Unraveling the Differences of the Hydrolytic Activity of Trypanosoma cruzi trans-Sialidase and Trypanosoma rangeli Sialidase: A Quantum Mechanics–Molecular Mechanics Modeling Study

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ABSTRACT: Chagas’ disease, also known as American trypanosomiasis, is a lethal, chronic disease that currently affects more than 10 million people in Central and South America. The trans-sialidase from Trypanosoma cruzi (TcTS) is a crucial enzyme for the survival of this parasite: sialic acids from the host are transferred to the cell surface glycoproteins of the trypanosome, thereby evading the host’s immune system. On the other hand, the sialidase of T. rangeli (TrSA), which shares 70% sequence identity with TcTS, is a strict hydrolase and shows no trans-sialidase activity. Therefore, TcTS and TrSA represent an excellent framework to understand how different catalytic activities can be achieved with extremely similar structures. By means of combined quantum mechanics–molecular mechanics (QM/MM, SCC-DFTB/Amber99SB) calculations and umbrella sampling simulations, we investigated the hydrolysis mechanisms of TcTS and TrSA and computed the free energy profiles of these reactions. The results, together with our previous computational investigations, are able to explain the catalytic mechanism of sialidases and describe how subtle differences in the active site make TrSA a strict hydrolase and TcTS a more efficient trans-sialidase.

Introduction

American trypanosomiasis, also known as Chagas’ disease, is caused by the parasite Trypanosoma cruzi (T. cruzi) and is considered by the World Health Organization (WHO) to be one of the 17 neglected tropical diseases. It is endemic in Central and South America where it affects 10–12 million people, killing over 15,000 each year and infecting hundreds of thousands worldwide.1,2 It is transmitted to humans through the feces of triatomine bugs known as “kissing bugs”, and also between humans by blood transfusion and from mother to infant.3 The acute phase of the disease is usually asymptomatic while the chronic phase is characterized by the development of cardiac and digestive pathologies.1,2 While most trypanosomatisid parasites remain in blood, T. cruzi is an intracellular parasite. This fact seriously hinders the development of drugs for the treatment of Chagas disease.4–8 To date, Nifurtimox and Benznidazole, are the only two approved drugs for the treatment of the infection despite their low efficacy and their severe side effects.9 Consequently, investigations of new and more effective drugs as well as the characterization of novel targets are required. The trans-sialidase from T. cruzi (TcTS) is essential for the parasite infectivity in the human body, and combined with the fact that TcTS is not present in humans, it is currently considered a promising biochemical target for developing new molecules to control Chagas’ disease.10–16

Sialic acids are O- and N-substituted derivatives of neuraminic acid, a nine-carbon monosaccharide, usually located on cell surface glycoproteins and glycolipids. These acids play a key role in biology by regulating antigenic expression and molecular interactions and providing structural support and protection to cell membranes.17–19 Sialidases are a family of enzymes that catalyzes the removal of sialic acid from various glycoconjugates and constitute virulence factors of numerous viruses and prokaryotic and eukaryotic microorganisms.20 These enzymes are expressed in different parasites including Trypanosoma brucei, the causative agent of African Trypanosomiasis, Trypanosoma rangeli (T. rangeli), and T. cruzi. Both the sialidase of T. rangeli (TrSA) and TcTS are part of the sialidase family, but while the former is a strict hydrolase, the latter preferentially displays a trans-sialidase activity catalyzing the transfer of sialic acid residues from the host glycoconjugates to the parasite surface mucins.21,22 As a result, the parasite is protected from the host’s immune system and gains the ability to adhere to and invade host cells.23

TcTS and TrSA have a 70% sequence identity and the overall Cα root mean squared deviation (RMSD) value is only 0.59 Å. Both enzymes fold into two structural domains. The N-terminal catalytic domain shows a β-propeller fold which is connected by a long α-helical segment to the C-terminal domain displaying a β-barrel lectin-like topology.24,25 Despite the high structural
similarity, no \textit{trans}-sialidase activity is present in TrSA, which exclusively catalyzes the hydrolysis of sialic acid residues from sialyl-glycoconjugates.\textsuperscript{25,26} Furthermore, good inhibitors of TrSA (e.g., DANA and taminflu) are only very weak inhibitors of TcTS,\textsuperscript{24,25,27} indicating that small differences in the sequence and structure can give rise to substantial changes in the catalytic mechanism.

In this work, the residue numbers of both TrSA and TcTS will be referred to those of the crystal structure of the TcTS complex with sialyllactose (PDB ID: \textit{1S0I}).\textsuperscript{28} The active sites of TcTS and TrSA contain several residues conserved in other microbial sialidases. Those active site residues include the following: the catalytic residues Tyr342, Glu230, and Asp59 (involved in proton shuffling and nucleophilic attack); an arginine triad (Arg35, Arg245, and Arg314), which interacts with the carboxylate group of the sialic acid; and Asp96, which is H-bonded with O11 and N12 of sialic acid. In addition, in TcTS, Trp312 and Tyr119 display a hydrophobic lactose-binding region. In TrSA, Tyr119 is substituted by Ser119 leading to a less suitable region for the accommodation of the lactose ring.

The catalytic mechanisms of the hydrolysis and transfer reaction are depicted in Scheme 1.

Structural and kinetic studies revealed a double-displacement (ping-pong) mechanism with formation of a covalent sialyl-enzyme intermediate via Tyr342.\textsuperscript{26–32} After the formation of the Michaelis complex in the presence of lactose (MC\textit{lac}), both enzymes are able to cleave the host sugar–sialic acid bond leading to the covalent intermediate, with the leaving group (donor sugar) still present at the active site (CI\textit{lac}). Once the CI\textit{lac} is reached, TcTS completes its \textit{trans}-sialidase activity by taking the reverse step but in this case transferring the sialic acid to a parasite sugar (acceptor group) leading to a new MClac. In this reaction, the anomeric carbon of the sialic acid is attacked by the lactose hydroxyl oxygen, which is deprotonated by Asp59 while the Tyr342 phenolic oxygen is protonated by Glu230. In contrast, TrSA is unable to achieve the transfer reaction of sialic acid to the parasite surface glycoconjugates. In this case, the donor sugar in CI\textit{lac} is replaced by a water molecule (CI\textit{wat}). The hydrolysis of CI\textit{wat} to MC\textit{wat} is achieved by a nucleophilic water attack. Although this second path can be accomplished by both enzymes, TcTS was shown to be significantly more efficient in transferring than in hydrolyzing sialic acids.\textsuperscript{25}

A great deal of effort has been applied to identify the determinants of the \textit{trans}-sialidase activity in TcTS in contrast...
with the strict hydrolase activity of TrSA.\textsuperscript{11,25,33\textendash}35 The active site of TcTS shows a narrower and more hydrophobic binding pocket than TrSA. This was confirmed by molecular dynamics (MD) simulations performed by our group that showed that the TrSA CI\textsubscript{wat} adopted a more open catalytic cleft compared to that of the TcTS, facilitating the access of water molecules.\textsuperscript{36} Therefore, the trans-sialidase activity in TcTS can, in part, be favored by the exclusion of water molecules and the establishment of nonpolar interactions between the active site residues and the lactose group.\textsuperscript{24,37} Moreover, we have recently found that when lactose is not present in the active site, the catalytic couple Tyr\textsubscript{342}-Glu\textsubscript{230} is dissociated when TcTS is found in its covalent intermediate (CI) form. However, that behavior was not observed in TrSA.\textsuperscript{38} These facts support the idea of a long-lived CI that could be involved in the sialyl-transfer mechanism of TcTS.\textsuperscript{28,39,40} Recently, five TrSA key residues were mutated by Paris et al. (Met\textsubscript{95}-Val, Ala\textsubscript{97}-Pro, Ser\textsubscript{119}-Tyr, Gly\textsubscript{248}-Tyr and Gln\textsubscript{283}-Pro) leading to a quintuple mutant that showed around 1\% trans-sialidase activity.\textsuperscript{27} However, despite the numerous experimental and theoretical studies carried out thus far, the reasons for the different catalytic activities between both enzymes are still unclear.

Molecular modeling has become one of the most important tools to gain atomic insight and quantitative understanding of enzymatic reactions.\textsuperscript{41} Computational enzymology provides detailed features of the reaction pathways acting as a complement to experimental techniques.\textsuperscript{42,43} Recently, we studied the CI\textsubscript{wat} formation in TcTS\textsuperscript{34} and in TrSA, together with specific mutations that provided important insight into the trans-sialidase activity.\textsuperscript{33} In the present work, the hydrolytic activity of TcTS and TrSA was modeled using computer simulations and free energy calculations in order to complete the mechanistic studies in TcTS and TrSA and rationalize their distinct catalytic behavior.

\section*{METHODS}

**Setup of the Covalent Intermediate Models.** MD simulations of the CI\textsubscript{wat} of TrSA and TcTS (PDB IDs: 2A75 and 2AH2, respectively) were performed by our group and described in previous work.\textsuperscript{36} Representative conformations of TcTS and TrSA from unrestrained MD simulations were used as initial structures for the quantum mechanics/molecular mechanics (QM/MM) umbrella sampling simulations. Those systems included a water molecule present at a location suitable for the nucleophilic attack. In this conformation, a hydrogen bond is established between the water molecule and the carboxylic group of Asp\textsubscript{59}. The distance between the oxygen atom of the water molecule and the anomeric C in both covalent intermediates is less than 4 Å and an average hydroxyl oxygen (Tyr\textsubscript{342})\textendash anomeric C (sialic acid)\textendash O (water) angle of approximately 170° suggested an ideal position for an in-line attack on the anomeric C (sialic acid)\textendash hydroxyl oxygen (Tyr\textsubscript{342}) bond.

**QM/MM Umbrella Sampling Simulations.** Classical molecular mechanics can provide atomic insight into the active site of enzymes and contribute to identifying the determinants of reactivity. However, they are unable to treat processes taking place during enzymatic reactivity, including bond formation and breaking.\textsuperscript{44} In the hybrid QM/MM approach, the reactive part is treated using quantum mechanics while the rest of the system is treated with a classical force field (MM).\textsuperscript{41,45}

For each CI\textsubscript{wat}, the QM region included Glu\textsubscript{230}, Asp\textsubscript{59}, sialic acid, Tyr\textsubscript{342}, Arg\textsubscript{245}, Arg\textsubscript{314}, and Arg\textsubscript{35} and the water molecule while the rest of the enzyme residues were treated using the Amber99SB force field (Figure 1).\textsuperscript{46,47}

Hydrogen link atoms were used to treat the covalent bonds between the side chain of the catalytic residues (QM region) and the backbone of the protein (MM region). The self-consistent charge density functional tight binding (SCC-
DFTB) method, as implemented in AMBER, was employed to study the quantum region. SCC-DFTB was used successfully in many biomolecular simulations and chemical reactions and was found to be in good agreement with QM/MM calculations developed at higher levels of QM theory, such as MP2. This method was also shown to provide the best semiempirical description of five- and six-membered carbohydrate ring deformations. We have previously shown that the energy barriers obtained using DFTB and MP2 for the trans-sialidase activity of TcTS are very similar.

The reactions of the hydrolysis of the CIwat to MCwat were modeled by means of umbrella sampling using conditions similar to those previously used in TcTS. The distances modeled by means of umbrella sampling using conditions used to calculate the long-range Coulomb forces. The 2-D reaction coordinate is defined by RC1 and RC2: RC1 = d₁ − d₄ and RC2 = −d₁ − d₄.

|Scheme 2. Illustration of the Reaction Coordinates Chosen To Simulate the Hydrolysis of the Sialyl-Enzyme Covalent Intermediate*|
|---|
|The 2-D reaction coordinate is defined by RC1 and RC2: RC1 = d₁ − d₄ and RC2 = −d₁ − d₄.|

The energy decomposition method was employed to investigate the functional roles of TcTS and TrSA active site residues during the hydrolysis of the sialic acid CIwat. This method has been widely applied to identify the importance of different amino acids in the catalytic mechanism of enzymes and was recently employed by our group in different studies. We used it here to investigate the stabilization pattern of TcTS and TrSA residues on TSwat with respect to the CIwat. The QM region included the catalytic residues Asp59, Glu230, and Tyr342, a water molecule, and the sialic acid. A total of 400 configurations in the CIwat and TSwat configurations were selected in each case. For each configuration, every residue from the MM subsystem within 10 Å to the sialic acid was individually mutated to Gly. The averaged quantum mechanical energy was computed in each step in the wild-type proteins and for each mutant structure. The influence of a particular residue on the energy of a particular conformation was measured taking into account the difference of energies when a particular residue is present (denoted by i in eq 1) or when it is replaced by Gly (i − 1 in eq 1).

\[ \Delta E_i = [E_{Q}^{\text{QM/MM}} + E_{Q}^{\text{QM/MM}}] - [E_{i-1}^{\text{QM/MM}} + E_{i-1}^{\text{QM/MM}}] \]  

The contribution of each residue to the stabilization of TSwat during the hydrolysis of the CIwat to the MCwat was obtained by measuring the difference between the stabilization patterns of these residues in TSwat and CIwat.

\[ \Delta E_i = \Delta E_i^{\text{TSwat}} - \Delta E_i^{\text{CIwat}} \]  

Thus, a positive/negative value of \( \Delta \Delta E_i \) indicates that residue \( i \) exerts a higher destabilization/stabilization electrostatic effect on TSwat than on CIwat. This selective stabilization pattern occurs because the analyzed residue adjusts its position and/or because the electrostatic distribution of the QM subsystem changes when the system goes from CIwat to TSwat. Thus, the \( \Delta \Delta E_i \) values represent useful information on how the enzyme selectively stabilizes a particular state (i.e., TSwat) with respect to another. It should be noted that the relationship between the \( \Delta \Delta E \) and how a mutation can help to further stabilize the reaction is not direct. In fact, if we modify a specific residue, not only the electronic distribution of the QM subsystem, but also the positioning of the other residues are affected, and thus all of the pattern is modified.

More O’Ferral–Jencks Diagram. In order to describe the mechanism, a reaction space plot based on a two-dimensional More O’Ferral–Jencks diagram was created for both TrSA and TcTS enzymatic reactions. The reactant (CIwat) is located on the lower left corner, the product is on the upper right, and the \( x \) and \( y \) axes represent the Pauling bond order along the MFEP for O (water)–sialic acid bond making and the breaking of the sialyl–Tyr342 bond, respectively. The Pauling bond order, \( n_b \), was determined using the following equation:

\[ n_b = n_0 \exp \left( \frac{R_0 - R_b}{0.6} \right) \]

where \( n_0 \) denotes the bond order of the fully formed bond of length \( R_0 \) (in this case \( R_0 = 1.4 \) Å, and \( n_0 = 1 \) and \( R_b \) is the...
average distance between the leaving group (Tyr342) or nucleophile (water) and the anomeric C.

Analysis of the Molecular Dynamics Trajectories. Three-dimensional structures and trajectories were visually inspected by using the computer graphics program PyMOL. Interatomic distances and angles were monitored by using the ptraj module in AmberTools.

RESULTS AND DISCUSSION

Description of the Hydrolysis Mechanism. The analysis of the 2-D FES describing the hydrolysis of the CIwat by TcTS and TrSA showed a clear MFEP connecting CIwat to MCwat (Figure 2).

For both enzymes MFEPs proceed diagonally indicating that the reaction coordinates RC1 (involving Tyr342−sialic acid bond cleavage) and RC2 (associated with the water activation and nucleophilic attack on the anomeric C) are highly correlated. To better describe the mechanism, a “reaction space” plot, based on a More O’Ferral−Jencks style diagram, is presented in Figure 3.

For both systems, at the transition state (TSwat), the Tyr342−sialic acid bond is almost cleaved (Pauling bond order ∼ 0.1) while the Csial−Owat bond formation is beginning (Pauling bond order ∼ 0.26), showing that the hydrolysis reaction follows an A_ND dissociative mechanism of dissociative type.

Interestingly, the same behavior was observed for the trans-sialidase activity of both enzymes. Taking into account all of these data, it can be concluded that, for TcTS and TrSA, the mechanism is A_ND dissociative, regardless of the nature of the nucleophile or the leaving group. In others words, the sialidase and trans-sialidase activities share a common mechanism in both cases.

The last paragraph highlights common features of the hydrolysis mechanism of TcTS and TrSA. However, it is worth noticing some subtle aspects that differ, such as the conformations of the reaction critical points, depicted in Figure 4.

Significant conformational changes of the sialic acid ring were observed during the hydrolysis of the CIwat to MCwat. In the CIwat, the sialic acid adopted a 3C3 (half-chair) conformation. The structures sampled at the TSwat adopted a 4H5 half-chair conformation where atoms O5, C1, C4, and C5 form the reference plane. Finally, when the MCwat is reached, the sialic acid ring conformation migrates to a B2,5 boat. We can follow this process by computing the pyramidalization dihedral around the anomeric carbon of sialic acid (Figure 5).

The torsion moved from +40° (CIwat) to −40° (MCwat) describing a reverse conformation change compared to the one observed during the trans-sialylation reaction in TcTS, resulting in an overall retention of the configuration of the sialic acid ring. The evolution of the anomeric C−O13 bond distance along the MFEP was also analyzed. The distance is shortened

Figure 2. Free energy surfaces for the hydrolysis of the CIwat catalyzed by TcTS (left) and TrSA (right). Results are in kilocalories per mole. The white dashed lines illustrate the minimum energy path to reach MCwat from CIwat.

Figure 3. Reaction space for the hydrolysis of the CIwat during the sialidase activity of TcTS (red) and TrSA (black).
gaining a partial double bond character at the TS\textsubscript{wat}, after which the anomeric C\textsuperscript{−}O\textsubscript{13} bond recovers the single bond character reproducing the effects observed during the transfer activity in TcTS. This is the expected behavior for an oxocarbenium transition state.

Energy Profiles for TcTS and TrSA. The energy barrier ($\Delta$G,\textdegree) to reach the MC\textsubscript{wat} from the CI\textsubscript{wat} in TcTS is 26.8 kcal/mol, which is considerably higher than the barrier obtained for TrSA (16.4 kcal/mol), indicating that the hydrolysis of CI\textsubscript{wat} is clearly more favored in TrSA. These data are in good agreement with the experimental evidence that identifies TrSA as a more efficient hydrolase than TcTS.\textsuperscript{25} Although TcTS and TrSA share very similar structural components, the active site shows important differences that could account for the different hydrolysis rates of both enzymes. Moreover, the modifications observed in TcTS are able to overcome the fact that the water concentration in the active site is several orders of magnitude higher than that of lactose such that hydrolysis should be theoretically favored over the transfer reaction.\textsuperscript{74}

The calculated energetic values of the hydrolysis catalyzed by TcTS can be compared with those from the trans-sialidase activity obtained in our previous works so that we can complete the rationale of the different activity of both enzymes (Figure 6).

In TcTS, the calculated energy barrier to bind a second lactose to the covalent intermediate during the trans-sialidase

| Table 1. Average Reaction Coordinate Distances (Å) at MC\textsubscript{wat}, TS\textsubscript{wat}, and CI\textsubscript{wat} in TcTS and TrSA\textsuperscript{a} | CI\textsubscript{wat}\textsuperscript{b} | TS\textsubscript{wat}\textsuperscript{c} | MC\textsubscript{wat}\textsuperscript{d} |
|---|---|---|---|
| | TcTS | TrSA | TcTS | TrSA | TcTS | TrSA |
| $d_1$ | 3.1 | 3.2 | 2.1 | 2.1 | 1.4 | 1.4 |
| $d_2$ | 2.1 | 2.1 | 1.8 | 1.4 | 1.0 | 1.0 |
| $d_3$ | 1.4 | 1.4 | 2.5 | 2.7 | 3.1 | 3.1 |
| $d_4$ | 1.7 | 1.5 | 1.3 | 1.2 | 1.0 | 1.0 |

\textsuperscript{a}All standard deviations corresponding to these calculations were below 0.3 Å. \textsuperscript{b}CI\textsubscript{wat} = covalent intermediate. \textsuperscript{c}TS\textsubscript{wat} = transition state. \textsuperscript{d}MC\textsubscript{wat} = Michaelis complex.
reaction is 20.8 kcal/mol—6 kcal/mol lower than the hydrolysis reaction. This agrees with the experimental findings confirming that TcTS behaves preferentially as a trans-sialidase rather than as a sialidase. Furthermore, the free energy difference ($\Delta G^\circ$) between the product of the hydrolysis (MCwat) and reactant (CIwat) in TcTS is 9.7 kcal/mol, indicating that the CIwat is much more stabilized than the MCwat. On the other hand $\Delta G^\circ$ between the product of trans-sialylation (MClac) and the reactants (CIlac) is 0.9 kcal/mol making the CIlac approximately equienenergetic with the MClac.34 These values suggest that although TcTS can behave as a sialidase at a much lower rate, the formation and stabilization of a CI is strongly preferred over its hydrolysis to the MCwat. These findings attest to the formation of a long-lived CI in TcTS as it is required during the trans-sialidase activity by retaining the sialic acid longer, which favors the binding of new lactose to sialic acid.

The hydrolysis and trans-sialylation energy barriers were also compared for TrSA. We noticed that the hydrolysis reaction barrier is roughly 10 kcal/mol lower than that of the transfer reaction. As a consequence, the hydrolysis in TrSA will be strongly preferred over the trans-sialylation. In addition, $\Delta G^\circ$ between MCwat and CIwat is 3.7 kcal/mol, whereas $\Delta G^\circ$ between MClac and CIlac is 10.9 kcal mol$^{-1}$, making MCwat a much more stable complex than MClac in TrSA. These results suggest that the reactions are kinetically controlled and explain why TrSA does not show any trans-sialidase activity and behaves as a strict hydrolase. Moreover, as it was shown for TcTS, the formation of a CI in TrSA is favored due the lower energy barriers calculated to reach the CI from the MC. In TrSA however, the reaction will proceed following the hydrolysis pathway.

The trans-sialidase activity energy values of TcTS were also compared to the hydrolysis activity measurements in TrSA. According to experimental data, the hydrolysis activity of TrSA is around twice the trans-sialylation rate catalyzed by TcTS.25 Our theoretical data also support these conclusions since the energy barrier of the TrSA hydrolysis is 4 kcal/mol lower than TcTS trans-sialylation and the final products of the reaction have similar energies.

**Energy Decomposition.** Finally, the energy decomposition method was employed to identify the influence of individual active site residues on the energy barrier of the hydrolysis of the CIlac (Figure 6).

![Figure 6. Free energy profiles for the trans-sialidase (top) and sialidase (bottom) activities catalyzed by TcTS and TrSA.](image)

As expected, all of the residues that showed a significant effect on the energy barrier had an opposite behavior to the one observed in our previous work during the formation of the CIlac from the MClac.34 The residues that stabilized the TS in one reaction are responsible for a destabilizing effect on the other and vice versa. This is due to the fact that the nucleophile and the leaving group are inverted in both reactions. In the former, the nucleophile was the Tyr342 hydroxyl and the leaving group was the lactose molecule. In the present study, a water molecule represents the nucleophile while Tyr342 is the leaving group. Two out of the three arginines (Arg245 and Arg314) of the arginine triad together with Glu362 considerably contribute to stabilize the TSwat whereas Arg35 has a destabilizing effect. Two additional arginines, Arg53 and Arg251, also display an important effect, increasing and lowering the energy barrier, respectively. In addition, Asp247, which interacts with Arg245, has a significant destabilizing effect. All of those arginines remain hydrogen-bonded to the carboxylate group of the sialic acid except for Arg53, which is interacting with Asp59. The stabilizing or destabilizing behaviors brought about by those residues are common to both TrSA and TcTS although the energy barriers are larger in TrSA, which can be explained considering the following facts. Configurations of the MCwat complex of both TrSA and TcTS are very similar, but this is not the case for the TSwat structures. As it was mentioned, $d_2$ is shorter for TrSA whereas $d_1$ is larger. Those differences account for a higher ionic character of both substrates in TrSA (i.e., the water molecule resembles more a hydroxyl group and the sialic acid

![Figure 7. Relative stabilization pattern of the most relevant active site residues on the TSwat considering the CIwat as reference.](image)
adopts a higher oxocarbenium character). As a consequence, the interaction between the active site residues and the enzyme substrates is stronger in TrSA than in TcTS which, in turn, is seen as a larger stabilizing/distabilizing effect of the common active site residues.

Important effects are observed in other residues from the catalytic cleft, though the contributions to the stability of the TS$_\text{wat}$ are not as large as the ones presented by the former arginines. While TcTS has a tyrosine in position 119, TrSA contains a serine. Interestingly, Ser119 contributes to lowering the energy barrier in TrSA ($\Delta\Delta E = -3.0$ kcal/mol) while the tyrosine shows the opposite effect in TcTS ($\Delta\Delta E = +3.7$ kcal/mol). This finding, together with the fact that Ser119 favors the solvent exposure of the catalytic cleft and the stabilization of the water molecule in the active site of TrSA, helps to rationalize why TrSA strictly displays hydrolase activity, contrary to TcTS. Moreover, when Ser119 was mutated to tyrosine in TrSA, the sialidase activity was reduced by 50%.$^{25,75}$ On the contrary, in TcTS, the aromatic side chain of Tyr119, together with Trp312, forms a hydrophobic pocket that excludes water from the active site,$^{14}$ hindering the hydrolysis reaction on one hand and favoring the transfer activity on the other by establishing stacking interactions with the incoming lactose.

# CONCLUSION

The CI$_\text{wat}$ hydrolysis reactions catalyzed by TcTS and TrSA were simulated using QM/MM calculations combined with umbrella sampling simulations. The FES obtained showed a clear MFEP between reactants (CI$_\text{wat}$) and products (MC$_\text{wat}$). In both enzymes the reaction proceeds by means of a $\text{A}D_{\text{X}}$ dissociative mechanism. The free energy calculations presented here together with our previous studies of the TS reactions allow us to obtain the following conclusions: (i) The estimated reaction barrier for the hydrolysis of the CI$_\text{wat}$ by a water molecule catalyzed by TrSA is 16.4 kcal/mol, i.e., 10 kcal/mol lower than the same reaction catalyzed by TcTS (26.8 kcal/mol), and explains why TrSA is a more efficient hydrolase than TcTS. (ii) In TcTS, the energy barrier to accomplish the transfer reaction is 6 kcal/mol lower compared to the sialidase reaction. TcTS would then preferentially behave as a trans-sialidase rather than a sialidase. Moreover, the free energy difference also accounts for the establishment of a long-lived CI favoring the TcTS trans-sialidase activity. (iii) In TrSA, the hydrolysis reaction barrier is 10 kcal/mol lower than the transfer reaction, indicating that TrSA would have a more important role as a sialidase.

In addition, the energy decomposition allowed us to identify that, due to differential conformations, some residues (particularly Arg245 and Arg314) show a distinct stabilization pattern on TS$_\text{wat}$ in TrSA and TcTS. Besides, the presence of Ser119 in TrSA slightly stabilizes the TS$_\text{wat}$ while Tyr119 has the opposite effect in TcTS.

Besides the energetic dissimilarities shown above for the catalytic mechanism of TcTS and TrSA, other characteristics such as differential exclusion of water molecules from the active sites, stability of the active site residue conformations at the CI stage, and others aspects should also be considered to fully understand the distinctive properties of these highly similar enzymes.

Results presented here provide new insights into the catalytic mechanisms of TcTS and TrSA that complement our previous observations. Taken together, they help us to rationalize from different perspectives why TrSA behaves as a strict hydrolase whereas TcTS preferentially acts a trans-sialidase. We hope this work can contribute to a deeper understanding of enzyme catalysis and to the development of new drugs leading to the inhibition of TcTS toward a possible treatment of the expanding Chagas’ disease.

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The authors declare no competing financial interest.

# ACKNOWLEDGMENTS

This work was funded by National Institutes of Health Grant R01AI073674. We thank the High-Performance Computing Center at the University of Florida for providing computational resources and Johan F. Galindo and Jason M. Swails for providing programming support.

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