

Characterization of a Novel Trans-sialidase of *Trypanosoma brucei* Procyclic Trypomastigotes and Identification of Procyclin as the Main Sialic Acid Acceptor

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Summary

Here we report the presence of a trans-sialidase on the surface of *Trypanosoma brucei* culture-derived procyclic trypomastigotes. The enzyme is not detected in lysates of bloodstream trypomastigotes enriched for either stumpy or slender forms. The trans-sialidase catalyzes the transfer of $\alpha(2-3)$ -linked sialic acid residues to lactose. β -galactopyranosyl residues are at least 100 times better acceptors for sialic acid than α -galactopyranosyl residues. In the absence of efficient acceptors, the purified enzyme transfers sialic acid to water, i.e., it acts as a sialidase. Although the *T. cruzi* and *T. brucei* trans-sialidases have very similar donor and acceptor specificities, they are antigenically distinct. Sodium dodecyl sulfate–polyacramide gel electrophoresis under nonreducing conditions and silver staining of the purified trans-sialidase reveals a single band of 63 kD. When the surface membrane of live procyclic trypomastigotes is trans-sialylated, using radioactive sialyllactose as the donor substrate, it appears that the only sialylated surface molecule is procyclin. Pronase treatment of live parasites removes only part of the surface sialic acid, in agreement with recent data showing that the glycosylphosphatidylinositol anchor of procyclin is sialylated (Ferguson, M. A. J., M. Murray, H. Rutherford, and M. J. McConville. 1993. *Biochem. J.* In press).

Trypanosoma brucei has a complex life cycle that involves extracellular multiplication in body fluids of mammalian hosts, and in the gut and salivary glands of the insect vector, the tsetse fly (1). During transformation from bloodstream trypomastigotes to insect procyclic trypomastigotes, the parasites undergo biochemical and morphological changes. Within 48 h of their ingestion by the fly, bloodstream trypomastigotes shed their surface coat, the variant surface glycoprotein (VSG),¹ and replace it with the procyclic acidic repetitive protein (PARP, or procyclin) (2–5). Both VSG and procyclin are anchored to the plasma membrane by glycosylphosphatidylinositol (GPI) (6, 7). VSG represents a parasite adaptation to life in the mammalian host, providing it with a mechanism to escape from immune effector mechanisms (1). The

function of the antigenically conserved (3, 8) *T. brucei* procyclin is unknown.

Recent data document a striking difference between the structure of the GPI anchor of procyclin and that of most other membrane proteins (including VSG) (9), i.e., the procyclin GPI anchor contains sialic acid (9a). Sialylated molecules have also been identified on the surface membrane of trypomastigotes of *Trypanosoma cruzi* (10). The sialic acid of *T. cruzi* is not synthesized by the parasite, but transferred from extrinsic host-derived macromolecules in a reaction catalyzed by an unusual trans-sialidase (10).

In this paper we investigate the origin of the sialic acid of *T. brucei* procyclic trypomastigotes. We demonstrate that the transformation of *T. brucei* blood trypomastigotes into culture-derived forms is associated with the expression of a trans-sialidase sharing several characteristics with the *T. cruzi* enzyme and, in addition, that procyclin is the main endogenous sialic acid acceptor.

¹ Abbreviations used in this paper: BSM, buffered semidefined medium; GPI, glycosylphosphatidylinositol; VSG, variant surface glycoprotein.

Materials and Methods

Parasites. The TREU 667 stock of *T. brucei brucei* (11) was used. Procyclic trypomastigotes were grown in buffered semidefined medium (BSM) (12) containing 10% FCS (HyClone Laboratories, Logan, UT) at 26°C. Bloodstream trypomastigotes enriched 85–90% for slender or stumpy forms (13) were purified from blood of infected rats by anion exchange chromatography (14). Y strain *T. cruzi* trypomastigotes (15) were from LLC-MK₂ cell cultures (CCL-7; American Type Culture Collection, Rockville, MD) in DME containing 10% FCS, as described previously (10).

Sialic Acid Measurement. This was done on: (a) untreated cell-derived *T. cruzi* trypomastigotes or *T. brucei* procyclics; (b) procyclics treated with sialidase (see below); (c) procyclics first treated with *Vibrio cholera* sialidase and then with either 1 mM α (2-3)-sialyllactose (Boehringer Mannheim Biochemicals, Indianapolis, IN) or α (2-6)-sialyllactose (Sigma Chemical Co., St. Louis, MO) in BSM containing 0.1% gelatin (Bio-Rad Laboratories, Richmond, CA) (BSM-G), for 40 min at 26°C; (d) procyclics treated with pronase (see below); and (e) supernatants of pronase-treated procyclics. The parasites were washed six times with cold BSM-G and incubated in 0.1 M sulfuric acid for 1 h at 80°C before being assayed for sialic acid by the HPLC-thiobarbituric method (16).

Enzyme Assays. Trans-sialidase activity was assayed as previously described by measuring the transfer of sialic acid from sialyllactose to radioactive lactose (10). Briefly, samples were assayed in a total volume of 50 μ l of 20 mM Hepes, pH 7 (Sigma Chemical Co.), containing 50 nmol of α (2-3)-sialyllactose and 0.36 nmol of [D -glucose-1-¹⁴C]lactose (60 Ci/mol) (Amersham Corp., Arlington Heights, IL). This mixture was incubated for 40 min at room temperature and the reaction terminated by the addition of 1 ml of water and passage through a 0.5-ml QAE-Sephadex A50 column preequilibrated with water, which retains the sialyllactose and allows lactose to elute. Under these conditions, the formation of radiolabeled sialyllactose was linear with respect to enzyme concentrations. Activity was expressed as cpm (bound sialyl[¹⁴C]lactose) eluted from the columns with 0.5 ml of 1 M ammonium formate solution. In some assays, the sialyl(α 2-3)lactose was substituted with 0.25–25 nmol of sialyl(α 2-6)lactose (Boehringer Mannheim Biochemicals), sialyl(α 2-9)sialyl(α 2-3)lactose-ceramide (kindly supplied by Dr. A. Hasegawa, Gifu University, Gifu, Japan), *N*-acetylneuraminic acid, colominic acid, or 4-methylumbelliferyl-*N*-acetylneuraminic acid (Sigma Chemical Co.) to test their potential ability to serve as sialic acid donors. In other assays, 40–4,000 nmol of lactose, stachyose, melibiose, Gal(β 1-6)Gal, α -methyl-D-galactopyranoside, β -methyl-D-galactopyranoside (Sigma Chemical Co.), Gal(β 1-4)[Fuc(α 1-3)]Glc (Oxford Glycosystems, Inc., Rosedale, NY), or 10 μ M of cupric nitrate or mercuric acetate were added to the reaction mixture to assess their possible ability to serve as sialic acid acceptors or trans-sialidase inhibitors. Results from these latter assays were expressed as the percentage of cpm reduction in relation to control reactions carried out in the absence of nonradioactive saccharides or inhibitors.

Trans-sialidase activity was also demonstrated by measuring the transfer of [³H]sialic acid residues from sialyllactose to different saccharides. [Sialic-9-³H](α 2-3)-sialyllactose was prepared by incubating 25 μ l of [sialic-9-³H]CMP-sialic acid (26.2 Ci/mmol; NEN Research Products, Boston, MA) with 0.15 M lactose in the presence of porcine submaxillary α (2-3)-Gal β (1-3)-GalNac sialyltransferase (17). 15 nmol of the labeled sialyllactose and 100 nmol of potential sialic acid acceptors were incubated for 210 min at room temperature in 15 μ l of 20 mM Hepes, pH 7, in the presence of purified trans-sialidase. The products of the reactions were isolated

by elution from QAE-Sephadex columns as described above, lyophilized, and subjected to TLC on silica gel 60 plates (Macherey-Nagel, Düren, Germany) using ethanol, *n*-butanol, pyridine, water, acetic acid (100:10:10:30:3 [vol/vol]). The sialylated compounds were visualized by spraying the TLC plates with EN³HANCE (NEN Research Products), followed by fluorography. Saccharide purity was assessed by silica-gel TLC analysis of 1- μ mol samples, followed by staining with the orcinol-ferric chloride reagent (18). Single bands were observed with melibiose, α (2-3)sialyllactose, α -methylgalactose, β -methylgalactose, and Gal(β 1-6)Gal.

Sialidase activity was determined by measuring the fluorescence of 4-methylumbelliferone resulting from the hydrolysis of 4-methylumbelliferyl-*N*-acetylneuraminic acid (initial concentration of 1 mM in 50 μ l of 20 mM Hepes buffer, pH 6.7), as described elsewhere (19).

Immunoprecipitation. Parasites were lysed in 1.5% NP-40, 50 mM Tris-HCl, pH 7.4, 1 mM PMSF, and 5 μ g/ml of antipain, pepstatin, and leupeptin (1 ml/10⁹ parasites). The lysates were cleared by centrifugation at 10,000 *g* for 5 min at 4°C. 40- μ l fractions of the lysates were incubated, with mixing, for 1 h at 4°C with 3, 9, and 27 μ l of protein A-agarose (Sigma Chemical Co.) bearing adsorbed mAb 39 (anti-*T. cruzi* trans-sialidase [19]). 80- μ l volumes of lysate were similarly incubated with protein A-agarose-bearing polyclonal antibodies against purified *T. cruzi* trans-sialidase or against a synthetic peptide corresponding to the first 19 NH₂-terminal amino acid residues of the *T. cruzi* trans-sialidase (20). These antibodies were from rabbits immunized intramuscularly with 60 μ g of purified *T. cruzi* trans-sialidase (19), or with 1 mg of synthetic peptide coupled to KLH (Sigma Chemical Co.) (21), in CFA, followed by two subcutaneous boosters of the same antigens in IFA, at 21-d intervals.

In addition, lysates were prepared from *T. brucei* radiolabeled with [³H]sialic acid residues (see below) in the presence of 1% BSA (Ultrapure; Boehringer Mannheim Biochemicals). These lysates were mixed with equal volumes of 1 M Tris/HCl, pH 8.6, 2% BSA, and left for 30 min at 56°C to inactivate trans-sialidase/sialidase activities. 60- μ l fractions of these lysates were incubated with 20 μ l of protein A-agarose bearing 20 μ g of mAb 137 (IgG1 anti-procyclicin; kindly supplied by Dr. T. Pearson, University of Victoria, Victoria, Canada [3]) or 20 μ g of mAb 3C9 (IgG1 anti-*T. cruzi* Ssp-3 [22]) for 40 min at 4°C. The beads were then centrifuged, washed three times with 50 mM Tris/HCl, pH 8.6, 285 mM NaCl, 0.3% BSA, and the bound antigen was eluted with 0.1 M HCl/glycine, pH 3.1. The amount of radioactivity remaining in the extracts and in the eluates from the beads was measured in a β counter.

Enzyme Purification. Trans-sialidase was purified from trypomastigote lysates, prepared as described above, by affinity chromatography on Con A-Sepharose (Pharmacia-LKB Biotechnology, Inc., Piscataway, NJ), followed by anion-exchange chromatography on a Mono-Q FPLC HR5/5 column (Pharmacia-LKB Biotechnology, Inc.), as described for the *T. cruzi* trans-sialidase (19). A sample of the purified enzyme was concentrated on a Centriprep-10 concentrator (Amicon Corp., Beverly, MA) and further subjected to sizing chromatography on Superose 12 HR 10/30 and 6 HR 10/30 columns (Pharmacia-LKB Biotechnology, Inc.), connected in series (19), and preequilibrated with 40 mM Tris/HCl, pH 8, containing 0.1% NP-40.

Protease and Sialidase Treatment. Trypomastigotes were washed once with BSM and treated with 250 μ g/ml of trypsin (Sigma Chemical Co.) in DME for 20 min at 37°C, or with 1,250 U/ml of pronase (Calbiochem-Behring Corp., San Diego, CA) in DME for either 15 min at 37°C, or 30 min at room temperature, as indicated in the text. The trypsin digestion was terminated by the ad-

dition of soybean trypsin inhibitor (final concentration of 500 $\mu\text{g/ml}$; Sigma Chemical Co.). The parasites were further washed in DME containing 2 mg/ml BSA and 100 $\mu\text{g/ml}$ soybean trypsin inhibitor. Pronase was removed by the addition of 50 vol of ice-cold DME containing 30% FCS followed by washing with DME containing 15% FCS, at 4°C. NP-40 lysates of the protease-treated parasites were assayed for trans-sialidase activity. In controls, the trypsin was added to parasites in the presence of soybean trypsin inhibitor, or the pronase added to the parasites concomitantly with the addition of the DME with FCS. In addition, the assays for trans-sialidase activity in lysates containing pronase were done at 4°C in the presence of 60 mg/ml of BSA.

Trans-sialidase, partially purified by Con A affinity chromatography, was treated with 100 $\mu\text{g/ml}$ of proteinase K (Sigma Chemical Co.), 250 $\mu\text{g/ml}$ of trypsin, or 1,250 U/ml of pronase in DME for 20 min at 37°C. The proteinase K and the trypsin digestions were, respectively, terminated by the addition of either PMSF (2 mM) and BSA (20 mg/ml), or soybean trypsin inhibitor (500 $\mu\text{g/ml}$) and BSA (30 mg/ml). After pronase digestion, BSA (60 mg/ml) was added to the reaction mixture, and assays for trans-sialidase activity were performed immediately, at 4°C, in the presence of 75 mg/ml BSA.

Trypomastigotes washed once with BSM-G were incubated for 2 h with 0.33 U/ml *V. cholera* sialidase (Boehringer Mannheim Biochemicals) in BSM-G, pH 5.5, and washed five times with BSM-G. Some lysate samples were incubated with equal volumes of 1 U/ml sialidase, or of sialidase buffer, for 15 min at 37°C, before being subjected to SDS-PAGE (see below).

Labeling of Surface Components with Sialic Acid. Trypomastigotes (1.5×10^8) were washed once with cold BSM-G, left for 2 h at 26°C in BSM-G, washed four times more with cold BSM-G, and resuspended in 250 μl of BSM-G containing 45 nmol of [^3H]sialyllactose. After a 25 min incubation at room temperature, 30 nmol

of additional [^3H]sialyllactose were added to the parasites and the incubation continued for 3 min at room temperature. The trypomastigotes were then washed three times with cold BSM-G and lysed with NP-40 for SDS-PAGE and immunoprecipitation.

SDS-PAGE and Western Blotting. Samples of FPLC fractions, or of cell lysates containing the equivalent of 5×10^6 parasites labeled with [^3H]sialic acid, were applied to 7.5% SDS-PAGE gels under both reducing and nonreducing conditions (23). Gels were either silver stained (24) or impregnated with 1 M sodium salicylate (25), stained with coomassie blue, and subjected to fluorography using an intensifying screen.

For Western blot analysis, separated proteins were transferred to nitrocellulose membranes and probed with mAbs 39 and 137. Bound antibodies were detected with an alkaline-phosphatase conjugate.

Results

Presence of Sialic Acid in *T. brucei* Procyclic Trypomastigotes. Initial experiments demonstrated the presence of sialic acid associated with the surface membrane of cultured, extensively washed *T. brucei* procyclic trypomastigotes. The total sialic acid content of parasite extracts, as compared with those of *T. cruzi* cell-derived trypomastigotes, is shown in Table 1. *T. brucei* contains more sialic acid than *T. cruzi*. Most of the sialic acid is surface associated since it was removed from the live parasites by pronase or by sialidase treatment (Table 1). Enzymatic treatments did not affect the motility of the parasites, as determined by light microscopy. These results, however, do not address the question of the origin of the surface-bound sialic acid. To investigate its possible acquisition from

Table 1. Presence of Sialic Acid in *T. brucei* Procyclic Trypomastigotes and Its Removal by Enzymatic Treatment

Parasite	Treatments	Source of sialic acid	Amount of sialic acid per 10^6 parasites*
<i>T. brucei</i> procyclics	None	Parasite lysate	177
	Sialidase [†]	Parasite lysate	49
	Sialidase; 3'-sialyllactose [§]	Parasite lysate	163
	Sialidase; 6'-sialyllactose [§]	Parasite lysate	71
	Pronase	Parasite lysate Supernatant [¶]	38 82
<i>T. cruzi</i> cell culture trypomastigotes	None	Parasite lysate	22

* As assessed by the thiobarbituric method and HPLC.

[†] Trypomastigotes were washed once with BSM-G, incubated for 2 h at 26°C with 0.33 U/ml of *V. cholera* sialidase in BSM, pH 5.7, and washed five times with BSM-G.

[§] Sialidase-treated parasites were washed twice with BSM-G, incubated with 1 mM of sialyllactose in BSM-G for 40 min at 26°C, and washed five times with BSM-G.

^{||} Prepared from trypomastigotes that had been washed five times with BSM, incubated for 30 min at room temperature with 1,250 U/ml of pronase in DME, pH 7.5, and washed once with BSM-G.

[¶] Containing material released by treating trypomastigotes with pronase.

Table 2. Stage Specificity of the *T. brucei* Trans-sialidase

Parasite species	Enzyme source	No. of parasites*	Trans-sialidase activity [†]
			<i>mean cpm</i>
<i>T. brucei</i>	Procyclic trypomastigote lysate	10 ⁶	2,044
		3 × 10 ⁶	5,300
	Slender bloodstream trypomastigote lysate	10 ⁶	3
		10 ⁷	- 6
	Stumpy bloodstream trypomastigote lysate	10 ⁶	8
		10 ⁷	15
	Procyclic trypomastigote culture supernatant [‡]	-	253
<i>T. cruzi</i>	Cell-derived trypomastigote lysate	10 ⁶	11,186
	Cell-derived trypomastigote culture supernatant [‡]	-	12,366

* Total number of lysed parasites in the aliquot assayed.

† All samples were assayed in duplicate; the background value (111 cpm, obtained in the absence of trans-sialidase) was subtracted. Variation between the duplicate values was <7% of the mean (lysates) or <18.6% of the mean (culture supernatant).

‡ 30- μ l volumes of supernatants from a procyclic trypomastigote culture (10⁷ parasites/ml), or from a Y-strain *T. cruzi* culture on LLC-MK₂ cells (5 × 10⁶ parasites/ml), were added to the reaction mixtures.

exogenous sources, trypomastigotes were treated with sialidase, reincubated for 30 min at room temperature with α (2-3)-sialyllactose, and extensively washed. As shown in Table 1, their sialic acid content was restored. When α (2-6)-sialyllactose was substituted for α (2-3)-sialyllactose, however, much less sialic acid was found on the parasites (Table 1), indicating that the sialylation reaction is specific and documenting the efficiency of the washing procedure to remove remaining free sialic acid, or sialic acid loosely bound to the parasites.

Presence of Trans-sialidase in *T. brucei* Procyclic Trypomastigotes. In the following experiments, we assayed for the presence of trans-sialidase in NP-40 extracts of *T. brucei* blood-stage slender and stumpy trypomastigotes, and of *T. brucei* procyclic trypomastigotes. Enzymatic activity was measured by the ability to transfer sialic acid from sialyllactose to radio-labeled lactose, forming labeled sialyllactose. As shown in Table 2, only the extracts of procyclics were active. The trans-sialidase activity per parasite was approximately six times less than that of Y strain cell-derived *T. cruzi* trypomastigotes (Table 2). No significant activity was found in supernatants of procyclic cultures (Table 2).

Enzyme Purification. NP-40 lysates of *T. brucei* procyclics were first subjected to Con A affinity chromatography and elution with α -methyl-D-mannoside. Approximately 40% of the trans-sialidase activity was recovered. This material was then subjected to anion-exchange FPLC. Most enzymatic activity was eluted between 70 and 130 mM of NaCl (Fig. 1 A), with a recovery of \sim 85%. Reduced SDS-PAGE of this

purified trans-sialidase showed two major bands of 73 and 77 kD, and a faint band of \sim 48 kD (Fig. 1, B, inset, left lane).

To assess the molecular mass of its native form and further purify the trans-sialidase, Mono-Q fractions corresponding to the peak of enzymatic activity were pooled, concentrated by retention on a 10-kD-cutoff membrane filter, and subjected to sizing chromatography on FPLC Superose 12 and Superose 6 columns connected in series. In several experiments, using different trans-sialidase preparations, enzymatic activity was detected in fractions corresponding to molecular masses ranging from \sim 66 to >700 kD. In the experiment illustrated in Fig. 1 B, the two major broad active peaks have molecular masses of \sim 180 and \sim 660 kD. In another run, a 66-kD peak replaced the 180-kD peak (not shown). On one occasion, a fresh lysate produced only the 660-kD peak (not shown). These data suggest that the trans-sialidase has a propensity to self-aggregate.

Reduced SDS-PAGE gels of fractions corresponding both to the 180- and 660-kD peaks, stained by silver, revealed a major band of \sim 73 kD (Fig. 1 B, inset). In the fraction corresponding to the high molecular mass peak, a less intense band of \sim 77 kD could also be seen. Two faint bands corresponding to molecular masses of \sim 63–67 kD could also be seen. Under nonreducing conditions, however, a single band of \sim 63 kD was observed (not shown).

Protease Sensitivity and Surface Localization of the Trans-sialidase. Con A-purified *T. brucei* trans-sialidase was relatively resistant to treatment with 250 μ g/ml of trypsin or with

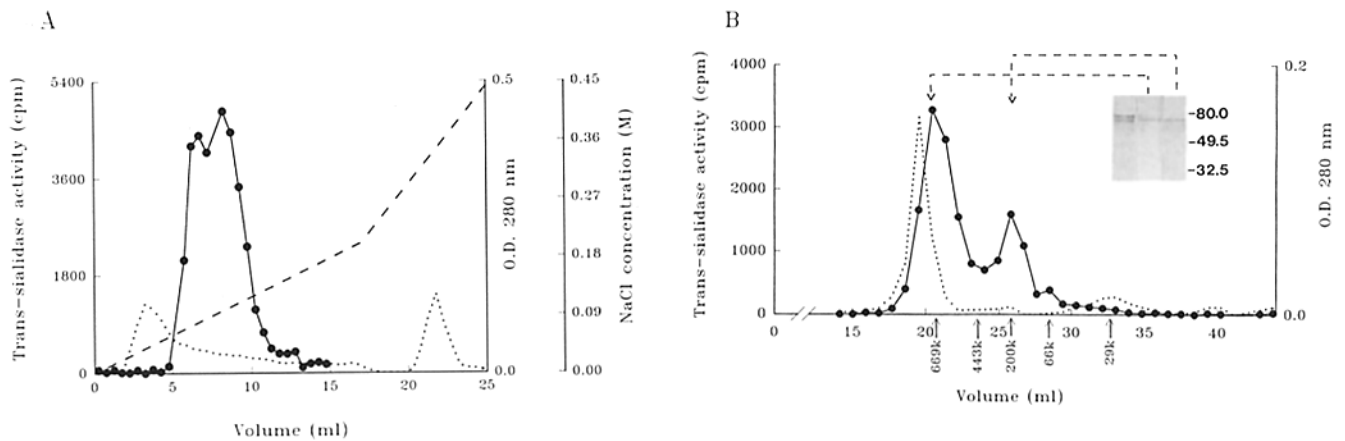


Figure 1. Purification of the *T. brucei* trans-sialidase on FPLC columns. Trans-sialidase activity and OD at 280 nm of fractions are represented by solid and dotted lines, respectively. (A) Fractions eluted with a NaCl gradient from a Mono-Q column, preequilibrated with 20 mM Tris-HCl, pH 8, containing 0.1% NP-40. The input was an enzyme sample purified from an NP-40 lysate by Con A affinity chromatography. The NaCl gradient is represented by the dashed line. (B) Gel filtration on Superose 12-Superose 6 columns run in tandem of a sample of the *T. brucei* enzyme purified by the ion-exchange chromatography described above. The inset shows the result of SDS-PAGE and silver staining of the input sample (left lane) and of the fractions indicated by the hatched arrows. The positions of molecular mass standards are shown on the right of the inset.

100 $\mu\text{g/ml}$ of proteinase K for 20 min at 37°C. Enzymatic activity was destroyed, however, by treatment with 1,250 U/ml of pronase (Table 3). Accordingly, treatment of live parasites with 1,250 U/ml of pronase for 15 min at 37°C, but not

with 250 $\mu\text{g/ml}$ of trypsin for 20 min at 37°C, markedly reduced the trans-sialidase activity of subsequently prepared lysates (Table 3).

Substrate Specificity. The ability of different sialylated com-

Table 3. Protease Sensitivity and Surface Localization of the *T. brucei* Trans-sialidase

Trans-sialidase preparation	Treatment	Trans-sialidase activity*
Con A-purified enzyme [†]	None	2,130
	Trypsin	1,671
	Proteinase K	1,930
	Pronase	4
Lysate of untreated procyclics [§]	None	4,237
Lysate of trypsin-treated procyclics	None	4,843
Lysate of pronase-treated procyclics [¶]	None	1,616

* Samples were tested at 4°C, in duplicate. Variation between the duplicate values was in every case <8% of the mean. The background value (52 cpm, obtained in the absence of trans-sialidase) was subtracted.

[†] An NP-40 lysate of procyclic trypomastigotes was incubated for 30 min with Con A-Sepharose, the bound trans-sialidase eluted by overnight incubation with an excess of α -methyl-D-mannoside at 4°C, and extensively dialyzed against cold PBS. The purified enzyme was tested without treatment or after treatment with 250 $\mu\text{g/ml}$ of trypsin or 100 $\mu\text{g/ml}$ of proteinase K, followed by the addition of protease inhibitors and BSA.

[§] Procyclic trypomastigotes were kept for 15 min at 37°C and then mixed with 1,250 U/ml of pronase. DME containing 30% FCS was immediately added to the parasites, which were washed with DME containing 15% FCS and lysed with NP-40 in the presence of 60 mg/ml of BSA. This lysate had the same level of trans-sialidase activity as a lysate containing no proteases.

^{||} Procyclic trypomastigotes were treated with 250 $\mu\text{g/ml}$ of trypsin for 20 min at 37°C. At the end of the incubation, soybean trypsin inhibitor (STI) and BSA were added to the parasites, which were washed with BSM containing BSA and STI and lysed with NP-40.

[¶] Procyclic trypomastigotes were treated with 1,250 U/ml of pronase for 15 min at 37°C. At the end of the incubation, DME containing 30% FCS was added to the parasites, which were washed with DME containing 15% FCS and lysed with NP-40 in the presence of 60 mg/ml of BSA.

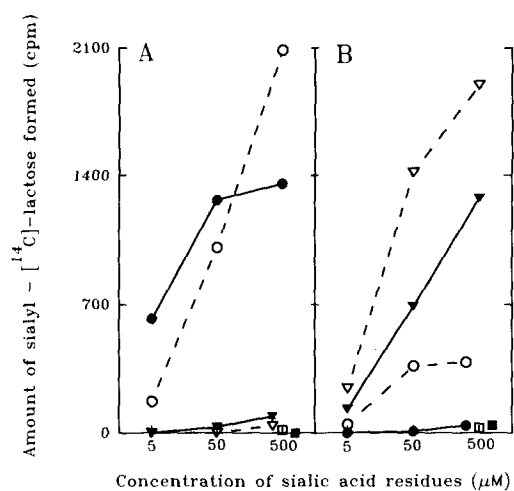


Figure 2. Ability of different compounds to donate sialic acid to lactose in the presence of *T. brucei* (filled symbols) or *T. cruzi* (open symbols) trans-sialidases. [¹⁴C]Lactose (0.36 nmol) was incubated with the indicated amounts of potential sialic acid donors and Con A-purified *T. brucei* or *T. cruzi* trans-sialidases, as described in Materials and Methods. (A) Sialyl(α2-3)lactose (circles), sialyl(α2-6)lactose (triangles), and colominic acid (squares). (B) Sialyl(α2-9)sialyl(α2-3)lactose-ceramide (circles), fetuin (triangles), and N-acetylneuraminic acid (squares).

pounds to serve as sialic acid donors for purified *T. brucei* and *T. cruzi* trans-sialidases was assessed. Both enzymes catalyzed the transfer to radiolabeled lactose of sialic acid from α(2-3)-sialyllactose, fetuin (Fig. 2), and 4-methylumbelliferyl-N-acetylneuraminic acid (not shown). In contrast, there was no sialylation of radiolabeled lactose when α(2-6)-sialyllactose, colominic acid [poly-α(2-8)-neuraminic acid], N-acetylneura-

minic acid, or α(2-9)-sialylsialyllactose were used as sialic acid donors (Fig. 2).

Next, various saccharides were assayed for their ability to inhibit sialic acid transfer by *T. cruzi* and *T. brucei* trans-sialidases. The addition of saccharides containing β-linked, but not α-linked, galactopyranosyl residues reduced the transfer of sialic acid to radiolabeled lactose (Table 4). β-methylgalactose, but not α-methylgalactose, also inhibited the reaction. Gal(β1-4)-[Fuc(α1-3)]Glc had an intermediate inhibitory activity on the formation of radiolabeled sialyllactose by both enzymes, when compared with the other disaccharides (Table 4). The dose-response curves comparing Gal(β1-4)Glc (lactose) with Gal(α1-6)Glc (melibiose), and β-methyl- with α-methylgalactose, showed that β-linked galactopyranosyl residues are at least 100 times more efficient in inhibiting the reaction than α-linked residues (Fig. 3).

To distinguish between competitive inhibition due to sialylation of cold saccharides and inhibition of catalysis, selected saccharides were incubated with [³H]sialyllactose in the presence of purified enzyme. The formation of new sialylated molecules was revealed by TLC on silica-gel plates, followed by fluorography. This experiment documented the sialylation of Gal(β1-6)Gal and β-methylgalactose (Fig. 4, lanes b and c) and, to a minor extent, of melibiose (Fig. 4, lane d). The lower efficiency of sialylation of melibiose was associated with an increased production of free sialic acid. Therefore, similarly to the *T. cruzi* enzyme, the sialidase activity increases in the absence of good sialic acid acceptors.

Absence of Reactivity of the Trans-sialidase with T. cruzi-specific Monoclonal and Polyclonal Antibodies. To reveal a possible immunological crossreactivity between the *T. brucei* and *T. cruzi* enzymes, we attempted to immunoprecipitate the *T. brucei*

Table 4. Effect of Potential Acceptors or Inhibitors on *T. brucei* and *T. cruzi* Trans-sialidase Activities

Chemical composition	Name	Reduction in sialyl- ¹⁴ C-lactose formation with trans-sialidase purified from: [‡]	
		<i>T. brucei</i>	<i>T. cruzi</i>
		%	
Gal(β1-4)Glc	Lactose	86 [§]	96
Gal(β1-6)Gal	-	51	90
Gal(α1-6)Gal(α1-6)Glc (β1-2)Fru	Stachyose	-7	-24
Gal(α1-6)Glc	Melibiose	0	9
β-Methyl-Gal	-	44	60
α-Methyl-Gal	-	11	5
Gal(β1-4)[Fuc(α1-3)]Glc	-	29	54

* Nonradioactive saccharides were added at a final concentration of 8 mM in a standard assay for trans-sialidase activity (1 mM sialyllactose and 7.2 nM ¹⁴C-lactose in 50 μl of 20 mM HEPES buffer, pH 7).

[‡] Trans-sialidase was purified from trypomastigotes by Con A affinity chromatography as described in Table 3.

[§] Percentage of reduction in cpm in relation to reactions carried out without nonradioactive saccharides (5,532 cpm for the *T. brucei* enzyme, 4,513 for the *T. cruzi* enzyme). All samples were assayed in duplicates, and the background value (72 cpm, obtained in the absence of trans-sialidase) was subtracted. Variation between the duplicate values was in every case <16% of the mean.

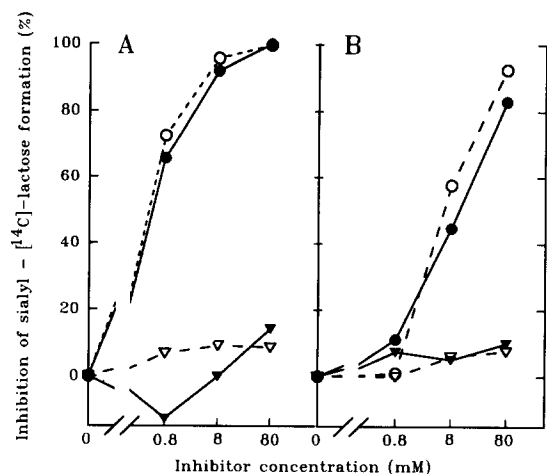


Figure 3. Inhibition of sialylation of radiolabeled lactose by saccharides. [¹⁴C]Lactose (7.2 μM), sialyllactose (1 mM), and the indicated amounts of nonradioactive saccharides were incubated with *T. brucei* (filled symbols) or *T. cruzi* (open symbols) trans-sialidases. Radioactivity associated with sialic acid was separated by anion-exchange chromatography and measured in a β counter. Trans-sialidases were purified from NP-40 trypomastigote lysates by Con A affinity chromatography. (A) Lactose (circles) and melibiose (triangles). (B) β-methyl-galactose (circles) and α-methyl-galactose (triangles).

trans-sialidase activity with monoclonal and polyclonal antibodies specific for *T. cruzi* trans-sialidase. Even when used in amounts 4.5–9-fold higher than that necessary to immunoprecipitate an equally active *T. cruzi* trans-sialidase preparation, the antibodies failed to remove *T. brucei* enzymatic activity (Fig. 5).

Identification of Procyclin as a Sialic Acid Acceptor. To identify the sialic acid acceptor(s) on the *T. brucei* surface, live procyclic trypomastigotes were incubated with [³H]sialyllactose. Fluorography of an SDS-PAGE gel carried out with a lysate of these parasites showed only one radioactive band (Fig. 6, lane d). Sialidase treatment of the lysate removed the radioactivity from the band (Fig. 6, lane e), while treatment with control buffer had little effect (Fig. 6, lane f), indicating that the sialic acid was covalently bound to surface molecules.

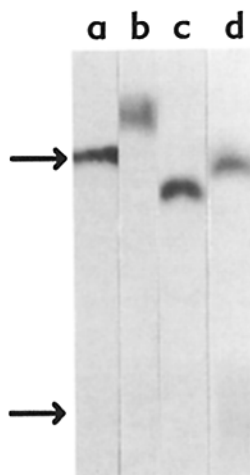


Figure 4. TLC on silica gel of different saccharides sialylated by *T. brucei* trans-sialidase. 100 nmol of lactose (lane a), β-methylgalactose (lane b), Gal(β1-6)Gal (lane c), or melibiose (lane d) was incubated with 15 nmol of [³H]sialyllactose in the presence of *T. brucei* trans-sialidase (purified by Con A affinity and anion-exchange chromatographies) for 210 min at room temperature. The products of the reaction were isolated by elution from a QAE-Sephadex column and analyzed by chromatography on silica gel, followed by fluorography. The arrows indicate the positions of free sialic acid (bottom) and sialyllactose (top).

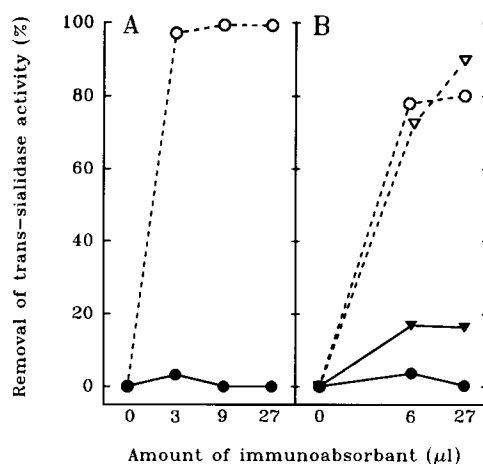


Figure 5. Lack of reactivity of *T. brucei* trans-sialidase with antibodies to *T. cruzi* trans-sialidase. *T. brucei* (filled symbols) or *T. cruzi* (open symbols) NP-40 lysates were immunoprecipitated with the indicated volumes of protein A-agarose beads bearing *T. cruzi* antibodies. The total volume of agarose beads was always brought to 27 μl by the addition of noncoated beads. (A) Immunoprecipitation with the anti-*T. cruzi* trans-sialidase mAb 39. (B) Immunoprecipitation with rabbit antibodies against purified *T. cruzi* trans-sialidase (circles) or with rabbit antibodies against a synthetic peptide corresponding to the first 19 NH₂-terminal amino acid residues of the *T. cruzi* trans-sialidase (triangles).

This band had the same molecular mass, intensity, and shape expected of a procyclin band (Fig. 6, lanes a–c) and reacted with a procyclin-specific mAb in Western blotting (Fig. 6, lane g).

An extract prepared with [³H]sialyllactose-labeled parasites was then immunoprecipitated with an antiprocyclin mAb. Most counts were immunoprecipitated, whereas no radioactivity was precipitated by a control mAb (Table 5).

Discussion

Here we report the isolation and characterization of a trans-sialidase in *T. brucei*. The enzymatic activity is stage specific: it is detected in the procyclic insect forms, but not on slender or stumpy blood-stage trypomastigotes. It is likely that the enzyme is expressed only during the transformation of the blood stages into procyclics in the insect midgut, but it cannot

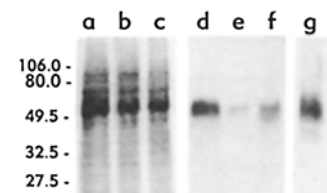


Figure 6. SDS-PAGE of *T. brucei* surface molecules sialylated by the addition of radiolabeled sialyllactose. Live *T. brucei* procyclic trypomastigotes were incubated with [³H]sialyllactose and lysed with NP-40. Lysate samples were untreated (lanes a and d), treated with sialidase (lanes b and e), or treated with sialidase buffer (lanes c and f) and then subjected to SDS-PAGE. The gel was impregnated with sodium salicylate and stained with coomassie blue (lanes a–c). The presence of radioactive molecules in the same gel was revealed by fluorography (lanes d–f). A lysate sample was subjected to Western blotting using an antiprocyclin mAb (lane g). The positions of molecular mass standards are shown on the left.

Table 5. Immunoabsorption with Procyclin-specific mAb of Molecules Sialylated by the Incubation of Live *T. brucei* Procyclic Trypomastigotes with [³H]Sialyllactose

mAb used in immunoabsorption*	Counts in supernatant		Counts in pellet	
	cpm ± SD	%	cpm ± SD	%
137†	3,755 ± 461	28.1	9,618 ± 709	71.9
3C9‡	15,293 ± 696	99.8	28 ± 8	0.2

Trypomastigotes were incubated for 28 min with sialic-9-[³H](α 2-3)-sialyllactose, washed six times with cold BSM-G, and lysed with NP-40.

* Lysates (60- μ l fractions) were incubated with 20 μ g of the indicated mAb adsorbed to 20 μ l of protein A-agarose, in quadruplicate. The beads were washed three times, centrifuged, and the amount of radioactivity in pellets or supernatants was measured.

† Procyclin-specific, IgG1 mAb.

‡ Unrelated IgG1 mAb.

be excluded that an inactive form of trans-sialidase is already present in the blood stages. Extracts of procyclics pretreated with pronase had diminished enzymatic activity, indicating that most of the enzyme is associated with the surface membrane of the parasites.

The trans-sialidase was purified by Con A affinity chromatography, followed by ion exchange and molecular sieving chromatography on FPLC. The activity of the purified enzyme was not affected by treatment with proteinase K. It is not clear whether proteinase K does not cleave the enzyme, or releases an enzymatically active fragment of the protein. SDS-PAGE and silver staining of the purified enzyme under nonreducing conditions revealed a single band of 63 kD, but under reducing conditions additional bands between 63 and 73 kD were also detected. Further investigation is necessary to determine whether some of these bands are contaminants, incompletely reduced molecules, products of different trans-sialidase genes, or represent differences in posttranslational modifications of a single polypeptide. By molecular sieving chromatography the apparent molecular mass of the active fractions varied considerably in different experiments, ranging from 66 to >700 kD, but we did not detect significant differences in the enzymatic activities of the monomeric and oligomeric forms. In *T. cruzi*, the trans-sialidase/sialidase is also multimeric (19, 20), the monomers varying from 120 to 180 kD in the cell-derived, bloodstream-like trypomastigotes (10, 19, 20) to 90 kD in the insect stages (L. B. Chaves, M. R. R. Briones, and S. Schenkman, manuscript submitted for publication).

The properties of the trans-sialidases of *T. cruzi* and *T. brucei* are similar. Both enzymes catalyse the transfer of α (2-3)-, but not α (2-6)-, α (2-8)-, or α (2-9)-linked sialic acid, and are equally inhibited by 10 μ M mercuric acetate or cupric nitrate (not shown). In both instances sialic acid is readily transferred to terminal β -galactopyranosyl residues (26; this paper). Terminal α -galactopyranosyl residues are at least 100 times less efficient acceptors (Fig. 3). Nevertheless, it appears that melibiose [Gal(α 1-6)Glc] can be sialylated if it is present in large excess in the incubation mixture (Fig. 4, lane *d*). By TLC we did not detect any contaminants in the melibiose

preparation, but we cannot exclude the possibility of a minor contaminant saccharide.

During the purification procedure, all fractions with trans-sialidase activity also had sialidase activity, as measured by the 4-methylumbelliferyl-*N*-acetylneuraminic acid assay (not shown). The observation that sialidase activity was inhibited by the presence of efficient acceptors (Fig. 4, lanes *a-c*) argues that the same enzyme displays both activities. It is well known that glycosidases can function as trans-glycosidases, depending on the relative affinity of the glycosyl residues for the saccharide acceptors or for water. In fact, a cloned gene product from *T. cruzi* has been shown to display both trans-sialidase and sialidase activities, depending on the presence or absence of appropriate acceptors in the incubation medium (27). It is very likely that, in the presence of the blood meal in the tsetse fly midgut, the transfer reaction will predominate and molecules on the parasite surface will be sialylated.

It appears that procyclin is the major (if not only) protein sialylated by the enzyme. One sialylated band was seen on SDS-PAGE of whole parasite lysates, and the position of the band coincided with that of procyclin. Moreover, mAbs to procyclin immunoprecipitate most of the sialylated molecules of the parasite surface membrane. This finding is agreement with recent data documenting the presence of sialic acid in the GPI anchor of procyclin (9a). The finding of sialic acid in supernatants of live trypomastigotes treated with pronase suggests that other N-linked or O-linked procyclin saccharides may also be sialylated.

T. brucei procyclics contain larger amounts of sialic acid than cell-derived *T. cruzi* trypomastigotes, but the saccharide density on the plasma membrane of the parasites may not be different, since by light microscopy the procyclics are much larger than *T. cruzi* trypomastigotes. Assuming that there are as many procyclin as VSG molecules (10⁷) per parasite (28), and that procyclin is the only sialylated molecule, we calculate that there are ~10 sialic acid residues per procyclin molecule.

What is the function of the trans-sialidase? In *T. cruzi* the membrane-associated sialylated epitope, Ssp-3, appears to be involved in cell adhesion and penetration (10). Procyclic

T. brucei trypomastigotes may need to interact with epithelial cells (or with the peritrophic membrane) during their onward migration to the proboscis, and penetration into the salivary glands. Perhaps the procyclin sialic acid, and tsetse lectins (29), are involved in these hypothetical interactions. Another possible function of the procyclin sialic acid relates to the complement system. In the blood stages, it has been postulated that VSG protects the parasite from the cascade (1, 30). The transformation of blood stages into procyclics, and the gradual substitution of VSG by procyclin (4, 5), occurs in the insect midgut in the presence of the blood meal and perhaps of an active complement system. This process takes 12–48 h (4, 5), and it is conceivable that during this time the cascade is activated, leading to the deposition of C3b on

the parasite. The high concentration of sialic acid on the parasite surface should increase the avidity of factor H for membrane-bound C3b, and prevent the assembly of C3-convertase (31, 32). Sialic acid could also mask terminal β -galactopyranosyl residues (33) on the parasite surface, preventing recognition of the surface components by mammalian (natural?) antibodies. These hypotheses are amenable to experimental verification.

While this paper was being prepared for publication, a protein of *T. brucei* procyclic trypomastigotes with sialidase activity was reported (34). This sialidase and the trans-sialidase here described may be the same enzyme since they have similar physico-chemical properties and substrate specificities.

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