mM EDTA, and protease inhibitors (5 mg/ml trypsin inhibitor, 0.1 \(\mu\)M iodoacetamide, 1 mM PMSF, 2 \(\mu\)g/ml leupeptin, and 30 \(\mu\)M E64) and sonicated. The lysed cells were cleared by centrifugation (32,816 \(\times\) g for 15 min at 4 °C), and the supernatant was loaded into three 5-ml HitTrap IMAC HP columns (GE Healthcare) that were charged with Ni\(^{2+}\) ions. Next, trans-sialidases were eluted using a stepwise imidazole gradient of up to 250 mM. Protein fractions were dialyzed against Tris-HCl buffer, loaded into a Mono S column, and eluted using a linear gradient of NaCl. Sample purity was assessed by SDS-PAGE. Proteins were concentrated using Amicon Ultra (50,000 nominal molecular weight limit; Millipore) as needed.

**TcTS Activity Measurements**—Sialidase activity was determined by measuring the fluorescence of 4-methylumbelliferone released by the hydrolysis of 4-methylumbelliferyl-\(\alpha\)2,6-sialylacetamide (Sigma) as described by Ribeirão et al. (25). The hydrolysis reaction was carried out in 100 \(\mu\)l of 20 mM phosphate buffer, pH 7.4 containing variable concentrations of 4-methylumbelliferyl-\(\alpha\)2,6-sialylacetamide (0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 3.0, and 4.0 mM) and TcTS proteins as follows: TS, 5 \(\mu\)g/ml; TS\text{Y342H}, 1 \(\mu\)g/ml; and TS\text{D247A}, 1 \(\mu\)g/ml. The fluorescence emission of 4-methylumbelliferone at 450 nm was monitored for 1 h at 37 °C under 365-nm excitation in a Spectramax M5e instrument (Molecular Devices). Kinetic parameters were determined by direct fit of the rate versus substrate concentration data to the Michaelis-Menten equation using GraphPad Prism version 5 (GraphPad Software, San Diego, CA).

Specific trans-sialidase activity was measured by following the transfer of sialic acid from 3-SL (Carbosynth, Berkshire, UK) to acetyl-\(\alpha\)-1,4-lactosamine ([\(^{14}\)C]LacNAc; American Radiolabeled Chemicals, St. Louis, MO) as described by Ribeirão et al. (25). The reactions were performed in 50 \(\mu\)l of 20 mM phosphate buffer, pH 7.4, 0.5 mg/ml BSA (Sigma), 5 mM 3-SL, 2.5 mM N-acetyllactosamine (LacNAc; Sigma), and 0.52 nmol (10.5 \(\mu\)m) of [\(^{14}\)C]LacNAc (50 mCi/mmol; American Radiolabeled Compounds). The reactions were started by adding the enzymes TS, TS\text{Y342H}, and TS\text{D247A} at 5 \(\mu\)g/ml, 2 mg/ml, and 1 mg/ml, respectively. The reaction volumes were incubated for 10 min at room temperature and stopped by the addition of 1 ml of deionized water.

The sialylated [\(^{14}\)C]LacNAc was purified by chromatography in 0.5 ml of Dowex 1X4 (Sigma) in acetate form equilibrated with 20 ml of deionized water. Unreacted [\(^{14}\)C]LacNAc was washed out by 20 ml of deionized water. Bound sialylated [\(^{14}\)C]LacNAc was eluted with 3 ml of 1M ammonium acetate and quantified by liquid scintillation.

The specific activities of each enzyme were determined as the rate of reaction product by time and protein concentration. The significance was assessed by one-way analysis of variance using GraphPad Prism 5.

**System Setup for Molecular Dynamics**—The structure of TS was modeled according to previous work (19) because there are nine missing residues (DPAAASSSS) between amino acids Ala399 and Gly409 in the Protein Data Bank 1MS3 structure (24). The selected model was validated by a Ramachandran plot obtained through the PROCHECK server (26). Mutations TS\text{Y342H} and TS\text{D247A} were made using Swiss-PdbViewer (27) on the modeled TS to generate the TS\text{Y342H} and TS\text{D247A} variants.

The coordinates of ligands 3-SL and Lac were extracted from Protein Data Bank code 1SOI. The structure geometries of both oligosaccharides were optimized using a Monte Carlo algorithm in GHEMICAL (28). The lower energy structures of the ligands were semiempirically optimized using the RM1 Hamiltonian (29) in the MOPAC 2009 software (30). Charges were calculated by GAMESS-US utilizing the base 6-31G* and the RESP.pl Perl script from the R.E.D. III server. Finally, the molecules were parameterized in the Optimized Potentials for Liq-
uid Simulation—All Atoms (OPLS/AA) force field (31, 32) with MKTOP software (33).

Molecular Dynamics Simulations—Five systems were prepared: TS with 3-SL, TS with 3-SL and Lac, TSD/247A with 3-SL, TS Y342H with 3-SL, and TSY342H with 3-SL and Lac. The coordinates of systems with 3-SL were obtained by overlapping the constructed model and the crystal structure from Protein Data Bank code 1S0I in which the TcTS mutant D59A was co-crystallized with 3-SL. For systems containing Lac, the coordinates of this molecule were obtained using molecular docking (as described in the next section). All systems were analyzed with the PROPKA server (34) to find the local protonation state of all residues at pH 7.4.

The MD simulations were run by the GROMACS 4.0.3 computational package (35, 36) using the OPLS/AA force field. Systems were simulated keeping temperature and pressure constants at 310 K and 1 bar, respectively. Proteins with or without the ligands were immersed in dodecahedral boxes with periodic boundary conditions and filled by Transferable Intermolecular Potential 3P (TIP3P) water molecules and chloride anions for neutralization of the total charge of the systems. All systems were energy-minimized using a steepest descent algorithm with and without position restraint followed by conjugate gradient and quasi-Newton methods until a force of less than 41.84 kJ mol⁻¹ nm⁻¹ was reached. The systems were equilibrated for 500 ps with position-restrained MD for heavy atoms followed by 20 ns of free MD. We also used the particle mesh Ewald method, 6–12 Lennard-Jones potential with a 1-nm cutoff, and the Linear Constraint Solver (LINCS) algorithm in the MD steps.

Molecular Docking—Lac was docked on the protein-3-SL complex. For the protein, Gasteiger charges were calculated using AutoDock Tools (37), and for 3-SL, restrained electrostatic potential charges were calculated using the RESP.pl script downloaded from the R.E.D. III server. Coordinates with respective charges were added to the docking input containing the coordinates of the protein. We utilized a grid of 26 × 36 × 36 nm (XYZ), which was centered at the active site. One hundred possible conformations were calculated by AutoDock Vina (38), and the best ones were selected by considering the docking energy, the number of conformers in each cluster, and the distance from and location of key residues for interaction in the pocket.

Diffusion Ordered Spectroscopy (DOSY)—3-SL, methyl-S-(5-acetamide-3,5-dideoxy-D-glycero-ß-D-galacto-2-nonulopyranosyl-(2-3)-ß-galactopyranosylacid (NeuSGal), methyl-4-O-ß-D-galactopyranosyl-ß-D-glucopyranoside (Me-Lac), lacto-N-neotetraose, and BSA were dissolved in deuterated PBS, pH 7.5 (not corrected for isotope effects). Solutions of TS, TSY342H, and TSD/247A in 20 mM Tris-HCl were exchanged with deuterated PBS using four cycles of concentration with Amicon Ultra (50,000 nominal molecular weight limit; Millipore) and diluted in deuterated PBS. One hundred microliters of 0.1 mM protein solution (TcTSs or BSA) was titrated with the sialoside (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, and 2.0 mM) or lacto-N-neotetraose (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.9 mM) as a negative control. The solution containing 0.1 mM protein plus sialoside was further titrated with Me-Lac (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.9 mM) in Shigemi tubes. NMR spectra were obtained at a probe temperature of 293 K on a Bruker DMX 600-MHz spectrometer equipped with a 5-mm self-shielded gradient triple resonance probe. All DOSY experiments were performed with a stimulated echo using bipolar gradient pulses for diffusion, one spoil gradient, and the 3-9–19 pulse sequence with gradients for water suppression according to the Bruker pulse program stebpp1s19. The gradient duration was set to δ = 10 ms (protein-only experiments) and 20 ms (protein–carbohydrate complexes experiments), and the diffusion time was set to ∆ = 40 ms. Gradient strength was linearly varied in 16 steps from 0.6 to 6.7 G/cm. For each of the 16 gradient amplitudes, eight transients of 192 complex data points were acquired to a 10.0-ppm spectral width. The pulse repetition delay between each scan was 3 s. The data acquisition and analysis were performed using the spectrometer software Topspin 3.2 (Bruker Corp.) and GraphPad Prism 5.

Isotopic Protein Labeling—A freshly transformed bacterial colony with the His-tagged TS insert was grown at 37 °C in 15N-2H-enriched M9 minimal medium to obtain 15N-2H-labeled protein samples according to Tugarinov et al. (39). Protein expression was induced with 1.0 mM isopropyl β-D-thiogalactoside for 12 h. After harvesting, cells were resuspended in buffer containing 50 mM Tris-HCl and 0.5 mM NaCl, pH 8.0 and disrupted by sonication. Following centrifugation at 5000 × g for 10 min, isotopically labeled protein was purified and concentrated as described for unlabeled TcTSS.

The 2H-15N-labeled TS sample was dissolved in 20 mM phosphate buffer, pH 7.5 prepared with 2H2O, providing a final protein concentration of ~200 µM. NMR spectra of isotopically labeled samples with or without 2 mM NeuSGal or with 2 mM NeuSGal and 2 mM Me-Lac were recorded at 303 K on a Bruker DMX 600-MHz spectrometer equipped with a cryoprobe (5-mm CPTCI 1H). All 1H-15N heteronuclear single quantum coherence spectra were recorded by setting up a resolution of 1024 × 128 points and spectral window of 10.822 and 3.040 kHz for H and 15N, respectively, according to the trosyetb3gpsi pulse sequence. All NMR data were processed and analyzed using Topspin 3.2.

RESULTS

Characterization of Enzyme Kinetic Parameters—To assess the extent of enzyme activity loss due to the mutations, we tested the enzyme variants for both hydrolysis and trans-sialidase activities. Sialidase activity was assayed by continuous

| Enzymes | $K_{m}$ (mM) | $V_{max}$ (µM/min) | $K_{cat}$ (µM/min) | $K_{cat}/K_{m}$ (µM⁻¹/min⁻¹) |
|---------|----------|------------------|-----------------|----------------------|
| TS      | 0.537 ± 0.062 | 0.798 ± 0.168 × 10⁻³ | 13.747 ± 3.025 | 26.282 ± 3.888 |
| TSY342H | 2.213 ± 0.159 | 0.063 ± 0.017 × 10⁻³ | 0.003 ± 0.001 | 0.682 ± 0.257 × 10⁻³ |
| TSD247A | 1.659 ± 0.015 | 0.395 ± 0.212 × 10⁻³ | 0.036 ± 0.019 | 0.027 ± 0.007 |

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fluorometric detection of 4-methylumbelliferone, the product of hydrolysis of 4-methylumbelliferyl-\(N\)-acetylneuraminic acid (25). \(TSD_{247A}\) and \(TS_{Y342H}\) showed higher \(K_m\) values than TS (Table 1), suggesting that the mutant proteins have lower sialic acid affinity than TS. The turnover constants and catalytic efficiencies of mutant proteins were found to be more than 300 times lower than those of TS (Table 1), supporting their residual hydrolytic activity.

The sialic acid transfer was assessed as described by Ribeirão et al. (25). \(TS_{Y342H}\) and \(TSD_{247A}\) were close to inactivity with specific activities 13,000 and 240 times lower than that of TS, respectively (Fig. 1). Using this method, we did not observe a detectable reaction product in a continuous manner for \(TS_{Y342H}\) and \(TSD_{247A}\) in contrast to the high activity displayed by TS. To comprehend the molecular basis of the loss of activity of the mutated enzymes, we performed MD simulations of TS, \(TS_{Y342H}\), and \(TSD_{247A}\) complexed to 3-SL.

\(Y342H\) Mutation Affects the Structure of Protein-Carbohydrate Complex—Given that histidine is a residue frequently found in reaction mechanisms, functioning either as a nucleophile (40) or as a basic catalyst (41, 42), the inactivation of enzyme

![FIGURE 2. Alterations in hydrophobicity in the catalytic cleft of \(TS_{Y342H}\). Initial (A) and final (B) frames of MD simulations of the \(TS_{Y342H} + 3\)-SL system are shown. 3-SL and His-342 (center of images) are represented by sticks colored in cyan; the residues of the cleft at 3 Å away from His-342 are represented in lines and balls colored according to polar character (nonpolar residues in white, polar in green, negatively charged in red, and positively charged in blue). Distances of the imidazole ring from His-342 to 3-SL (C) or to Asp-59 (D) throughout the simulation time are shown. E, solvent-accessible surface (SAS) of residues from the active site during MD. F, conditional distribution function of water molecules relative to Asp-59 from TS and \(TS_{Y342H}\) binary systems. Data relative to the TS + 3-SL system are represented by blue lines; those relative to the \(TS_{Y342H} + 3\)-SL system are represented by red lines.](image-url)
mation activity due to the Y342H mutation was unexpected although extensively reported (6, 43–45).

Throughout the MD simulation, His-342 was observed to internalize into the active site, losing interaction with polar residues, such as Arg-35, Glu-230, and Ser-285 (Fig. 2A), and approaching nonpolar residues like Leu-36 and Ala-179 (Fig. 2B). The novel interaction network of His-342 prevented it from binding to sialic acid of 3-SL, the distance from which increased from 5 up to 8 Å during the simulation (Fig. 2C). The inner position of His-342 placed it apart from Asp-59 with the distance reaching 11 Å at the end of the simulation (Fig. 2D). Such a gap would impair both TcTS activities because the supposed nucleophile could not reach the donor substrate. Besides, the position adopted by His-342 also contributed to its reduced solvation in contrast to that observed for Tyr-342 within the position adopted by His-342 placed it apart from Asp-59 with the distance reaching 11 Å at the end of the simulation (Fig. 2).

The D247A mutation also disrupted the hydrogen bond formed between the guanidine motif of Arg-245 and the Asp-247 carboxyl group observed in TS (Fig. 3D). The D247A mutation also disrupted the hydrogen bond formed (Fig. 3B). The D247A mutation, however, disrupted these interactions (Fig. 3A) because it altered both the side chain length and the charge of the residue. Therefore, in TS, the sialic acid glycerol moiety was separate from Ala-247, which resulted in the complete absence of hydrogen bonds between them (Fig. 3B).

Another series of remarkable residues influenced by the D247A mutation is the arginine triad, which is composed of Arg-35, Arg-245, and Arg-314. These residues are responsible for fixing the sialoside carboxyl group in the proper position for catalysis (24). Analysis of the TS and TSD247A MD simulations showed important differences in the network of interactions (Fig. 3). In the TS + 3-SL system, the carboxyl motif of 3-SL was close to Arg-245 (Fig. 3C), whereas it shifted toward Arg-35 in the complex containing TSD247A (Fig. 3D), which suggests that 3-SL was not correctly positioned for catalysis. The D247A mutation also disrupted the hydrogen bond formed between the guanidine motif of Arg-245 and the Asp-247 carboxyl group observed in TS (Fig. 3E and F). Meanwhile, loss of the charge of Asp-247 favored hydrogen bond formation between Arg-245 and another nearby acid residue, Glu-230 (Fig. 3G and H). This feature may reduce the basic character of Glu-230, which in turn could reduce the nucleophilic character of Tyr-342.

We also observed that the D247A mutation altered the solvation of important residues in the catalytic cleft. In particular, the radial distribution function of solvent relative to Asp-59 suggests that this residue was less solvated in the complex containing the mutant TSD247A than in TS-3-SL (data not shown). Such limited contact of this aspartate with water molecules would prevent their activation, a required step for the occurrence of the hydrolysis reaction.
Moreover, the D247A mutation impaired the interaction between Tyr-248 and Trp-312 observed in the TS/H110013-SL simulation (Fig. 4, A and B). In the TSD247A complex, Tyr-248 moved 5 Å away from Trp-312 (Fig. 4C), which increases Trp-312 motility as shown by its root mean square deviation (r.m.s.d.; Fig. 4D). In the TSD247A + 3-SL MD, Tyr-248 shifted away from the catalytic site, whereas in the TS + 3-SL system, the interaction between Tyr-248 and Trp-312 moved this last residue away from Tyr-119 (Fig. 4E) and caused Tyr-119 to be closer to 3-SL (Fig. 4B).

**Surprises of Protein-Carbohydrate Interaction: a New Cavity Revealed**—Trp-312 movement also contributed to the formation of a second cavity in the TS/H110013-SL complex that was not present in the TSD247A + 3-SL system. By the trajectory analysis of the MD simulations, we identified pronounced structural differences among TS, TSY342H, TSD247A, and the Protein Data Bank 1S0I crystal structure. Many residues from the TS and TSY342H complexes were shifted away from 3-SL, which favored the opening of a second cavity adjacent to the donor substrate (Fig. 5, A and B, respectively) not observed in the crystal structure (Fig. 5C). The Tyr-119 movement is in agreement with previous works (14, 24). Furthermore, the Asp-59 residue of TS and TSY342H underwent a great conformational change, being shifted 7 Å away from the 3-SL glycosidic bond (Fig. 5E). The new position of Asp-59 in TS + 3-SL and TSY342H + 3-SL restrained its role as a basic catalyst for both enzyme reactions. Interestingly, the additional cleft was not observed in the TSD247A MD (Fig. 5D) where Asp-59 was close to the donor substrate (Fig. 5E).

The second cleft of TS was suggested previously (25, 44) because binding of the acceptor substrate was only achieved when the donor substrate was present (23, 24). Such an argument is strengthened by the observation that the inactive mutant TSD247A did not display the second cleft during the MD simulations, which suggests that binding of the acceptor substrate is imperative for the trans-sialidase reaction to take place.

**Does the Second Site in TS and TSY342H Support Acceptor Substrate Finding?**—The second cleft observed in TS and TSY342H complexed with 3-SL was 8 × 12 × 13 Å, which should be large and deep enough to lodge the acceptor substrate. To address this hypothesis, we docked a Lac molecule into the new site of TS + 3-SL and TSY342H + 3-SL systems and performed MD simulations on the ternary complex.

During the simulation, we perceived that the Lac remained stable in the second cleft, which favored stabilization of 3-SL in a more likely position for catalysis as observed from its r.m.s.d.
in the ternary complex compared with the binary complex (Fig. 6A). Also, we observed that the r.m.s.d. of 3-SL was larger and more unstable in the binary system than in the ternary system (2 Å on average). The same pattern was shown by the binary and ternary complexes formed by TSY342H and its ligands; however, 3-SL achieved notable stabilization just later in the MD simulation time (Fig. 6B).

Other residues were in a more favorable situation for the transfer reaction in the ternary system than in the binary system, such as Asp-59. The carboxyl group of Asp-59 moved toward the Lac 3-OH, which favored its proton abstraction. Lac binding also reduced Asp-59 solvation in the TS ternary complex (Fig. 6C), which suggests that the acceptor substrate displaced water molecules from the catalytic site and supported the high rates of transfer reaction exhibited by TS.

It is noteworthy that Trp-312 had a remarkable shift during MD of the TS ternary complex. Initially close to the sialoside galactosyl ring (Fig. 7A), Trp-312 disrupted the interaction with the acceptor substrate and moved toward the acceptor substrate (Fig. 7B). These changes were evident in the analysis of distances of Trp-312 C2 and β-Galp C1 from 3-SL and of Trp-312 and D-glucopyranoside C4 from Lac (Fig. 7C). Besides, the movement of Trp-312 resulted in a radical alteration in the dihedral angle formed by carbons α and β (Fig. 7D).

**Experimental Binding of TcTSs to Donor and Acceptor Substrates by NMR**—The results obtained so far suggest that sialoside binding induces a conformational change in the architecture of the TS active site to enable the binding of acceptor substrate. The same cooperative binding profile was observed for TSY342H. For the inactive variant TSD247A, however, the opening of a second cavity was not observed. Because this protein has all catalytic residues intact, its inactivity may be due to the inability of the acceptor substrate to bind to the catalytic cleft in the presence of sialoside. To support the MD findings,
we performed binding experiments using DOSY. DOSY-NMR experiments provide the observed diffusion constants ($D_{obs}$) of the ligands that represent a sum among its molar fractions bound to the protein or not. By the conversion of the molar fractions to molar concentrations, it is possible to calculate the dissociation constants ($K_d$) as a measure of the binding affinity of the protein/ligand complex (46).

Because $T_{D247A}$ and $T_{Y342H}$ showed only residual sialidase activity, even a hydrolyzable donor substrate, such as 3-SL, would be adequate for our binding studies. Nevertheless, binding studies with TS required a donor substrate resistant to enzymatic hydrolysis. It is known that some sialidases are not able to cleave thiosialosides (47). Thus, we explored the resistance of the thiosialoside NeuSGal (Fig. 8A) to TS hydrolysis by monitoring the reaction using one-dimensional $^1$H NMR. After 24 h of incubation (Fig. 8, B and C), the resonances corresponding to H3eq and H3ax signals of the $\alpha$- and $\beta$-anomers of free sialic acid at 2.72 and 1.62 ppm and 2.21 and 1.84 ppm, respectively (3), were not detectable. Neither was the signal corresponding to H1 of free methyl galactoside at 4.4 ppm. These data demonstrated that NeuSGal was a suitable substrate for DOSY assays with TS.

Our data showed a regular binding profile for the TS-NeuSGal complex without ligand saturation even at the higher dose tested (Fig. 9A). NeuSGal displayed a $K_d$ of 0.456 mM for TS binding (Table 2), and notably, Me-Lac was able to bind to TS only in the presence of NeuSGal with a $K_d$ of 0.015 mM (Table 2). During Me-Lac titration, $D_{obs}$ values from sialoside did not change significantly, suggesting that there was no displacement of sialoside from the active site by Me-Lac. The same binding pattern was observed for the complexes formed among $T_{Y342H}$ and its ligands (Fig. 9B) with $K_d$ values of 0.234 and 0.052 mM for 3-SL and Me-Lac (Table 2), respectively. When bound to $T_{D247A}$, 3-SL displayed a $K_d$ of 0.107 mM (Table 2), lower than that observed when complexed to NeuSGal (Fig. 9C). In the presence of BSA (used as negative control), Me-Lac presented a $D_{obs}$ of $2.82 \times 10^{-10}$ m$^2$/s, similar to that for the titration of Me-Lac to the binary $T_{D247A}$ complex. These results support the MD data showing that lactoside does not bind to the $T_{D247A}$-3-SL complex. Together, DOSY and MD simulation data show a differential cooperativity among ligands and $TcTS$ variants, supporting the existence of a ternary complex during TS catalysis.

**DISCUSSION**

Once restricted to Latin America, Chagas disease, which is caused by the parasite $T. cruzi$, is a worldwide challenge that has now spread to North America, Europe, and the western Pacific region (52). Despite the health and economic costs of this disease, its chemotherapeutic treatment is still notably unsatisfac-
tory (53), and designing new drugs is an urgent matter. The key roles of TcTS in the pathogenesis combined with its absence in mammalian hosts have suggested TcTS as a promising target for preventing the invasion of host cells by the parasite.

Both the trans-sialidase and sialidase reactions of TcTS have interested researchers all over the world because it has been proven that T. cruzi incorporates sialic acid through a mechanism different from that of mammalian sialyltransferases (54), which catalyze the sialylation with CMP-activated sialic acid substrates. However, almost 30 years of research has not been sufficient to unveil the reaction mechanism of this enzyme completely. In this study, NMR spectroscopy and molecular modeling were applied to the trans-sialidase theme to elucidate the molecular basis of its reaction mechanism.

The analysis of MD simulations has shown that upon 3-SL binding TS and TSY342H undergo conformational changes that open a second cleft in the catalytic site adjacent to the sialoside binding cleft. Such a cavity was not observed in TSD247A, which kept the sialoside closely bound to the active site. However, almost 30 years of research has not been sufficient to unveil the reaction mechanism of this enzyme completely. In this study, NMR spectroscopy and molecular modeling were applied to the trans-sialidase theme to elucidate the molecular basis of its reaction mechanism.

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Docking of Lac into TS + 3-SL and TSY342H + 3-SL systems was further followed by MD. From the solvated MD analysis of the ternary system, we perceived that Lac remained stable in the second cleft by placing the Asp-59 carboxyl group in the proper position for proton abstraction of the 3-OH in Lac, which favored the transfer reaction. Moreover, lactose binding reduced the solvent exposure of the catalytic site. These results do not support the suggested role of Asp-59 as an acid catalyst because in the ternary system this residue is too distant from the sialoside to protonate its aglycone. Such speculation is strengthened by the low theoretical pK_a presented by Asp-59 that was already suggested by other groups (55, 56). Whereas Amaya et al. (14) have argued that the Asp-59 pK_a tends to be higher due to its proximity to Tyr-119, we observed that these two residues were only in close proximity when the acceptor substrate was present in the cleft. This finding strongly suggests that Asp-59 acts as a basic catalyst only. In contrast, the Asp-96 residue has a theoretical pK_a high enough to act as an acid catalyst. This residue is highly conserved among viral and bacterial sialidases and acts as the acid/base catalyst in these enzymes (13, 57). Asp-96 was also considered to be a potential catalyst in the work of Buschiazzo et al. (16) and was cited together with Tyr-342 and Glu-230 as the conserved polar residues of the catalytic triad. However, Asp-96 interacted with the glycerol portion of 2,3-didehydro-2-deoxy-N-acetylneuraminic acid in the crystal structure of TcTS (24), whereas in a crystal structure released in 2004, a TcTS mutated at D59A and soaked with 3-SL revealed an interaction between the Asp-96 and the N-acetyl moiety of the sialoside (14). Despite the position of Asp-96, the TcTS crystals did not favor its role as an acid catalyst, and there is still no consensus on its role. One hypothesis is that before the nucleophilic attack the sialoside changes its ring conformation and adopts a configuration...
favorable for interaction with Asp-96, a position that was never captured in crystals.

The residue Trp-312 orchestrates the conformational change in the TS catalytic site after the binding of 3-SL. Other works have already attributed a role to this tryptophan as a gateway that provides the opening/closure motion of the TcTS binding cleft (17, 19). When the acceptor substrate is bound, however, Trp-312 moves far away from the sialoside toward the acceptor, which can place it in the best position for catalysis following its ejection after the reaction takes place and corroborates the lever/shovel function reported by Mitchell et al. (18).

The interaction observed in MD between \( \text{H9252-Gal} \) rings from both substrates is in line with the experimental results of Todeschini and co-workers (58), who showed transference of saturation among the protein and the H1, H3, and H4 from the donor \( \text{H9252-Gal} \) and the H1 and H3 protons from the acceptor \( \text{H9252-Gal} \) using saturation transfer difference-total correlation spectroscopy; the same protons were observed in this study to interact with Trp-312. This ability of the tryptophans to interact closely with the Gal rings has been widely observed (59–62). Such an interaction is suggested to be stacked CH/π based on quantum calculations (63). Taken together, these data contribute to unveiling the role of Trp-312, whose importance was observed previously in the inactive TSW312A mutant (21), in TS catalysis.

Comparison of the structure of TS with the structure of TSY342H clarified the internalization of His-342 into the active site toward hydrophobic residues. The new chemical environment experienced by this residue hampers the access of water molecules to the sialoside, impairing the hydrolysis reaction. This result provides an explanation for the reduction of the hydrolytic activity of TSY342H and is consistent with the residual sialidase activity demonstrated here and by another group (45).

**TABLE 2**

| Enzymes    | Sialoside \( K_d \) | Lactoside* \( K_d \) |
|------------|---------------------|----------------------|
| TS         | 0.456 ± 0.002       | 0.015 ± 0.001        |
| TSY342H    | 0.234 ± 0.004       | 0.052 ± 0.002        |
| TSD247A    | 0.107 ± 0.002       | NB                   |

* \( K_d \) of lactoside in presence of sialoside.
* No binding was observed.
The great distance between His-342 and Glu-230 impedes proton abstraction from the imidazole ring (His-342). Thus, although histidine would be a better nucleophile than tyrosine because it would have a negative ionic form stabilized by resonance, such a form becomes unlikely to occur as there are no basic residues close by that can act as a base. These data help to clarify how the Y342H mutation can affect the catalytic activity of TS.

The effect of the D247A mutation on TS activity was demonstrated previously (19), and the enzyme kinetics are detailed here. From our MD data, the role of the Asp-247 residue for the active site architecture can be dimensioned. This residue establishes hydrogen bonds with Asp-247 and Glu-230 while holding Arg-245 apart from Glu-230. Hydrogen bond formation between Arg-245 and Glu-230 would reduce the acidic character of this glutamate and hamper proton abstraction from Tyr-342, which is a crucial step of the trans-sialidase reaction. Furthermore, the Asp-247 residue itself is located in a narrow cleft, establishing hydrogen bonds with the 3-SL glycerol motif and assisting the orientation of sialoside into position for catalysis.

The glycerol portion of sialoside was observed to be a relevant group for ligand binding because saturation transfer difference NMR studies using sialoside derivatives showed that the sequential removal of hydroxyl groups reduced the binding of sialoside probes to TS (64). Buchini et al. (65) have observed that the cleft where the glycerol motif is located is larger and more hydrophobic than the equivalent from human cytoplasmic neuraminidase. These researchers have also demonstrated that the incorporation of bulky and hydrophobic substituents into C9 of sialic acid analogs, such as benzoyl and umbelliferyl, increases their specificity for TcTS against a human neuraminidase. These data are in line with ours and highlight the importance of Asp-247 for the structure of TS and its interaction with ligands, which suggests that this residue has a direct role in trans-sialidase specificity and should be considered for the design of new drugs targeted to this enzyme.

Using DOSY-NMR spectroscopy, we demonstrated that TS_{Y342H} and TS, but not TS_{D247A}, interacted with Me-Lac only after sialoside binding, which supports our MD data and previous work (6). Furthermore, the conformational switch triggered by sialic acid donor binding in TS was indeed confirmed by the differences in chemical shifts observed in H-15N heteronuclear single quantum coherence spectra. Surface plasmon resonance results obtained by Buschiazzo et al. (24) showed that Lac bound to an inactive TS_{D59N} mutated enzyme in the presence of 3-SL, which is consistent with our theory. However, the authors were unable to show the existence of two distinct binding sites when monoclinic crystals of the active TS were soaked in Lac and 2,3-didehydro-2-deoxy-N-acetylneuraminic acid, which indicated that the Lac binding site was too narrow to accommodate the Lac moiety of the donor and acceptor substrates simultaneously. The results of our work demonstrate that additional conformation rearrangements occur in the TS_{Y342H} and TS such that they can bind the acceptor substrate. In previous results using inactive TS_{Y342H} incubated with a2,6-sialyllactose in the presence of lacto-N-neotetraose, we showed that incorrect fitting of sialoside into the binding site of TS did not trigger β-Galp binding, which corroborates our double induced site hypothesis. These data suggest that the interaction of 2,3-didehydro-2-deoxy-N-acetylneuraminic acid with TcTS demonstrated by Buschiazzo et al. (24) is not sufficient to induce the required conformational rearrangement to accommodate acceptor and donor substrates simultaneously. Conformational fluctuations at the catalytic cleft of TcTS have been suggested previously by our group and others (17–20, 66). Indeed, MD simulations have led to a better understanding of

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**FIGURE 10.** TcTS undergoes conformational changes triggered by the binding of donor and acceptor substrates. A, overlap of 1H-15N heteronuclear single quantum coherence spectra of TS in the absence (blue) or presence of NeuSGal (green) and NeuSGal and Me-Lac (red). B, C, and D, detail of boxed regions of the spectra indicated in A.
the importance of protein flexibility in the catalytic mechanism of several enzymes (67) and were recently used together with NMR to demonstrate the opening of two sialoside binding sites in influenza virus neuraminidase (68).

The structural data of TcTS reported in this study reveal a remarkable alteration in the TS catalytic site, showing for the first time an acceptor substrate binding site in TcTS, which has been suggested to exist by many groups but has not been captured in crystal structures to date. Our data support the formation of a ternary complex during the trans-sialidase reaction because acceptor binding was shown to displace water molecules from the active site by adjusting the position of the sialyl donor in the cleft to favor the transfer. These data support the prevalence of trans-glycosylation over hydrolysis.

Our data suggest that trans-sialylation by TcTS follows neither a classical sequential nor a ping-pong mechanism (69) but a hybrid of these two systems in which the enzyme intermediate is formed after the ternary complex. Many examples of enzymes that do not fall into the strict classification of sequential or ping-pong mechanisms but lie somewhere in between these two systems have been reported (70, 71). Furthermore, a hybrid ping-pong sequential mechanism fits all of the data published to date (11, 15, 25, 69, 72).

In conclusion, this work showed, for the first time, the acceptor substrate binding site in TcTS, which has been suggested to exist by many groups but has never had its structure revealed. Our experimental and structural results strongly support the formation of a ternary complex for the trans-sialidase reaction of this enzyme. Taken together, our results put forward a coherent dynamic framework to understand how glycosidase evolved to become a highly efficient trans-glycosidase and suggest that the structure of TS with two cavities would be a better model for rational drug design addressed to trans-sialidase.

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TcTS Substrate-induced Binding Site

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