THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 11, Issue of March 18, pp. 7970-7975, 1994
Printed in U.S.A.

A Proteolytic Fragment of *Trypanosoma cruzi trans*-Sialidase Lacking the Carboxyl-terminal Domain Is Active, Monomeric, and Generates Antibodies That Inhibit Enzymatic Activity*

(Received for publication, September 9, 1993, and in revised form, December 9, 1993)

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***trans*-Sialidase isolated from trypomastigote forms of *Trypanosoma cruzi*, the protozoan parasite that causes Chagas' disease, is multimeric and heterogeneous in size. We show here that limited proteolysis of *trans*-sialidase with papain yields a single monomeric polypeptide chain of 70 kDa that conserves full enzymatic activity on soluble and membrane-bound substrates. The papain fragment lacks most of the 12-amino acid repeats of the carboxyl-terminal domain that comprises about 50% of the native *trans*-sialidase. When injected into rabbits, the papain-generated fragment induces antibodies that inhibit *trans*-sialidase activity and trypomastigote sialylation. The repeats are also not required for the stability of the enzyme or for the correct folding during the biosynthesis in *Escherichia coli*, but seem essential for *trans*-sialidase oligomerization. We conclude that *trans*-sialidase is composed of two structurally and functionally independent domains.**

trans-Sialidase (TS)[1] is an enzyme that preferentially transfers sialic acid linked by glycosidic bonds to carbohydrate acceptors instead of water (1). In contrast, most of the sialidases and glycosidases, although able to catalyze *trans*-glycosidase reactions (2, 3), preferentially transfer the saccharides to water, in what is known as the hydrolysis reaction. TS has been found only in parasitic trypanosomes, such as the agents of African trypanosomiases, caused by *Trypanosoma brucei* and related species (4, 5), and South American trypanosomiases, caused by *Trypanosoma cruzi* (6–8). Trypanosomes do not synthesize sialic acid (9), but use TS to acquire it from host glycoconjugates. The sialic acid is transferred to surface glycoproteins, which apparently are required for survival of the parasites in the host (10–13).

Two distinct forms of TS have been characterized. One is monomeric, has low molecular mass (60–90 kDa), and is found in parasite stages growing in the insect vectors of both species of *Trypanosoma* (5, 14–16). The other is multimeric, has high molecular mass (>400 kDa), and is found in the bloodstream *T. cruzi* trypomastigotes (1, 17, 18).

T. cruzi TS is encoded by a multigene family. The members of this gene family have in common a conserved amino-terminal domain, displaying SXDXGXTW motifs (17, 19–21), found in bacterial neuraminidases (22), and a distinctive carboxyl-terminal region composed of a long tandem repeat of 12 amino acids (LTR) (17, 19). The number of encoded LTRs varies between the members of the gene family (23). Trypomastigote TS migrates in SDS gels as multiple bands between 80 and 240 kDa, and all isoforms have the same amino-terminal sequence (24). It appears, however, that only some isoforms are enzymatically active, since the products of some cloned TS genes expressed in heterologous cells do not display TS activity (20). The relevant differences between the active and inactive forms reside in the amino-terminal region, which is assumed to contain the catalytic site. The function of the LTRs is not known. Because they are immunodominant (25), it has been suggested that they deviate the immune response of the host, prevent recognition of the amino-terminal domain of TS, and thus enhance parasite survival (26).

In the present paper we have studied the role of the LTR using two independent approaches. We have attempted to remove the LTR by limited proteolysis. By treating purified TS with papain, we isolated a homogeneous product of 70 kDa (TS-pap) which retained full enzymatic activity. In parallel, we have studied the expression in *Escherichia coli* of a TS gene lacking the sequences encoding the LTRs.

MATERIALS AND METHODS

TS Purification—TS was purified from the extracellular medium of *T. cruzi* Y-strain trypomastigotes derived from cultures of $LLCMK_2$ cells (ATCC-CCL-7) grown in low glucose Dulbecco's modified Eagle's medium with penicillin and streptomycin (Life Technologies Inc.), containing 10% fetal bovine serum at 37 °C, 5% CO_2, as described (16). Briefly, 0.1% Nonidet P-40 were added to pooled supernatants from cultures of *T. cruzi*-infected $LLCMK_2$, the supernatants filtered through a 0.22-μm filter, and concentrated by precipitation with 50% $(NH_4)_2SO_4$. The precipitates were dialyzed against phosphate-buffered saline, and then passed through a tresyl-agarose (Schleicher and Schuell) column containing immobilized anti-TS monoclonal antibody 39 (1). The column was washed with phosphate-buffered saline, followed by 10 mM sodium phosphate, pH 6.5, and TS eluted with 3.5 M $MgCl_2$, 10 mM sodium phosphate, pH 6.0. The fractions eluted from the column were immediately filtered through a Sephadex G-25 column equilibrated with 20 mM Tris-HCl, pH 8.0, to remove $MgCl_2$. TS was further purified by passage through a Mono Q column equilibrated in 20 mM Tris-HCl, pH 8.0, and eluted with a linear gradient from 0 to 1 M NaCl in the same buffer.

Limited Proteolysis—Purified TS at 20–100 μg/ml was incubated with 10% (v/v) agarose-immobilized papain (Pierce Chemical Co.) in 0.1 M Tris-HCl, pH 7.4, containing 10 mM EDTA and 10 mM cysteine. After 1 h at 25 °C with agitation, the resin was removed by centrifugation,

* This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil, the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases, The Rockefeller Foundation, The John D. and Chaterne T. MacArthur Foundation, Whitehead Presidential Fellowship from New York University, and National Institutes of Health Grant RO3 TW00227-01. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TS, *trans*-sialidase; LTR, long tandem repeat; TS-pap, 70-kDa fragment of TS; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.

and the supernatant diluted 5 times with a buffer containing 20 mM Tris-HCl, pH 8.0. The diluted sample was then centrifuged 30 min at 10,000 × *g*, and loaded into a Mono Q column equilibrated with 20 mM Tris-HCl, pH 8.0. TS containing fractions were eluted with a linear gradient of NaCl in the same buffer as described above.

Activity Measurements—TS activity was determined in a final volume of 50 µl containing 20 mM Hepes, pH 7.0, 0.2% bovine serum albumin (BSA), ultrapure (Boehringer Mannheim GmbH, Germany), 100 nmol of sialyllactose (65%, α2,3-sialyllactose, Boehringer), and 0.36 nmol of D-glucose-1-[^{14}C])lactose (60 mCi/mmol) (Amersham). After an appropriate incubation time, usually 30 min in the standard reaction, the volume was increased by addition of 1 ml of H_2O, and each sample filtered through a 0.5-ml QAE-Sephadex A-25 column also equilibrated with H_2O. The columns were then washed with 8 ml of H_2O, and the produced [^{14}C]sialyllactose eluted with 1 ml of 1 M ammonium formate. The recovered radioactivity was quantified by liquid scintillation counting. We define 1 TS unit as the amount of enzyme able to transfer 0.36 nmol of sialic acid to [^{14}C]lactose in 30 min, at 37 °C in the presence of 100 nmol of sialyllactose. As the transfer reaction is linear to about 25% of the total transfer (0.36 nmol, or 30,000 cpm) we diluted the enzymes to achieve no more than 7,500 cpm in each reaction. Activity was also assayed by using TS immobilized in 5-mm disks of nitrocellulose, or by using saline-washed human erythrocytes as a sialic acid source, or by using human erythrocytes pretreated with *Vibrio cholera* neuraminidase (Boehringer) (27) as acceptor and (9-sialic acid)-[^{3}H]sialyllactose (28) as sialic acid donor.

Gel Electrophoresis, Immunoblot, and Immunodepletion Experiments—Samples were boiled in a sample buffer of 2% SDS, 50 mM Tris-HCl, 5% 2-mercaptoethanol, 10% glycerol, 0.01% bromphenol blue, and run in 7.5% polyacrylamide-SDS gels. The gels were directly silver stained, or transferred to nitrocellulose membranes and probed with rabbit antisera diluted 100–200 times with 1% BSA in 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4. Antisera were obtained from rabbits immunized with a synthetic peptide, DSSAHSTPSTPADSSAH, based on the repeated sequence of the carboxyl terminus of TS (anti-LTR), or with a synthetic peptide, MVAIADARYETSNDNSLID, based on the residues 48–66 of the amino-terminal of mature TS (anti-NH_2-terminal) (24). The peptides were coupled to keyhole limpet hemocyanin with glutaraldehyde and used as immunogen. Antisera was also prepared by immunizing rabbits with 20 µg of purified papain fragment of TS in complete Freund's adjuvant. High titer antisera were obtained after 4 subcutaneous boosters, monthly spaced, of 10 µg of papain fragment emulsified in incomplete Freund's adjuvant. Bound antibodies were detected with anti-rabbit IgG conjugated to alkaline phosphatase, or with protein A labeled with ^{125}I.

For the immunodepletion experiments, 25 µl of protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) was suspended in 200 µl of 50 mM Tris-HCl, 0.1 M NaCl, pH 8.5, and mixed with the different antibody preparations. After 30 min of incubation with constant agitation, the antibodies attached to protein A beads were centrifuged, washed with 50 mM Tris-HCl, 0.1 M NaCl, pH 8.5, and incubated with 50 µl of the enzyme preparation diluted in 50 mM Tris-HCl, pH 8.5, 0.1 M NaCl, and 0.2% BSA. For inhibition experiments, the antibodies were purified on protein A-Sepharose columns, dialyzed in phosphate-buffered saline, or in 10 mM sodium phosphate, 10 mM EDTA for digestion with immobilized papain (Pierce) to prepare Fab fragments according to the manufacturer's instructions.

Isoelectric focusing was performed in the Fast System (Pharmacia LKB) using the Phastgel IEF 3–9 gels and using the ElectroFocusing Calibration Kit (Pharmacia LKB) as pI standards. The samples were applied after a pre-run of 75 volts/h, and focused 410 volts/h at 15 °C. Part of the gels were silver stained, and part sliced and incubated for 1 h in the standard reaction conditions to measure TS activity.

Generation of Exonuclease III Deletions—The TS gene from plasmid pSII-154 (20) was transferred into the expression vector pQE-60 (Qiagen) as follows: a 5′ oligonucleotide primer (5′-GCCCATGGCACCCGGATCGAGCCGAGTT-3′) was designed to insert an ATG codon (as part of an *Nco*I restriction site), just ahead of the apparent signal sequence cleavage site in the active form of the TS enzyme (24); a 3′ oligonucleotide primer (5′-GGCGTCGACAGCACACGCACACAGAAGAGG-3′) was designed to eliminate the glycosylphosphatidylinositol tail attachment recognition site. Polymerase chain reaction amplification of the entire encompassed open reading frame with these primers yielded a product smaller than expected (about 1.7 kilobases), which was cloned into pQE-60 by filling in the 3′ end of the product as well as the *Bgl*II site of the vector, digesting both with *Nco*I and then ligating. The cloned DNA fragment contained all the expected restriction sites as far 3′ as the *Kpn*I site, but the protein product expressed from this construct had neither enzymatic activity nor COOH-terminal repeats. This partial gene construct was then used as the source of the 5′ end of the gene up to the *Hin*dIII site, and pSII-154 was used as the source of the 3′ *Hin*dIII segment to reconstruct the entire gene. Expression of the resulting construct, pTS, yielded a full length (approximately 160 kDa) protein product with both neuraminidase and TS activities, and COOH-terminal repeats as recognized by monoclonal antibody 39 (1).

To eliminate defined amounts of the repeat coding region, the pTS vector was cut with *Nhe*I at a site 3′ to the end of the gene, and the overhang was filled in with α-phosphorothioate dNTPs. The plasmid was then cut at the *Bpu*1102I site found in the vector between the *Nhe*I site and the 3′ end of the gene, and subjected to exonuclease III digestion for various lengths of time, treated with S1 nuclease, Klenow fragment plus dNTPs, and T4 DNA ligase. The resulting deletion plasmids were examined by releasing the 3′-half of the gene at the *Kpn*I site found just 5′ of the repeat coding region, and at the *Xba*I site found in the vector 3′ to the *Nhe*I site. Informative plasmids were induced for protein expression and the products assayed for TS and neuraminidase as described (20). The end points of the deletions were determined by sequencing out the 3′ end of the genes using a primer (AGGTACCACTTGGTTCTTACGATG) which overlapped the *Kpn*I site. Standard dideoxy termination reactions and Sequenase were used as described by the manufacturer (U. S. Biochemical Corp.).

Immunofluorescence Experiments—Trypomastigotes released from $LLCMK_2$ cells in Dulbecco's modified Eagle's medium containing 0.2% BSA (Boehringer) were centrifuged, washed in Hanks' balanced saline, and resuspended to 1 × 10^8 parasites/ml of Hanks' balanced saline. Ten microliters of the parasite suspension was added to immunofluorescence slides, and after 10 min excess parasites were removed. The live and adsorbed parasites were washed with Hanks' balanced saline and incubated with diluted antisera or purified IgG. After 15 min, the parasites were washed, fixed 1 h with 4% paraformaldehyde, washed 3 times with Hanks' balanced saline, resuspended with 0.2% BSA/Dulbecco's modified Eagle's medium, and incubated 30 min with 50 µg/ml monoclonal antibody 3C9, which recognizes the sialylated epitope Ssp3 (8). Bound antibodies were detected with a 1/200 dilution of anti-mouse IgG-fluorescein isothiocyanate (Boehringer). Controls were run in parallel without 3C9 and no fluorescence was detected in any case.

RESULTS

Affinity purified TS migrates in SDS gels as multiple bands of molecular mass from 83 to 240 kDa (Fig. 1*A, lane a*). An unidentified doublet of 29 and 30 kDa was also detected in this preparation. However, these bands are not recognized by antibodies to LTR or by any other anti-TS sera, and most likely are contaminants. The 50–55-kDa bands in the gel are artifacts of the silver nitrate staining procedures. Upon treatment of TS with immobilized papain, there is no decrease in activity. All the bands of high molecular mass disappear and a single sharp 70-kDa band becomes visible in the gels (*lane b*). No decrease in molecular mass was observed by incubating TS in the same buffer without papain. Most of the native, untreated TS elutes from Sephacryl S-300 as a broad peak of about 400 kDa. A small fraction elutes as a 70/80-kDa protein, which may represent a degradation product similar in nature to that obtained by papain treatment. In contrast, TS-pap elutes from Sephacryl S-300 in a 70-kDa peak (Fig. 1*B*). The undigested TS and TS-pap were then further purified by anion-exchange chromatography. TS-pap elutes in a sharp peak preceding the undigested enzyme (Fig. 1*C*), indicating that at pH 8.0 TS-pap is less negatively charged than the undigested TS. The specific activity of purified TS-pap and undigested TS using sialyllactose as a substrate is identical, *i.e.* 20 ± 2 units/µg. These values were obtained by estimating the amount of protein by absorbance at 280 nm.

To further characterize TS-pap, an aliquot of the sample eluted from the Mono Q column was subjected to isoelectric focusing, and one part of the gel stained by silver nitrate, and the other sliced to measure TS activity. As show in the Fig. 2, most TS-pap focuses in a band at pH 6.3. This band contains more than 90% of the activity recovered in the gel. A minor band focuses at pH 6.9 and corresponds to the remaining 10%

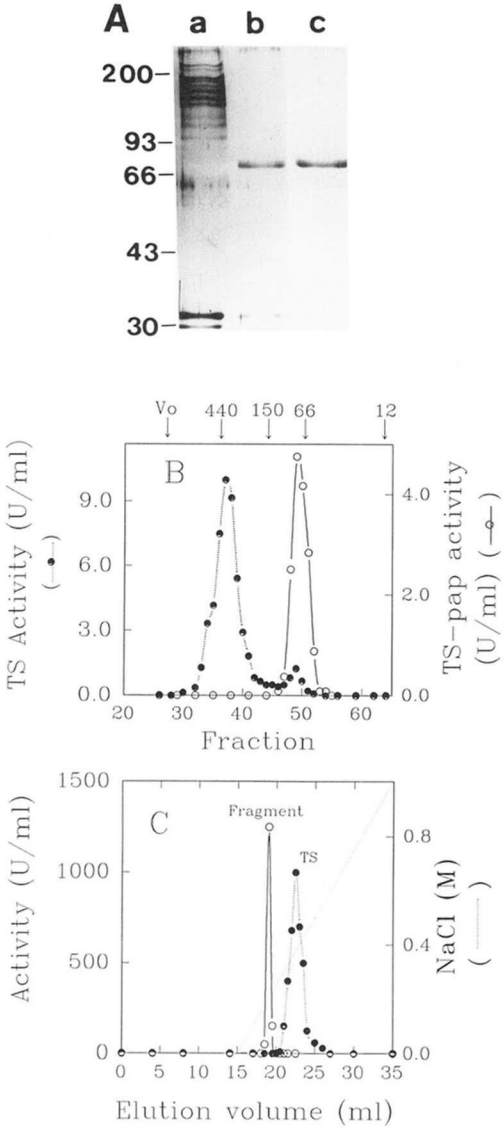

FIG. 1. **Digestion of TS with papain.** *A*, silver-stained SDS gel electrophoresis of affinity purified TS (*lane a*), TS treated with papain (*lane b*), the TS-pap after purification in Mono Q column. The *numbers* in the *left* indicate the molecular mass of standards in kDa. *B*, elution profile of affinity purified TS (●), or the papain fragment (○) applied into a Sephacryl S-300 superfine column (1.5 × 90 cm), equilibrated with 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.02% sodium azide. The column was eluted at 8.4 ml/h, and the activity determined in 2.1-ml fractions. The *arrows* indicate the position of elution of blue dextran (V_0), apoferritin (440 kDa), IgG (150 kDa), BSA (66 kDa), and cytochrome *c* (12.5 kDa). *C*, elution profile of affinity purified TS (●) or TS-pap (○) in a Mono Q column (HR5/5) equilibrated with 20 mM Tris-HCl, pH 8.0. The *dotted line* indicates the NaCl concentration.

of activity. In contrast, undigested TS focuses as a diffuse band below pH 5.5.

The fact that the multiple SDS-PAGE bands of TS are converted to a single component with a higher pI indicates that acidic regions of variable size are removed from TS by papain. The most acidic region of TS resides in the LTR of the carboxyl terminus, which contains 1 aspartic acid and 1 histidine in each repeat unit. To analyze the structure of TS-pap, we used antibodies specific for either the LTR domain or the amino terminus of TS. As shown in Table I, more than 90% of undigested TS activity is precipitated by protein A-Sepharose bearing anti-LTR antibodies, whereas most of the activity of the TS-pap remains in the supernatant. Both activities are immunoprecipitated by an antiserum against TS-pap. As also shown in the table, both TS-pap and TS bind to concanavalin A. In immunoblots TS-pap is not recognized by anti-LTR (Fig. 3, *lane b*), but does react with an antiserum raised against TS-pap, and with an antiserum to a synthetic peptide representing a region of TS close to the amino terminus. Some preparations of TS-pap reacted weakly with anti-LTR, suggesting that they may still contain a few repeats.

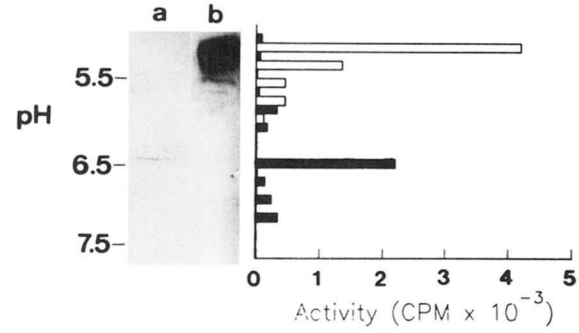

FIG. 2. **Isoelectric focusing of TS and TS-pap.** TS and TS-pap purified by Mono Q were subjected to isoelectric focusing as described under "Materials and Methods." *Lane a* of the silver-stained gel, and the *black bars*, correspond to TS-pap. *Lane b* and *open bars* correspond to undigested TS.

TABLE I
Immunoprecipitation of TS and TS-pap with immobilized antibodies and concanavalin A

Appropriate dilutions of TS and TS-pap purified by Mono Q were incubated with the indicated antibodies preadsorbed to protein A-Sepharose, or concanavalin A-Sepharose. After 30 min of incubation under agitation each sample was centrifuged and the remaining activity measured in the supernatant. The values in cpm are means of duplicate determinations, and the numbers in parentheses correspond to the activity relative to normal rabbit serum or Sepharose 4B.

Sepharose adsorbent	Activity remaining in the supernatant	
	TS	TS-pap
	cpm	
Anti-LTR	600 (16%)	2400 (92%)
Anti-TS-pap	175 (5%)	339 (13%)
Normal rabbit serum	3600 (100%)	2600 (100%)
Concanavalin A	270 (8%)	120 (3%)
Sepharose 4B alone	3600 (100%)	2700 (100%)

These results, taken together with the gel filtration experiments, suggest that the LTRs are necessary for the oligomerization of the TS subunits. This oligomerization could influence the ability of TS to transfer sialic acid to membrane-bound glycoconjugates. To test this possibility, we compared the activity of TS-pap and TS toward erythrocytes. We used the same number of units of each enzyme form, determined with soluble substrates, to transfer sialic acid to the surface of erythrocytes. As shown in Fig. 4, TS-pap is even more active than TS in transferring sialic acid from sialyllactose to desialylated human erythrocytes (A), or from erythrocytes to [^{14}C]lactose (Fig. 4*B*). These observations are compatible with the notion that all subunits arising from the oligomeric form of the enzyme are active, since in this arrangement they are unable to make the same number of productive contacts with the substrate as does the monomeric enzyme.

We investigated whether LTRs were essential for the correct folding of the enzyme during biosynthesis. For this purpose the TS gene from plasmid pSII-154 (20) was inserted into the expression vector pQE-60. Bacteria containing this plasmid (pTS) express proteins from 120 to 160 kDa that are recognized in immunoblots by antibodies to LTR and to TS-pap (Fig. 5*A*, *lanes b* and *h*, respectively), and have TS activity. Several deletions were prepared by exonuclease III digestion of pTS, as indicated in Fig. 5*B*. Whereas, some deletions partially remove the LTR domain, resulting in proteins with TS activity (p2D,

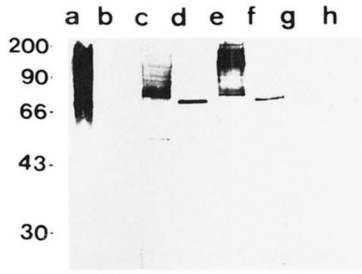

FIG. 3. **TS-pap is not recognized by anti-LTR in immunoblots.** Affinity purified TS (*lanes a, c, e,* and *g*), and the TS-pap purified by Mono Q (*lanes b, d, f,* and *h*) were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with: *1*) anti-LTR (*lanes a* and *b*); *2*) anti-TS-pap (*c* and *d*); *3*, anti-NH_2 terminus (*e* and *f*); and *4*, normal rabbit serum (*g* and *h*). Bound antibodies were detected with anti-rabbit IgG conjugated with alkaline phosphatase. The *numbers* in the *left* indicate the molecular mass of standards in kDa.

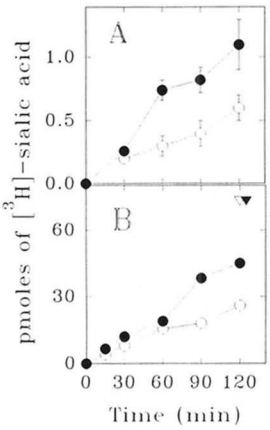

FIG. 4. **TS-pap catalyzes the transfer of sialic acid to and from erythrocytes.** Desialylated human erythrocytes (*A*) and untreated human erythrocytes (*B*) at 5% were incubated with constant agitation, respectively, with 50 μM [^{3}H]sialyllactose and 7.2 μM [^{14}C]lactose in the presence of 20 units of TS (○) or TS-pap (●). In *A*, aliquots were removed at the indicated times, the cells washed three times in saline and the [^{3}H]sialic acid incorporated into the erythrocytes was determined by liquid scintillation. In *B*, the amount of [^{14}C]sialyllactose formed was measured after QAE-Sephadex chromatography by the standard assay. The values are means ± S.D. of triplicates (*A*) or means of duplicates (*B*). The *triangles* in *B* indicate the amount of [^{14}C]sialyllactose formed in the presence of 0.1% Triton X-100 to lyse the cells.

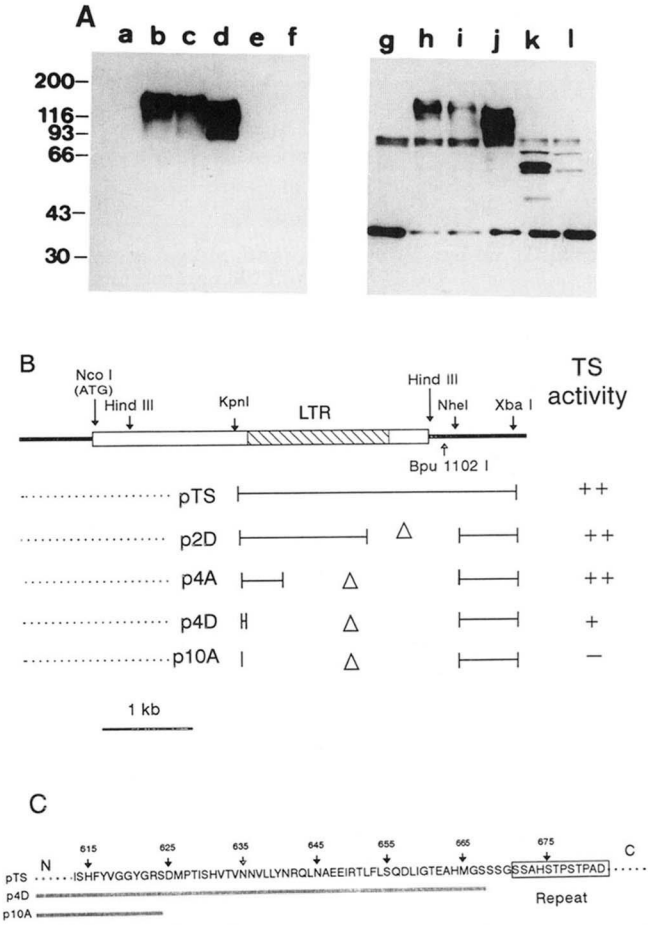

FIG. 5. **Expression of proteins and activity by recombinants containing different sizes of the LTR domain.** *A*, immunoblot of extracts of *E. coli* expressing each construct reacted with anti-LTR (*lanes a-f*) and anti-TS-pap (*lanes g-h*). Bacteria containing the plasmid PQE-60 (control) were loaded on *lanes a* and *g*; pTS on *lanes b* and *h*, p2D on *lanes c* and *i*, p4A on *lanes d* and *j*, p4D on *lanes e* and *k*, and p10D on *lanes f* and *l*. Bound antibodies were revealed by incubation with ^{125}I-protein A and autoradiography. The *numbers* on the *left* are the molecular mass of standards in kDa. *B*, scheme of the TS genes indicating the exonuclease deletions and their activity. The ATG indicates the putative initiation site, the *stripped bar* the position of LTR. The deletions are indicated by the *interrupted line* with a Δ symbol. *C*, predicted amino acid sequences (single letter codes) of clones p10D, p4D, and p4A showing the truncated regions generated by exonuclease III digestion. The *bars* on the *p4D* and *p10A* lines represent identity with the *pTS* amino acid sequence shown above. The *numbers* refer to the amino acid position considering the longest open reading frame of the entire TS gene.

p4A), deletion p4D expresses proteins that completely lack the LTRs, but still conserves TS activity. Sequence determination of the deletions end points confirm that the LTR repeats are not present in p4D (Fig. 5*C*). The predicted TS protein ends 3 amino acids before the repeated sequence would have started, and is instead fused with 33 amino acids encoded by an open reading frame present in the pQE-60 vector. In addition, the 70-kDa product encoded by p4D is not recognized by anti-LTR (Fig. 5*A, lane e*), and is degraded to polypeptides of 65-, 60-, and 50-kDa bands, all of which are recognized by the anti-TS-pap antisera (*lane k*). On the other hand, construct p10D, lacking LTRs and the sequence coding for the 46 amino acids preceding the repeats, has no TS activity. This construct expresses a 61-kDa protein, smaller than the protein of 65 kDa of p4D, as well as a protein of 59 kDa, both of which are recognized by the anti-TS-pap antisera (*lane l*). The doublet bands migrating around 90 kDa and the band of 40 kDa recognized by anti-TS-pap are also found in extracts of bacteria containing only the pQE-60 plasmid (*lanes a* and *g*), and are therefore unrelated to TS. These results rule out the possibility that the presence of LTR is necessary for the correct folding of the amino-terminal domain, and expression of TS activity. The immunoblots also show that all the plasmid-encoded products appear to undergo proteolysis with a regular pattern, independent of the presence or size of the LTR. Therefore, the amino-terminal domain is also susceptible to proteolysis when the TS is expressed in bacteria.

Next, we asked whether the presence of LTRs, or the oligomerization state, could influence the thermal stability of TS. Both enzyme forms were preincubated at different temperatures for 10 min and the remaining activity assayed at 37 °C. No differences in activity as a function of inactivation temperature were detected (Fig. 6). Therefore, the LTRs do not influence the thermal stability of the catalytic site.

As shown in Table II, anti-TS-pap inhibits 100% of TS activity, and about 80% of TS-pap activity. Anti-LTR and anti-NH_2-terminal peptide antibodies do not inhibit these activities. To rule out artifacts due to enzyme aggregation that might occur in a fluid-phase assay, IgG and Fab fragments were purified from the anti-TS-pap, and used to inhibit TS, TS-pap, or TS immobilized on nitrocellulose disks. In every case, specific,

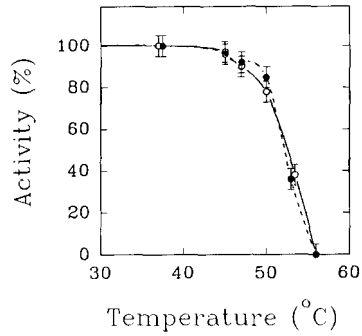

FIG. 6. **TS and TS-pap have similar thermal sensitivity.** Appropriate dilutions of TS (○) and TS-pap (●), both purified by Mono Q column, were preincubated for 10 min at the indicated temperature and assayed at 37 °C for 30 min using the standard reaction conditions. The results are means of triplicates ± S.D. and correspond to the percentage of activity relative to the activity of both enzyme forms preincubated at 4 °C.

TABLE II
Inhibition of TS, TS-pap, and T. cruzi sialylation by immune sera

Antisera	Dilution (final)	% of inhibition		Sialylation of *T. cruzi*[b]
		TS activity[a]	TS-pap activity	
Anti-TS-pap	1/10	100	80	Inhibited
Anti-TS-pap	1/100	100	NT[c]	NT
Anti-LTR	1/10	25	12	Not inhibited
Anti-N-terminal	1/10	0	26	NT
Normal serum	1/10	0	0	Not inhibited

[a] Five microliters of purified TS or TS-pap were preincubated with 5 μl of the different antisera for 10 min. Then, 40 μl of a reaction mixture containing sialyllactose and [^{14}C]lactose, buffer and BSA as in the standard reaction were added and the incubation proceeded for 30 min. The results show means of replicate percentages of inhibition of [^{14}C]sialyllactose formation as compared to controls incubated in the presence of normal rabbit serum.

[b] The degree of *T. cruzi* sialylation was estimated by the presence of Ssp-3 in trypomastigotes by indirect immunofluorescence labeling with monoclonal antibody 3C9 of live parasites attached to glass slides, as described under "Materials and Methods."

[c] NT, not tested.

dose-dependent inhibition was observed (Fig. 7). We also tested whether the anti-TS-pap antibodies inhibit *T. cruzi* sialylation. When trypomastigotes are released from infected mammalian cells in medium without a source of sialic acid, they contain very small amounts of this saccharide. Upon incubation with serum the parasites become rapidly sialylated and the stage-specific epitope Ssp3 is formed (8). In the experiments summarized in Table II, we have used the antisera themselves as a source of sialic acid. As shown, Ssp3 is not formed in the presence of anti-TS-pap serum, but it is formed in the presence of the anti-LTR serum. Similar effects are observed by using purified anti-TS-pap IgG plus up to 30 μM sialyllactose as a source of sialic acid. Higher concentrations of sialyllactose, however, overcome the antibody-mediated inhibition (not shown).

DISCUSSION

In this paper we identified and characterized a proteolytic fragment of *T. cruzi* TS. It is a 70-kDa polypeptide that contains the amino-terminal domain including the catalytic site, but none, or at most, a few tandem repeats of the carboxyl terminus. Digestion with papain of the affinity purified TS, which is highly heterogeneous and multimeric, results in a monomeric polypeptide. It migrates as a sharp 70-kDa band in SDS-PAGE, and with a pI of 6.3 in isoelectric focusing. This TS fragment retains full enzymatic activity, indicating that the only function of the LTRs is to maintain the native TS in an oligomeric state. The secondary structure prediction for the LTR domains sug-

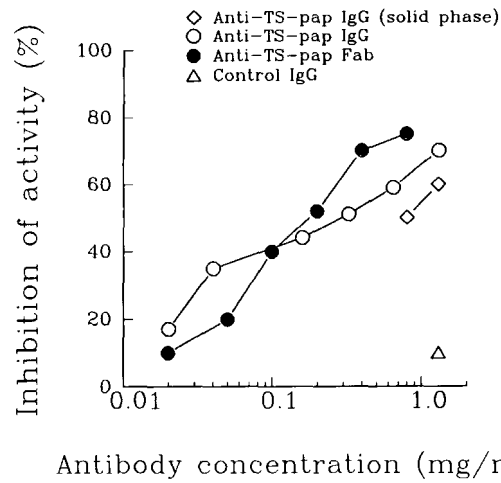

FIG. 7. **Inhibition of TS activity by IgG and Fab of the anti-TS-pap.** Anti-TS-pap IgG (○) and Fab (●) and normal rabbit IgG (△), were preincubated with TS-pap for 10 min. The substrates were added to each tube, as in the standard assay, and the incubation proceeded for 30 min at 25 °C. Shown are the percentages of inhibition of TS activity relative to control without IgG, measured in duplicate. The ◇ indicates the percentage of inhibition of undigested TS adsorbed to nitrocellulose disks after 4 h of incubation.

gests the presence of β-turns (29). Since each consensus tandem repeat has 2 charged residues, 1 an aspartic acid, and the other a histidine, 4 serines, 2 threonines, and 2 prolines, we speculate that these turns could generate a periodic structure and promote interactions between adjacent chains and TS oligomerization. There is no decrease in the total activity after papain hydrolysis. As less protein is apparently detected in the silver-stained gel (Fig. 1*A*), it is possible that the presence of LTR yield an overstaining of the gels. In addition, the specific activity of TS is also overestimated when its concentration is estimated from optical densities since the LTR domain is devoid of tyrosine, tryptophan, and phenylalanine.

The precise cleavage sites made by papain were not identified in this study. Nevertheless, based on the predicted primary sequence of TS, on the removal of the leader peptide (24), and on the presence of at least one glycosylation site (17), cleavage at the beginning of the LTR would result in a 70-kDa fragment. TS-pap is glycosylated, since it binds to concanavalin A, and is weakly, or not recognized by anti-LTR antibodies. On the other hand, TS-pap reacts with antibodies to the amino terminus of the native TS. Therefore, TS-pap may correspond to most of, or the entire, amino-terminal domain of TS.

The present results also show that the heterogeneity of the native TS bands in SDS-PAGE must be associated with variations in size of the C-terminal region containing LTRs. We have previously found that antibodies to the antigenic determinant of the carbohydrate portion of the glycosylphosphatidylinositol anchor reveal several bands of TS in immunoblots (1), showing that the differences in TS size are not likely to result from proteolysis within the LTR domain. Most likely, the heterogeneity observed in TS purified from parasite cultures reflects the expression of several genes encoding products containing variable numbers of repeats, but very similar amino-terminal domains.

As shown here, the presence of LTR promotes only TS oligomerization. It does not contribute to stability of the amino-terminal domain, or to an increased enzymatic activity toward membrane bound substrates. These findings indicate that the LTRs and the rest of the enzyme form entirely independent structural and functional domains. Moreover, the presence of LTRs is not required for enzyme assembly during its biosyn-

thesis, at least in *E. coli*. Conversely, based on the deletion experiments, we show that the 43 amino acids preceding the LTRs are required for TS activity. These amino acids, defined by deletions of p4D and p10A, might be important to maintain the structure, or to promote the correct folding of the catalytic domain of TS. These experiments also show that the proteolysis of recombinant TS in *E. coli* is independent of size and presence of LTRs. The sites of proteolysis are in the amino-terminal domain, because: (*a*) the deletions containing different numbers of repeats, or lacking the repeats, yield in SDS-PAGE multiple bands with the same pattern of gradually diminishing molecular weights; and (*b*) the antibodies to the amino-terminal peptide of TS reveal on immunoblots only the upper molecular weight bands of each deletion (not shown). The fact that a single 70-kDa band is obtained by papain digestion argues that this type of proteolysis does not occur in *T. cruzi*.

Trypomastigote TS is attached to the parasite membrane via a glycosylphosphatidylinositol anchor in the carboxyl terminus (15). We speculate that the LTR domain might link this membrane anchor to the active and globular domain, forming a stalk-like structure that would provide a long separation of the active site from the membrane. The significance for this separation, and of TS oligomerization, is still unknown. It could have a unique role in the sialylation of the bloodstream trypomastigotes of *T. cruzi*, since LTRs are not present in the TS enzymes expressed by *T. cruzi* and *T. brucei* developing in their insect vectors (16). For example, it could be important for the physical interaction of trypomastigote forms with the sialic acid donors during cell invasion (30–32). It could also be required for the sialylation of trypomastigote acceptor molecules that are larger than the sialic acid acceptors of other parasite forms (8, 33).

The present work also shows that the 70-kDa fragment, lacking LTRs, elicits antibodies that can inhibit TS. Most of the antibodies directed to TS obtained in natural *T. cruzi* infections, or by immunization with purified TS, appear to recognize the LTRs, and were shown to be ineffective at inhibiting TS (25). The inhibition achieved with anti-TS-pap is not due to enzyme precipitation or aggregation following the interaction with antibodies. Monovalent Fab fragments and whole IgG inhibit equally well the activity of multimeric, monomeric, or immobilized TS. Therefore, the inhibition may reflect a direct binding of antibodies to, or to epitopes near, the catalytic site of the enzyme, thus preventing TS interaction with the substrates. Alternatively, the antibodies could inhibit TS activity indirectly by binding to a site not involved in the catalysis and promoting enzyme inactivation. The anti-TS-pap antibodies also inhibit sialylation of live parasites. Under this condition, sialylation might be favored by the proximity of the enzyme with the acceptors on the parasite surface, explaining why the antibody inhibition can be overcome by increasing the concentration of sialic acid donors.

In summary, the identification of a proteolytical fragment conserving full enzymatic activity now permits the development of more powerful inhibitors of TS, such as specific antibodies. It should also facilitate structural studies, including the crystallization of the papain-generated fragment, or of a recombinant TS lacking repeats. These studies should allow us to understand more precisely the mechanism of action, and the role, of this unique enzyme in the life cycle of the parasite.

Acknowledgment—We thank Dr. Victor Nussenzweig for many suggestions and for critically reading the manuscript.

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