Enzymatically Inactive trans-Sialidase from Trypanosoma cruzi Binds Sialyl and β-Galactopyranosyl Residues in a Sequential Ordered Mechanism*

Adriane R. Todeschini‡, Wagner B. Dias‡, Murielle F. Girard§, Jean-Michel Wieruszeski†, Lucia Mendonça-Previato‡, and Jose O. Previato‡

From the ‡Instituto de Biofísica Carlos Chagas Filho, Centro de Ciências da Saúde-Bloco G, Universidade Federal do Rio de Janeiro, 21 944970, Cidade Universitária, Ilha do Fundão, Rio de Janeiro, Brasil, §Institut d’Épidémiologie Neurologique et de Neurologie Tropicale, Université de Limoges, 87025 Limoges, France, and ¶Laboratoire de RMN Synthèse, Structure et Fonction des Biomolécules, Institut Pasteur, 59019 Lille, France

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Host/parasite interaction mediated by carbohydrate/lectin recognition results in the attachment to and invasion of host cells and immunoregulation, enabling parasite replication and establishment of infection. Trypanosoma cruzi, the protozoan responsible for Chagas disease, expresses on its surface a family of enzymatically active and inactive trans-sialidases. The parasite uses the active trans-sialidase for glycoprotein sialylation in an unusual trans-glycosylation reaction. Inactive trans-sialidase is a sialic acid-binding lectin that co-stimulates host T cells through leucosialin (CD43) engagement. The co-mitogenic effect of trans-sialidase can be selectively abrogated by N-acetyllactosamine, suggesting the presence of an additional carbohydrate binding domain for galactosides, in addition to that for sialic acid. Here we investigated the interaction of inactive trans-sialidase in the presence of β-galactosides. By using NMR spectroscopy, we demonstrate that inactive trans-sialidase has a β-galactoside recognition site formed following a conformational switch induced by sialoside binding. Thus prior positioning of a sialyl residue is required for the β-galactoside interaction. When an appropriate sialic acid-containing molecule is available, both sialoside and β-galactoside are simultaneously accommodated in the inactive trans-sialidase binding pocket. This is the first report of a lectin recognizing two distinct ligands by a sequential ordered mechanism. This uncommon binding behavior may play an important role in several biological aspects of T. cruzi-host cell interaction and could shed more light into the catalytic mechanism of the sialic acid transfer reaction of enzymatically active trans-sialidase.

Trypanosoma cruzi is the etiologic agent of Chagas disease or American trypanosomiasis that affects ~18 million people in Central and South America. Another 100 million people are at risk of infection (1). Mammalian cell invasion is crucial for T. cruzi survival (2). Elucidation of molecular components regulating the initiation of the parasitic infection is critical for understanding the pathogenesis of Chagas disease and will enable the development of novel, effective, and selective treatments.

Initial communication between T. cruzi trypomastigotes and mammalian cells requires contact of soluble or membrane-bound parasite molecules with host ligands. T. cruzi expresses on its surface a family of glycosyolphosphatidylinositol-anchored active and inactive trans-sialidase (TS)1 proteins (3–5), which contain a Tyr or a His residue, respectively, at position 342 (4). The parasite uses active TS to sialylate its surface glycoproteins by a trans-sialidase reaction (6). Thus, TS activity is capable of extensively remodeling the T. cruzi cell surface by using host glycoconjugates as sialyl donor. Alternatively, the enzyme may sialylate host cell glycomolecules to generate receptors used by the trypanosome for adherence to and penetration of target cells. Results with sialic acid-deficient mutants of Chinese hamster ovary cells support this hypothesis (7–9). Sialic acid-deficient cells are less infected than wild type cells, suggesting that recognition of sialyl residues on Chinese hamster ovary cells is necessary during T. cruzi invasion.

Recently, we demonstrated that TS binds to α2,3-linked sialic acid from CD43 on murine T cells and initiates CD43-dependent co-stimulatory responses that increase mitogenesis, cytokine secretion, and promote rescue from apoptosis (10). The co-mitogenic effects of inactive TS on T cells were selectively abrogated by addition of N-acetyllactosamine, suggesting the inactive form of this glycosyltransferase is a sialic acid-binding lectin with an additional binding site for lactosides. In this work, we demonstrate by using NMR spectroscopy that inactive TS possesses two sugar-binding sites, one for α2,3-sialic acid-containing molecules and a second for β-galactosides. We also show that inactive TS recognizes its ligands in a sequential ordered mode. Sialoside binding is necessary to trigger a conformational modification of the inactive TS, allowing it to interact with β-galactosides. Knowledge of binding properties of TS could clarify its role in the molecular mechanism of T. cruzi/host cell interaction.

EXPERIMENTAL PROCEDURES

Materials—Most of the chemical products used were from Sigma or Fisher. The following materials were obtained from other sources: pre-

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† To whom correspondence should be addressed: Instituto de Biofísica Carlos Chagas Filho, CCS-Bloco G, Universidade Federal do Rio de Janeiro, Cidade Universitária-Ilha do Fundão, 21 944970, Rio de Janeiro-RJ, Brasil. Tel.: 55-21-2562-6646; Fax: 55-21-2280-8193; E-mail: luciapm@biof.ufrj.br.

1 The abbreviations used are: TS, trans-sialidase; βGalp, β-galactopyranose; Neu5Ac, N-acetylneuraminic acid; STD, saturation transfer difference; TOCSY, total correlation spectroscopy; PBS, phosphate-buffered saline.
Inactive TS containing a poly(His) tag was purified as described (11), was evaluated by 10% SDS-PAGE, and inactive TS was stored in 20 mM Tris-HCl buffer, pH 7.4, at 4°C. Bacteria were grown in supplemented TB medium in the presence of 100 μg/ml ampicillin. When the culture reached a OD600 of 1.5, 30 mg/liter isopropyl-β-D-thiogalactoside was added, and incubation was continued overnight.

Bacteria were lysed at 4°C in 20 mM Tris-HCl containing 2.0 mg/ml lysozyme, 2% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 5.0 μg/ml leupeptin, 1.0 μg/ml trypsin inhibitor, and 0.1 μM iodosacetamide. Inactive TS containing a poly(His) tag was purified as described (11), using Ni²⁺-chelating chromatography on a HiTrap column, eluted with imidazole gradient. The eluates were applied to Mono Q and Mono S columns and eluted with NaCl gradient. The homogeneity of the protein was evaluated by 10% SDS-PAGE, and inactive TS was stored in 20 mM Tris-HCl buffer, pH 7.4, at 4°C until used.

Frontal Affinity Chromatography Using Lactose-Sepharose Column—Frontal affinity chromatography was carried out using a lactose-Sepharose column (7.4 x 0.8 cm) equilibrated with PBS buffer in the absence or presence of α-2,3-sialyllacto-N-tetraose (2 mM). BSA and inactive TS were run at a flow rate of 0.5 ml/min at 4°C. Fractions of 1 ml were collected, lyophilized, and suspended in 200 μl and 20 μl were analyzed by SDS-PAGE (10%) under reducing conditions.

NMR Experiments—α-2,3-Sialyllactose, methyl β-lactoside, methyl β-melibioside, methyl α-mannoside, or Galβ1-3GlcNAcβ1-3Galβ1-4Glc (lacto-N-tetraose) were dissolved in deuterated PBS, pH 7.6 (not corrected for isotope effects). Inactive TS solution in 20 mM Tris-HCl was exchanged with deuterated PBS by gel filtration on a G-25 column. Twenty μl of a stock solution containing 10 mg/ml of inactive TS was added to a solution of sialylglycoside (2 mM final concentration), and the total volume was adjusted to 500 μl. NMR spectra were obtained at a probe temperature of 20°C on a Bruker DMX 600 equipped with a 5-mm self-shielded gradient triple resonance probe or on a Bruker DRX 600 with a 5-mm triple resonance probe.

One-dimensional Saturation Transfer Difference (STD)—One-dimensional STD experiments were performed by low power presaturation of the methyl region of the protein during the 2-s relaxation delay. The pulse scheme was as follow: relaxation delay with or without presaturation of the protein resonances, 90° pulse and acquisition. Two hundred and fifty six scans of 16,000 points over a 10 ppm spectral width were collect. Data were obtained with an interspersed acquisition of on-resonance and control spectra in order to minimize the effects of temperature and magnet instability.

Saturation Transfer Difference-Total Correlation Spectroscopy (STD-TOCSY)—STD-TOCSY spectra were recorded with a mixing time of 66 ms, 32 scans per t1 increment. 200 t1 increments were collected in an interlaced mode for on or off presaturation, as described under “Experimental Procedures.”

**RESULTS**

Recently, we applied STD-NMR techniques to demonstrate that enzymatically inactive *T. cruzi* TS is a sialic acid-binding lectin, allowing atomic resolution of the epitope involved in the interaction (12). By using the same methodology, the interaction of inactive TS with lacto-N-tetraose,
methyl-β-lactose, methyl β-melibiose, and methyl α-mannoside was studied.

Fig. 1 shows the one-dimensional STD-NMR spectra of inactive TS in the presence of α2,3-sialyllactose and lacto-N-tetraose. From an inspection in the spectra, it is immediately obvious that no proton from lacto-N-tetraose or methyl β-lactoside (results not shown) receives magnetization from the protein. The STD-TOCSY acquired under the same conditions confirmed these results (Fig. 2). Neither saccharide studied makes contact with the inactive TS, because no cross-peaks were observed (Fig. 2B).

Schudder et al. (13) and Ribeirão et al. (14) demonstrated by kinetic studies that active TS catalyzes the transfer of α-2,3-sialyllactose to terminal β-Galp-containing molecules through a bisubstrate sequential mechanism involving an enzyme-substrate ternary complex, indicating that the β-galactoside must bind to the enzyme at some stage in the catalytic cycle. If inactive TS exhibits the same binding properties, it must interact with lacto-N-tetraose or methyl β-lactoside when a sialoside is present. To test this hypothesis, we used STD experiments to probe the interaction of

![STD-TOCSY of α2,3-sialyllactose and lacto-N-tetraose bound to inactive TS. A, reference TOCSY spectrum of inactive TS in the presence of α2,3-sialyllactose and lacto-N-tetraose. B, STD-TOCSY spectrum. C, amplification of B. Spectra were recorded in PBS/D$_2$O, pH 7.6, 20 °C with mixing time of 66 ms, 32 scans per $t_1$ increment. 200 $t_1$ increments were collected in an interlaced mode for on or off presaturation, as described under “Experimental Procedures.”](image)

![Relative intensities of STD-TOCSY cross-peaks. The intensity is normalized to the intensity of the corresponding cross-peak in the reference TOCSY spectrum.](image)

![Frontal affinity chromatography using lactose-Sepharose column. Frontal affinity chromatogram of inactive TS through a lactose-Sepharose column equilibrated with PBS (trace a) and α2,3-sialyllacto-N-tetraose containing PBS (trace b). Each fraction was run on SDS-PAGE. Arrows show the molecular mass of inactive TS.](image)
inactive TS with lacto-N-tetraose in the presence of H9251-2,3-sialyllactose. Fig. 3 shows the reference TOCSY (Fig. 3A) and the STD-TOCSY (Fig. 3B and C) spectra of the lacto-N-tetraose incubated with inactive TS in the presence of α,2,3-sialyllactose. The STD-TOCSY spectrum indicates that the NAc and H3ax protons of Neu5Ac residue are in close contact with the inactive enzyme and that H3eq, H4, and H5 from the Neu5Ac residue and H1, H3, and H4 from the β-Galp ring receive saturation from the protein (Fig. 4). The contacts and intensities observed for H9251-2,3-sialyllactose interaction with inactive TS are in complete agreement with those found previously (12). In addition, Fig. 3C clearly shows additional cross-peaks at 4.47 and 3.68 ppm arising from H1 and H3 of β-Galp of the lacto-N-tetraose, respectively. Similarly, contact between the β-Galp residue and inactive enzyme was observed when methyl β-lactoside was incubated at the same conditions (results not shown).

Binding of inactive TS with β-lactoside in the presence of α,2,3-linked sialic acid was also verified by frontal affinity chromatography. When the inactive TS was loaded on the lactose-Sepharose column pre-equilibrated in PBS buffer with α,2,3-sialyllacto-N-tetraose, the enzyme was eluted in a higher elution volume in relation to the front found when the column was equilibrated in absence of the sialoside (Fig. 5).

These results demonstrate that binding of α,2,3-sialyllactose to inactive TS elicits a conformational alteration in the protein framework that allows the β-galactoside to bind. The α,2,3-sialyllactose and β-galactoside simultaneously accommodate in the inactive TS-binding pocket of inactive TS, suggesting that the inactive members of the TS family interact with their ligands by a sequential ordered mechanism.

Previously, we demonstrated that inactive TS binds only weakly to α,2,6-sialyllactose, because in contrast to α,2,3-sialyllactose, no contact between the protein and the β-Galp ring was detected (12). To investigate whether the conformational switch of inactive TS depends on the correct positioning of the sialoside in the enzyme-binding site, interaction of inactive TS with α,2,6-sialyllactose and lacto-N-tetraose was studied. STD-TOCSY spectrum (Fig. 6) contains no signals arising from the lacto-N-tetraose, suggesting that a proper positioning of sialylated ligand is necessary for the β-galactoside interaction.

To investigate whether inactive TS is specific for β-Galp residues, the enzyme was incubated with methyl β-mannoside (Fig. 7) or methyl β-melibiose (result not shown) in the presence of α,2,3-sialyllactose. Additional signals were not observed, demonstrating that, similar to its active analogue, inactive TS only interacts with terminal β-Galp residues. Taken together, our results show that correct positioning of the sialoside in the inactive TS-binding site is necessary for the protein
to undergo the conformational change that allows the β-galactoside to bind (Fig. 8).

**DISCUSSION**

Host/parasite interaction mediated by carbohydrates may be influenced by lectin properties. Lectins can behave either as receptors or ligands for parasite/host interactions, resulting in the recognition, attachment to, and invasion of host cells, and immunoregulation, enabling parasite replication and establishment of infection.

*T. cruzi* genome contains hundreds of genes encoding a family of cell surface and enzymatically active and inactive TS and sialic acid acceptor glycoproteins (mucin-like molecules) (5). Several reports (15) have suggested that these molecules could be either receptors or ligands during the process of *T. cruzi* infection. Previously, we showed that the enzymatically inactive TS is a sialic acid-binding lectin able to bind to and co-stimulate T cells through CD43 engagement, and that there is evidence for an additional binding site for β-galactopyranosyl residues, because the co-mitogenic effect was selectively abrogated by addition of N-acetyllactosamine (10). This possibility prompted us to investigate the interaction of inactive TS with β-galactosides. By using NMR spectroscopy, we demonstrated that inactive *T. cruzi* TS has a carbohydrate recognition domain for β-Galp residue that is formed only after a conformational switch triggered by prior sialoside binding. As far as we know, this is the first report of a lectin that recognizes its ligands by a sequential ordered mechanism. The binding behavior of inactive TS may play a significant role in the process of *T. cruzi* adhesion, invasion, and host immunoregulation, allowing the parasitic infection to be established.

Molecules of the TS family are glycosylphosphatidylinositol-anchored to the membrane and can be released into the serum in fairly high amounts during acute phase Chagas disease in humans (5). Therefore, TS family members can act as soluble modifiers of immune responses. The bivalent nature of inactive TS demonstrated here would promote glycan cross-linking, which is believed to be essential for cellular signal transduction. Both soluble and membrane-bound inactive TS could interact with sialo- and asialo-glycoconjugates expressed by host cells leading to exacerbated and immunopathologic reactions in Chagas disease.

The finding that inactive TS has two carbohydrate binding domains may explain some apparently contradictory results on the involvement of sialyl and galactosyl epitopes in *T. cruzi* host cell interaction. Whereas Schenkmann *et al.* (16) have shown that sialylation of Ssp-3 epitope of mammalian cell-derived trypomastigotes is required for target cell recognition, Yoshida *et al.* (17) reported that the removal of sialic acid from the surface of insect-derived metacyclic trypomastigotes enhances parasite/host interaction. The removal of sialic acid from *T. cruzi* glycoproteins and the concomitant exposure of cryptic β-Galp residues would favor inactive TS interaction with both host sialoglycoconjugates and terminal β-Galp-containing glycoproteins on the parasite surface, thus enhancing *T. cruzi*-host adhesion. This phenomenon was well characterized for CD22, a mammalian sialic acid-binding lectin (18–20). CD22 is masked on the surface of murine B cells, as evidenced by enhanced binding of specific sialyl probes after sialidase treatment of B cells (19). The removal of sialic acid and concomitant exposure of β-Galp residues from host cell glycans, which occurs as a result of the *T. cruzi* TS reaction, may therefore be physiologically significant by promoting parasite adherence to and penetration of host cells (7–9).

The findings that inactive TS recognizes its ligands with the same specificity as its active analogue may bring insights into the mechanism of catalytic transglycosylation by *T. cruzi* TS. *T. cruzi* TS is an unusual sialidase with a predominant transglycosylase activity (21). The catalytic mechanism of trypanosomal sialidase (11) seems to be similar to viral sialidase (22) which is thought to hydrolyze the sialyl glycosidic bond through an oxocarbenium ion intermediate, with formation of trace amount of 2-deoxy-2,3-didehydro-N-acetylneuraminic (Neu5Ac2en) as a by-product. The predominance of transglycosylation over hydrolysis can be explained by the presence of distinct binding sites for acceptor and donor substrates in the TS catalytic pocket. Our data support this hypothesis; we demonstrated that inactive TS binds α2,3-sialyllactose and lacto-N-tetraose or methyl-β-lactose in a sequential ordered manner to form a ternary complex.

In the same way as we have shown for inactive TS, Buschiazzo *et al.* (23) demonstrated recently that sialic acid binding induces a conformational modification in the crystal structure of active TS, allowing the acceptor substrate (lactose) to bind. The authors, however, were unable to show the existence of two distinct binding sites when monoclinic crystals were soaked in lactose and Neu5Ac2en, indicating that the lactose-binding site is too narrow to accommodate the lactose moiety of the donor and acceptor substrates simultaneously. Our results,
using inactive TS incubated with α2,3-sialyllactose in the presence of lacto-N-tetraose, show that incorrect fitting of sialoside into the binding site of inactive TS does not trigger β-Galp binding. These data suggest that Neu5NAc2en interaction with active TS (23) is not sufficient to induce the required conformational rearrangement to accommodate acceptor and donor substrates simultaneously. Consistent with our hypothesis are the surface plasmon resonance results showing that lactose binds to an inactive mutant of TS (Asp59 → Asn) in the presence of α2,3-sialyllactose (23).

Although the exact catalytic mechanism of trans-sialylation is still obscure, data obtained in this study may provide some clues. Correct binding of sialic acid donor to TS may trigger a conformational change in the enzyme that creates the conditions for formation of a ternary complex. Acceptor binding would displace the water molecules from TS catalytic cleft before formation of the oxocarbonium ion (11, 23) or sialyl-transfer reaction (Fig. 8). Thus, high efficiency of transfer of enzyme intermediate (24) takes place, facilitating the selective sialic acid to lactose by active TS could be explained by this model. Natural sialosides as α2-3-sialyllactose would fit correctly in the TS catalytic pocket, inducing the acceptor donor to bind and increasing transference rates, whereas synthetic donors, as 4-methylumbelliferyl-sialic acid and p-nitrophenyl sialic acid, would not be able to trigger a sufficient shifting in the enzyme framework to allow acceptor binding, being better substrates for hydrolysis reaction (25).

Taken together, our results suggest that enzymatically inactive molecules of the TS family are sialic acid- and β-Galp-binding proteins that can play an important role during the T. cruzi infection, and that inhibition of TS activity based on sialic acid analogues may transform T. cruzi binding proteins that can play an important role during the infection, and that inhibition of TS activity based on sialic acid- and β-Galp-binding lectins. Therefore, polyvalent galactosides/sialosides provide intriguing possibilities for the design and synthesis of selective TS inhibitors.

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