Two trans-sialidase forms with different sialic acid transfer and sialidase activities from *Trypanosoma congolense* *

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SUMMARY

Trypanosomes express an enzyme called trans-sialidase (TS), which enables the parasites to transfer sialic acids from the environment onto trypanosomal surface molecules. Here we describe the purification and characterization of two TS forms from the African trypanosome *Trypanosoma congolense*. The purification of the two TS forms using a combination of anion exchange chromatography, isoelectric focusing, gel filtration and, subsequently, antibody affinity chromatography resulted, in both cases, in the isolation of a 90 kDa monomer on SDS-PAGE which was identified as trans-sialidase using micro-sequencing. Monoclonal antibody 7/23, which bound and partially inhibited TS activity, was found in both cases to bind to a 90 kDa protein. Both TS forms possessed sialidase and transfer activity, but markedly differed in their activity ratios. The TS form with a high transfer to sialidase activity ratio, referred to as TS-form 1, possessed a pI of pH 4-5 and a molecular weight of 350-600 kDa. In contrast, the form with a low transfer to sialidase activity ratio, referred to as TS-form 2, exhibited a pI of pH 5-6.5 and a molecular weight of 130-180 kDa. Both TS forms were not significantly inhibited by known sialidase inhibitors and revealed no significant differences in donor and acceptor substrate specificities, however TS-form 1 utilized various acceptor substrates with a higher catalytic efficiency. Interestingly, GARP, the surface glycoprotein, was co-purified with TS-form 1 suggesting an association between both proteins.
INTRODUCTION

The flagellated protozoa, trypanosomes, the agents of several diseases, express a unique type of glycosyltransferase, called trans-sialidase (TS)\(^1\), which is believed to play an important role in maintaining pathogenicity of the parasites (1;2). Unlike typical sialyltransferases, which require CMP-activated sialic acid as the monosaccharide donor (3), TS catalyses the transfer of, preferably, \(\alpha2,3\)-carbohydrate-linked sialic acids to another carbohydrate forming a new \(\alpha2,3\)-glycosidic linkage to galactose or \(N\)-acetylgalactosamine. In the absence of an appropriate acceptor TS acts like a sialidase (SA), similar to viral, bacterial, mammalian and trypanosomal SA, hydrolyzing glycosidically linked sialic acids (1;2).

TS was first described in the bloodstream form of the American trypanosome \textit{Trypanosoma cruzi} (\textit{T.cr.}) (4), the pathogen of Chagas disease, afflicting millions of people in Latin America. TS has also been reported to occur in the procyclic insect forms of the African trypanosomes \textit{Trypanosoma brucei gambiense} and \textit{Trypanosoma brucei rhodesiense} (5), which are the cause of human sleeping sickness. Furthermore, TS has been found in procyclic forms of other African trypanosomes, such as \textit{Trypanosoma brucei brucei} (\textit{T.b.br.}) (6;7) and \textit{Trypanosoma congolense} (\textit{T.con.}) (5). These parasites are the agents of Nagana, the trypanosomiasis in African ruminants.

Trypanosomes are unable to synthesize sialic acids, instead they utilize TS to transfer sialic acid from the environment onto trypanosomal surface molecules. In the case of \textit{T.cr.}, TS is employed to acquire sialic acid from mammalian host glycoconjugates to sialylate mucin-like acceptor molecules in the parasite plasma membrane (8). Furthermore, TS sialylates host cell glycoconjugates to generate receptors, which are used for parasite adherence and subsequent entry into host cells (2). In the African species \textit{T.b.br.} and \textit{T.con.}, where TS is only expressed in the procyclic insect stage, the enzyme is used to sialylate the major cell surface glycoprotein of the parasites (e.g. PARP, GARP) in the vector (tsetse fly). Thus, a negatively
charged glycocalyx is formed, which is believed to protect the parasites from digestive conditions in the fly gut, or from immuno-competent substances present in the fly’s blood meal, and may enable them to interact with epithelial cells (6;9). Additionally, it has recently been reported, that T.cr.TS itself directs neuronal differentiation in PC12 cells (10), stimulates IL-6 secretion from normal human endothelial cells (11), as well as potentiating T cell activation through antigen-presenting cells (12).

Investigating TS has become increasingly attractive over the last years not only because of its involvement in trypanosomal pathogenicity, but also because of its biotechnological importance. That is, TS is a unique enzyme that, because of its ability to transfer Neu5Ac in a stereo- and regio-specific manner, can be utilized to synthesize a variety of biologically relevant structures of the type Neu5Acα2,3Galβ1-R (13;14).

To date, only two trypanosomal TS have been studied in detail, the American T.cr.TS (15-17) and the African T.b.br.TS (6;7;18), with different genes encoding T.cr.TS (19-21) and T.b.br.TS (22) being identified and analyzed. Furthermore, the SA expressed by Trypanosoma rangeli (T.r.), a non-pathogenic relative of T.cr., has been isolated and characterized biochemically (23) and genetically (24). Although the crystal structure of T. cruzi trans-sialidase has recently been published (25), a number of questions concerning the exact TS transfer mechanism remain un-answered. Since native and recombinant enzymes can differ in their glycosylation, antibody specificity and biochemical properties, it is important that the native enzyme be purified and characterized, with the subsequent aim of obtaining sequence information. This is especially important, as several genes encoding TS/SA enzymes, or even silent genes may exist in trypanosomes, as has been shown for T.cr. (26). Here we describe the purification and characterization of two TS forms from the African trypanosome T.con. and their identification using micro-sequencing. Moreover, we report on the production of monoclonal antibodies raised against both enzyme forms and their subsequent use in purification. Additionally, we present characterization studies which reveal significant
differences between both TS forms concerning their transfer to SA ratios and catalytic efficiencies using various acceptor substrates.

EXPERIMENTAL PROCEDURES

Materials

Unless otherwise stated analytical grade reagents from Sigma (Deisenhofen, Germany), Merck (Darmstadt, Germany), ICN (Eschwege, Germany) and Roche Diagnostics GmbH (Mannheim, Germany) were used throughout this study. Galβ1,4-[14C]GlcNAc was purchased from Hartmann Analytic GmbH (Braunschweig, Germany). Materials for chromatography including Q-Sepharose FF and Sephadex G150 SF were obtained from Pharmacia (Freiburg, Germany).

Substances

2′(4-methylumbelliferyl)lactoside (MULac) was provided by Dr T. Yoshino (Tokyo, Japan), 4-amino-Neu2en5Ac and 4-guanidino-Neu2en5Ac by Dr M. von Itzstein (Gold Coast, Australia), suramin was a gift from Dr P. Nickel (Bonn, Germany) and recombinant T.cr.TS and T.b.br.TS were a gift from Dr A.C.C. Frasch (Buenos Aires, Argentina). Neu5Acα2,3-lactose (α2,3-SL) and Neu5Acα2,6-lactose were isolated from cow colostrum according to Veh et al. (1981) (27). Neu5Acα2,3-N-acetyl-lactosamine was purchased from Dextra-Laboratories (Reading, UK) and fetuin from ICN. Sialyl-oligosaccharides from bovine and human milk, as well as glycomacropeptide and apolactoferrin were provided by Numico Research (Friedrichsdorf, Germany). Sialyl-Lewis\(^\wedge\)\(^\wedge\), N-acetyllactosamine, lacto-N-biose I, lacto-N-neotetraose, lacto-N-tetraose, lactose, lactitol, mannose, galactose, glucose, maltose,
galactose-α1,4-galactose and Neu5Ac were obtained from Calbiochem-Novabiochem GmbH (Bad Soden, Germany). Chondroitin sulfate A, heparan sulfate, dextran sulfate, heparin (high and low molecular weight), Neu5Ac, Neu2en5Ac, 2′(4-methylumbelliferyl)galactoside (MUGal) and N-(4-nitrophenyl)oxamic acid were purchased from Sigma.

**Antibodies**

Antiserum to *T.cr.*TS was generously provided by Dr I. Marchal (Lille, France). Anti-*T.con.* procyclin (GARP) mAb was purchased from Cedarlane (Toronto, Canada). Horseradish peroxidase-conjugated affinity-pure donkey anti-rabbit IgG antibody was from Dianova (Hamburg, Germany). Peroxidase-conjugated anti-mouse IgG antibody and biotin-conjugated anti-mouse IgG3 antibody from Southern Biotechnology Associates Inc., USA was purchased from Dunn Labortechnik GmbH (Asbach, Germany). Peroxidase-conjugated streptavidin was purchased from Roche (Mannheim, Germany).

**Cultivation**

Procyclic culture forms of *T.con.* (STIB 249; kindly provided by Dr Retro Brun from the Swiss Tropical Institute, Basel, Switzerland) were cultivated axenically in SM/SDM 79 medium (28), containing 10 % fetal calf serum (FCS, PAA Laboratories, Austria) and 0.001 % hemin. After three to four days of cultivation, the trypanosomes were transferred into new SM/SDM 79 medium without FCS and hemin. Following a further three days, the culture supernatant was harvested *via* centrifugation.
Assays

For all enzyme assays the formation of product was linear with respect to time and protein amount. In all activity tests controls were performed in the absence of enzyme sample or using heat-inactivated enzyme. For fluorescence detection a 96-well-plate fluorimeter (Fluorolite 1000, Dynatech Laboratories, U.S.A.) was used.

SA activity was routinely tested in the presence of 1 mM MU Neu5Ac in 20 mM Bis/Tris buffer, pH 7.0 (29). The reaction mixture was incubated for 120 min at 37 °C in black 96-well-plates (Microfluor, Dynex, U.S.A.). By the addition of 0.08 M glycine/NaOH buffer, pH 10, the reaction was terminated and the fluorescence of MU released measured immediately at an excitation and emission wavelength of 365 nm and 450 nm, respectively. The instrument was calibrated with MU standard solutions. One unit of SA activity equals one µmol of MU released per minute, which is equivalent to 1 µmol of sialic acid released per minute.

TS activity was routinely tested using the non-radioactive assay described by Schrader et al.2. Briefly, TS activity was monitored by incubating 25 µl of enzyme solution in 50 mM Bis/Tris buffer, pH 7.0, containing 1 mM α2,3-SL as the donor and 0.5 mM MUGal as the acceptor in a final volume of 50 µl at 37°C for 2 h. The reaction was terminated by the addition of ice cold water and, subsequently, applied to mini-columns containing Q-Sepharose FF. After washing, the sialylated product was eluted with 1 M HCl, hydrolyzed at 95°C for 45 min and cooled on ice. The samples were neutralized, adjusted to pH 10 and MU released was measured as stated above. One unit of TS activity equals one µmol of MU released per minute, which is equivalent to 1 µmol of sialic acid transferred per minute. During the course of this study the TS test described above was modified by applying the assay principle to a 96-well-plate format. Because of its enhanced throughput all TS tests for mAb screening, as well as kinetic experiments were performed using the 96-well-plate assay (Schrader et al.)2. Protein concentration was determined using either the BCA protein assay kit from Pierce (Cologne, Germany) or the Bio-Rad protein assay (30) from Bio-Rad (Munich, Germany), as
described by the manufacturer. All assays were performed in 96-well-plates employing BSA as the standard, and photometric determination were performed using a 96-well-plate photometer (Tecan Sunrise, Tecan Deutschland GmbH).

Total amounts of bound sialic acid were measured by the micro-adaption of the orcinol/Fe$^{3+}$/HCl reaction (31).

**Separation and purification of the two TS forms**

The crude culture supernatant was filtered (1.2 µm membrane, Millipore GmbH, Schwalbach, Germany) and concentrated in an Amicon ultrafiltration device (MWCO 20 kDa, Sartorius, Göttingen, Germany) prior to undergoing chromatography. Following all purification steps, fractions were concentrated with the aid of the following devices depending on the volume: Centrex UF-2 (MWCO 30 kDa, Schleicher&Suell, Dassel, Germany), Centricon Plus-20 (MWCO 30 kDa, Millipore, Eschborn, Germany) or an Amicon ultrafiltration device (MWCO 20 kDa). Unless otherwise stated all purification experiments were performed at 4°C.

The separation of two major TS activity peaks was provided by chromatography on Q-Sepharose FF. The concentrated culture supernatant was applied to a column (2 x 20 cm) of Q-Sepharose FF, equilibrated with 20 mM Bis/Tris buffer, pH 7.0, at a flow rate of 0.6 ml/min. Following extensive washing bound TS activities were eluted using a 600 ml continuous NaCl gradient (0 to 0.8 M) in 20 mM Bis/Tris buffer, pH 7.0. Fractions of 6 ml were collected and analyzed for transfer and SA activity. A larger Q-Sepharose column could not be employed due to poor separation of the two TS forms. Therefore, several Q-Sepharose runs were performed using the column size stated above, with separated TS-form 1 and TS-form 2 following each run being combined and further purified individually by isoelectric focusing (IEF).
Isoelectric focusing was carried out in a 16 ml Rotor cell (Rotofor Preparative Isoelectric Focusing Cell, Biorad) using ampholytes which provided a pH range between pH 4-6 (Biolyte pH 4-6, Biorad). The buffer contained in the collected fractions was immediately exchanged, fractions concentrated and activity determined. Active fractions were pooled and further purified by gel filtration chromatography.

Each individual TS form was applied to a column (1 x 90 cm) of Sephadex-G150 SF equilibrated with 20 mM Bis/Tris buffer, pH 7.0, containing 100 mM NaCl at a flow rate of 0.125 ml/min, which had been calibrated using the high molecular weight calibration kit (Pharmacia, Freiburg, Germany) as described by the manufacturer. Fractions of 500 µl were collected and analyzed for activity. Active fractions were pooled, concentrated and analyzed by SDS-PAGE.

T. congolense TS antibody production, detection, isolation and isotyping

BALB/c (H-2^d) mice, obtained from Harlan/Winkelmann (Borchen, Germany) and reared under conventional conditions, were used for the production of monoclonal antibodies (mAb). Female BALB/c mice 6 weeks of age were injected three times intraperitoneally with 25 µg of the partially purified TS forms adsorbed to 2 mg Al(OH)_3 (Imject® Alum, Pierce, Rockford, USA). Three days after the last injection spleen cells of one mouse was fused with non-secretor Ag8.653 myeloma cells (32) by the conventional polyethylene glycol-mediated fusion technique (33). After fusion, cells were plated in 288 wells of 24-well hybridoma plates (Greiner, Nürtingen, Germany) in RPMI 1640 supplemented with 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine. The medium was further supplemented with 10 % conditioned medium from the J774 cell line. Wells containing antigen-specific IgG-secreting hybridomas were identified via
ELISA using mouse-IgG-specific antiserum and an enzyme immunoassay (TS activity binding assay). Clones in positive wells were subcloned and reanalyzed.

The TS activity-binding assay was performed using Dynabeads M-450 Goat anti-Mouse IgG (Dynal, Hamburg, Germany) and a Magnetic Particle Concentrator for micro-centrifuge tubes (Dynal MPC-S, Dynal, Hamburg, Germany). Briefly, 200 µl of beads were washed twice with PBS buffer (phosphate-buffered saline) as described by the manufacturer. Following incubation with putative anti-\(T.con\).TS mAb at room temperature for 1 h the beads were washed again 5 times with 900 µl PBS buffer and further incubated with 200 µl of TS-containing solution at 4 °C for 1 h. The incubation was terminated by transferring the supernatant to a new cap and the beads were subsequently washed 5 times with 900 µl PBS buffer. In the supernatant, as well as on the beads, TS activity was determined and compared to a control provided by binding non-TS-specific IgG2b antibodies to the Dynabeads. The reduction of TS activity in the TS-containing solution in comparison to the control, as well as the activity detected on the beads, enabled the determination of a clone producing anti-\(T.con\).TS specific mAb.

Purification of the anti-\(T.con\).TS mAb from hybridoma supernatant was performed by affinity chromatography using rProtein A-Sepharose FF (Pharmacia, Freiburg, Germany) according to the manufacturer. The antibody concentration of the eluted preparation was determined with an enzyme immunoassay for the quantitative determination of mouse IgG (Roche Diagnostics GmbH, Mannheim, Germany). Immunoglobulin subclass determination was performed with the “Hybridoma subtyping Kit” (Roche Diagnostics GmbH, Mannheim, Germany).

**Immonoaffinity chromatography**

Purified anti-\(T.con\).TS (mAb 7/23, 24 mg) were incubated for 2 h with rProtein A-Sepharose FF (5 ml) and equilibrated with binding buffer (20 mM Na2HPO4, NaH2PO4, pH
Following washing with 70 ml of binding buffer, the matrix was further washed with cross-linker buffer (0.2 M triethylamine, pH 8.5) and, subsequently, incubated with 10 ml of cross-linker reagent (20 mM dimethyl pimelimidate in cross-linker buffer) at room temperature for 1 h. The cross-linking reaction was terminated by washing the column with 70 ml of 0.2 M ethanolamine, pH 9, followed by 70 ml of binding buffer and 70 ml of Na citrate buffer, pH 3.0. A flow rate of 1-1.25 ml/min was used at all stages of matrix preparation. The column was washed with binding buffer prior to immunoaffinity chromatography.

The immunoaffinity matrix equilibrated in binding buffer was incubated with the partially purified TS forms overnight at 4 °C. Unbound protein was removed by washing with binding buffer. TS activity was eluted stepwise with 70 ml of 100 mM Bis/Tris, pH 7.0; 100 mM Bis/Tris, pH 7.0, containing 1 M NaCl; 20 mM Na citrate, pH 4.5; and 20 mM Na citrate, pH 3.0; at a flow rate of 1-1.25 ml/min. The last fraction was immediately neutralized prior to TS activity determination.

**Micro-sequencing**

In-gel trypsin digestion and mass spectrometric analysis of peptides were performed by WITA GmbH, Berlin, Germany.

**Kinetic studies**

Kinetic data for both enzyme forms were obtained by making suitable modifications to the standard SA and TS assay. To measure transfer activity various concentrations of the acceptor substrates MUGal and MULac (0-2 mM) were used at a constant donor substrate (α2,3-SL) concentration of 1 mM. The kinetic parameters for α2,3-SL were obtained by varying α2,3-SL concentrations (0-3 mM) at a constant concentration of MUGal (0.5 mM). Various
concentrations of MUNeu5Ac (0-0.2 mM) were used to obtain kinetic data for SA activity. Apparent $V_{\text{max}}$ and apparent $K_{m}$ values were determined by non-linear regression using the computer program Enzfitter from Elsevier Biosoft. Additionally, the temperature and pH optima of both forms were investigated in the range of 5–55 °C and pH 4.5–10.5, respectively.

**Donor and acceptor substrate specificities and inhibitor studies**

A number of glycoconjugates, as well as mono- and oligosaccharides (Table 4) were assayed as potential donors using the TS assay described. Known viral and bacterial SA inhibitors, as well as salts (NaCl, KCl), cations (20 mM Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$; 5 mM Cu$^{2+}$, Zn$^{2+}$, Fe$^{2+}$, Co$^{2+}$) and other compounds including anti-*T.con.*TS mAb (0-20 µg/ml) were assayed for their ability to inhibit TS activity using essentially the standard TS assay described, except additives were pre-incubated in the assay mixture for 30 min at room temperature prior to starting the reaction. Potential TS acceptors (Table 4) were assayed in a similar manner to that described for potential inhibitors.

**SDS-Polyacrylamide Gel Electrophoresis (PAGE) and immunoblot analyses**

SDS-PAGE was performed according to Laemmli (34) in a Mini Protean II Cell (Bio-Rad, Munich, Germany) in the presence of a reducing agent (dithiotreitol). Polyacrylamide gels usually consisted of 8% resolving and 4% stacking gel, with the exception of gels used for immunoblot analyses using anti-*T.con.* GARP mAb, where the resolving gel was 12%. As molecular weight markers pre-stained SDS-PAGE standards from Bio-Rad (for immunoblotting) or SDS-PAGE Marker High Range from Sigma were applied (for staining). Gels were subsequently stained with either silver (35) or Coomassie brilliant blue R-250 (36). For immunoblot analyses, after SDS-PAGE, proteins were transferred onto a nitrocellulose
membrane (Schleicher and Schuell, Dassel, Germany) using a Mini-V 8-10 Blot Module (Life Technologies, Eggenstein, Germany) as described by the manufacturer.

For immunodetection, blots were blocked overnight at 4 °C in TBS (tris-buffered saline) buffer containing 0.05 % Tween 20 (TBST) and 5 % skim milk (blocking buffer), washed 6 times with TBST for 5 min and then incubated for either 24 h at 4 °C (antiserum to T.cr.TS, dilution 1:5000) or 1 h at room temperature (anti-T.con.TS mAb, dilution 1:3000 or anti-T.con. procyclin (GARP) mAb, dilution 1:1000) in blocking buffer solution containing the appropriate primary antibody. Following incubation, the blots were washed again 6 times with TBST buffer and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:10000). After washing 6 times for 10 min with TBST buffer, bands were visualized using the ECL immunoblotting detection reagent kit (Amersham, Braunschweig, Germany) as described by the manufacturer.

**Immunoprecipitation**

Immunoprecipitation was performed in a similar manner to that described for the TS activity-binding assay. Briefly, 30 µl of Dynabeads M-450 Goat anti-mouse IgG were washed twice with PBS buffer and incubated with either 30 µl of PBS buffer (control) or 30 µl of anti-T.con.TS mAb (mAb7/23) at room temperature for 1 h. Following incubation the beads were washed again 5 times with 900 µl PBS buffer and further incubated at 4 °C for 1 h with 200 µl of TS containing concentrated culture supernatant. The beads were washed 5 times with 900 µl PBS buffer, subsequently boiled in SDS-PAGE sample buffer (containing SDS and dithiothreitol) at 95 °C for 5 min and analyzed by SDS-PAGE and immunoblotting as described above. The detection of *T. conglobense* TS was achieved as described above. The detection of GARP was performed using anti-T.con. GARP mAb as the primary (dilution 1:1000) and biotinylated anti-mouse IgG3 mAb (dilution 1:4000) as the secondary antibody to
avoid cross-reactivity with the light chain of anti-\textit{T.con}.TS mAb. Following washing with TBST buffer, blots were incubated with streptavidin (dilution 1:5000) for 45 min at room temperature. After washing again with TBST buffer GARP was visualized using the ECL system as stated above.

RESULTS

\textit{Cultivation}

In axenic cultures the trypanosomes grow as flagellate forms called epimastigotes, which correspond to the forms found in the guts of blood-sucking vectors (procyclic or insect forms) (37). In contrast to the African trypanosome \textit{T.b.br.}, where TS activity was found to be only membrane-bound (6;7), TS activity in procyclic forms of \textit{T.con.} was detected both, in the culture supernatant and membrane-bound (5). During the cultivation of procyclic forms of \textit{T.con.} in FCS/hemin-containing media the cell number increased during 3 to 4 days from $1 \times 10^6$ cells/ml to $7 \times 10^6$ cells/ml, whereas the parasites died within 3 to 4 days when cultivated in FCS/hemin-free media. However, in both cases the parasites did produce enzymatic activity during cultivation. When cultivating the parasites in FCS/hemin-containing media 84 \% of transfer activity and 97 \% of SA activity were measured in the culture supernatant. In contrast, cultivation of the trypanosomes in FCS/hemin-free media resulted in only 25 \% of transfer activity and 60 \% of SA activity in the culture supernatant. In both cases the residual activity was bound to the cell pellet. Additionally, transfer and SA activity measured in the FCS/hemin-containing and FCS/hemin-free media were found to possess similar characteristics (data not shown). However, in the culture supernatant derived from the cultivation without FCS/hemin the specific enzymatic activity was 4 times higher than that seen in the culture supernatant obtained from FCS/hemin-containing media. Therefore, and
because of the quenching effect of hemin on enzyme activity assays, parasites were cultivated in FCS/hemin-free media.

Separation and purification of two TS forms from T. congolense

The separation and partial purification of the two TS forms, summarized in Table 1, was achieved by employing a combination of anion exchange chromatography, isoelectric focusing, gel filtration and, subsequently, immunoaffinity chromatography. Ion exchange chromatography was chosen as a first step in the purification cascade, mainly because of its high capacity, but also due to its ability to sufficiently separate the two enzyme forms (Fig. 1A). Activity-positive fractions eluting at a salt concentration higher than 0.3 M were combined and referred to as TS-form 1, whereas TS active fractions eluting at a salt concentration below 0.3 M were combined and called TS-form 2. As can be seen in Table 1, following Q-Sepharose a difference in the transfer to SA activity ratios for both TS forms could already be observed. That is, TS-form 1 and TS-form 2 exhibited a transfer to SA activity ratio of 17 and 2.4, respectively. Even though no significant enrichment of transfer and SA activity was obtained, this first chromatography step provided effective separation of TS-form 1 and TS-form 2.

The separation of both activity peaks following chromatography on Q-Sepharose was also observed when culture supernatant derived from FCS/hemin-containing media was used. This indicates that the described TS forms exist under different cultivation conditions and, therefore, represent major trans-sialidase forms.

Following ion exchange chromatography each form was treated independently. Further purification of TS-form 2 was achieved by IEF (Table 1), however IEF was not particularly effective at further enriching TS-form 1, instead only leading to a loss of TS activity, and was
therefore not used in the purification of TS-form 1. The isoelectric points obtained for TS-form 1 and 2 were found to be pH 4-5 and pH 5-6.5, respectively (Fig. 1C).

The two TS forms were further purified independently using gel filtration on Sephadex-G150 SF. As shown in Fig. 1B and Fig. 1D, the molecular weights of TS-form 1 and 2 were found to be 350-600 and 130-180 kDa, respectively. The transfer activities of TS-form 1 and form 2 were purified by 30- and 150-fold, respectively, but interestingly in the case of TS-form 1 only very low amounts of SA activity could be detected (Table 1). This shows that after complete separation and partial purification of two TS forms both possessed very different transfer to SA activity ratios (8000 for TS-form 1; 45 for TS-form 2). The SDS-gel in Fig. 2 depicts the protein pattern during the various stages of purification. For both TS forms a clear enrichment of a 90 kDa band on SDS-PAGE under reduced conditions was observed (Fig. 2, lane 1-3, 5-7). However, a number of contaminating proteins still remained. Therefore a further specific purification step was sort.

Production of anti-T. congolense TS monoclonal antibodies, and immunoblot analyses

Both partially purified TS forms were used for mAb production, with a combination of ELISA and the TS binding test, described under Experimental Procedures, being employed for the detection of TS-specific antibodies. Clone 7/23 was found, using the TS binding test, to reduce TS activity in a TS containing sample by 75 %. TS activity present on the Dynabeads was also detected for clone 7/23. Monoclonal Ab 7/23 was found to belong to the subclass IgG2b. Additionally, the V region of mAb 7/23 sequenced was analyzed (V_L sequence: AY198310; V_H sequence: AY198309). After resolving the concentrated supernatant, as well as the two separated TS forms on SDS-PAGE, immunoblotting with anti-T.con.TS mAb (mAb 7/23) led to the staining of a single protein band at about 90 kDa, which had been shown to be enriched after purification of the two TS forms (Fig. 2 and 3A).
contrast, anti-\textit{T.\,con.}TS mAb did not cross react with the 70 kDa band representing recombinant \textit{T.\,cr.}TS and the 80 kDa band representing \textit{T.\,br.}TS (Fig. 3B). Furthermore, immunoblotting analysis revealed that employing anti-\textit{T.\,cr.}TS antiserum a 70 kDa protein band representing recombinant \textit{T.\,cr.}TS was detected, whereas the 90 kDa protein band of the two \textit{T.\,con.}TS forms was not (Fig. 3C). These results are very similar to that seen for antiserum and mAb raised against \textit{T.\,cr.}TS which showed no cross-reactivity with \textit{T.\,br.}TS (6).

\textit{Immunoaffinity chromatography}

Immunoaffinity chromatography employing the anti-\textit{T.\,con.}TS mAb 7/23 was used for further purification of the two TS forms. For both forms the majority of TS activity was eluted using 20 mM Na citrate buffer, pH 3.0. The transfer activities of TS-form 1 and TS-form 2 were enriched by 1071- and 4200-fold, respectively, whereas the SA activity which could only be measured for TS-form 2, was enriched by approximately 28-fold. The two purified TS forms migrated with an apparent molecular weight of 90 kDa on SDS-PAGE (Fig. 2, lane 4 and 8). However, TS-form 1, which was purified to apparent homogeneity, provided a far greater recovery and was therefore used for micro-sequencing.

\textit{Micro-sequencing}

Following the purification scheme outlined in Table 1 the 90 kDa protein of TS-form 1 (Fig. 2, indicated with an arrow) was excised following SDS-PAGE and peptides were analyzed after in-gel trypsin digestion. Subsequently, a peptide with the amino acid sequence VVDPTVVAK, in which the mass data showed a high similarity with the mass data of a peptide from \textit{T.\,br.}TS, was obtained. Additionally, a BLAST database search revealed that the observed peptide showed 100\% sequence identity with a peptide from \textit{T.\,br.}TS (aa 193–
as well as with a peptide from one of two \textit{T.con}.TS gene sequences (\textit{T.con}.TS1) obtained using a PCR-based cloning approach\(^3\) (Table 2). With that, the 90 kDa protein of TS-form 1, as well as indirectly through immunoblot analysis the 90 kDa protein of TS-form 2, were identified as a TS. In contrast, the analyzed peptide did not show 100\% sequence identity with peptides from \textit{T.r}.SA, \textit{T.cr}.TS and a second \textit{T.con}.TS sequence\(^3\) (\textit{T.con}.TS2) (Table 2).

\textit{Immunoblotting with anti-\textit{T.congolense} GARP monoclonal antibodies}

GARP is the major surface glycoprotein of procyclic forms of \textit{T.con}. which is bound to the parasite membrane by a GPI (glycosylphosphatidylinositol) anchor (38). It has been shown that GARP acts as an excellent acceptor molecule for \textit{T.con}.TS and is therefore believed to be the major natural sialic acid acceptor on the surface of procyclic \textit{T.con}. (5). Therefore, and because of the differences in the molecular weight of both TS forms, the possibility that GARP might interact with TS was investigated.

Concentrated culture supernatant, and TS forms at various stages of purification, were analyzed by immunoblotting with anti-\textit{T.con}. GARP mAb. Under reducing conditions GARP migrates as a 28-32 kDa protein band on SDS-PAGE (39) and as can be seen in Fig. 4 a protein band at about 30 kDa was detected. Surprisingly, GARP was only detected in the concentrated culture supernatant and in two purification stages of the higher molecular weight TS form, TS-form 1, with the intensity of the signal increasing proportionally with specific TS activity (Fig. 4). This points towards an association between TS-form 1 and GARP under the mild purification conditions used in Q-Sepharose FF and Sephadex-G150 SF chromatography. However, immunoblot analysis of the immunoaffinity-purified TS-form 1 was unable to detect GARP (Fig. 4), indicating that the final purification step disrupted the possible association between GARP and TS-form 1. This may have been due to the intensive
washing steps (1 M NaCl and 20 mM Na citrate, pH 4.5) used during immunoaffinity chromatography. Interestingly, no bands reacting with anti-T.con GARP mAb were observed in all TS-form 2 samples investigated (Fig. 4). Nevertheless, the association of TS-form 1 with GARP must be specific and strong, because anti-T.con.TS mAb (mAb7/23) was able to co-immunoprecipitate TS and GARP from a concentrated culture supernatant sample (Fig. 5). The 90 kDa protein band visualized following immunoprecipitation represents TS (Fig. 5A, lane 2), and the 32 kDa protein band represents GARP (Fig. 5B, lane 2). The protein band at 55 kDa represents the heavy chain of the T.con.TS mAb that reacted with the secondary antibody, horseradish peroxidase-conjugated anti-mouse IgG (Fig. 5A, lane 2).

**Kinetic studies**

Kinetic studies were performed on the two TS forms following their separation and partial purification using ion exchange chromatography and gel filtration. Various donor and acceptor substrates, generally employed to determine SA and transfer activities, were utilized. As can be seen in Table 3, when using MUGal as the acceptor, TS-form 1 and 2 bind the donor substrate α2,3-SL with very similar affinities. The $K_m$ values for α2,3-SL of 0.3 mM and 0.2 mM for TS-form 1 and 2, respectively, are in good agreement with that reported for native T.b.br.TS ($K_m$: 0.5 mM) (7). Both TS forms were found to prefer MULac over MUGal as the acceptor, however, the catalytic efficiency (expressed as app. $V_{max}/K_m$) is three to four times higher for both acceptors in the case of TS-form 1. TS-form 2 was also found to bind MUNeu5Ac, the donor substrate routinely used to measure the hydrolyzing reaction, with high affinity. A $K_m$ value of 0.09 mM is very similar to that reported for the native T.b.br.TS ($K_m$: 0.16 mM) (7).

Kinetic parameters for the hydrolyzing reaction could not be determined in the case of TS-form 1, because of insufficient SA activity. Interestingly, in comparison to TS-form 1 which
possessed predominately transfer activity, TS-form 2 was found to hydrolyze sialic acid from MUNeu5Ac 5 times more efficiently than transferring sialic acid from the donor α2,3-SL to GalMU (Table 3). These results suggest that not only do the two TS forms consist of different transfer to SA activity ratios, but additionally that TS-form 2 hydrolyzes sialic acid more efficiently than TS-form 1. On the other hand TS-form 1, which is more efficient in transferring sialic acid, behaves similar to the previously reported native and recombinant *T.cr.* TS and *T.b.br.* TS (7;17;22;40).

Moreover, both TS-forms exhibited no differences in their pH and temperature optima, with a pH optimum of 7 and a temperature optimum of 37-40 °C being determined, similar to those reported for *T.b.br.* TS (6;7).

**Donor and acceptor substrate specificities**

A number of sialoglycoconjugates were tested as potential sialic acid donors for TS form 1 and 2 from *T. con.* (Table 4A). Both TS forms revealed no differences in their donor substrate specificities, with the exception of apolactoferrin. As has been previously observed for *T.cr.* TS (17;41), *T.b.br.* TS (7) and crude *T.con.* TS (5) both isolated *T.con.* TS forms preferably catalyze the transfer of α2,3-linked sialic acid (α2,3-SL and Neu5Acα2,3-N-acetyl-lactosamine). Sialic acids in α2,6 linkage also serve as reasonable donor substrates (Table 4A), which differs from the findings reported for *T.cr.* TS (41). However, the results are similar to those reported for *T.b.br.* TS which can also utilise α2,6-linked sialic acid, but at an lower rate in comparison to *T.con.* TS (7). However, as previously reported for the crude *T.con.* TS (5), fetuin served as a good donor substrate with a high transfer rate being observed for both *T.con.* TS forms. The presence of a fucose near the terminal galactose residue (sialyl-Lewis^x^) resulted in a decrease in *T.con.* TS activity. This has also been shown for *T.cr.* TS (17) and *T.b.br.* TS (6). Moreover, sialyl-oligosaccharides from bovine and human milk, as well as
the κ-casein glycomacropeptide, known to inhibit bacterial and viral adhesion (42), proved to be excellent sialic acid donors for both TS forms (Table 4A).

Various substrates were tested for their ability to act as sialic acid acceptors for *T. con.* TS (Table 4B). Both TS forms were found to possess a similar acceptor preference, however, some acceptor substrate specificity differences were observed between the two forms. In agreement with that reported for *T. cr.* TS (41), β1,4-linked galactose-containing substrates were better acceptors for both *T. con.* TS forms than β1,3-linked galactose-containing substrates (Table 4B). Lactose and its derivatives serve as good acceptor substrates. In agreement with earlier studies on *T. b. br.* TS (5;7), monosaccharides did not act as sialic acid acceptors for the *T. con.* TS forms, apart from a slight effect using galactose (Table 4B). Parodi *et al.* (1992) (20) reported that maltose and cellobiose can be sialylated by *T. cr.* TS, however, they did not serve as sialic acid acceptors for both *T. con.* TS forms, even at a concentration of 5 mM. Moreover, the lipooligosaccharides LNnT and LNT (lacto-N-tetraose and lacto-N-neotetraose), which prevent the adherence of bacteria to epithelial cells, showed good acceptor properties for both TS forms (Table 4B). This is of importance because the sialylation of these biological relevant structures increases their survival in blood serum (43).

However, for all acceptor substrates tested TS-form 1 was found to utilize the various acceptors at an about 2-fold higher rate. This mirrors the kinetic results summarized in Table 3, where it is shown that TS-form 1 utilized the various acceptors with a greater efficiency in comparison to TS-form 2.

*Inhibitor studies*

Several known viral and bacterial SA inhibitors (44) were tested for their ability to inhibit the SA and transfer activity of both *T. con.* TS forms. None of the compounds tested showed any significant inhibitory effect. Neu5Ac, Neu2en5Ac (a natural inhibitor of SA), 4-amino-
Neu2en5Ac, as well as the potent inhibitor of influenza virus SA (45), 4-guanidino-
Neu2en5Ac, exhibited no more than 20% inhibition of transfer activity for both TS forms at a
concentration of 2 mM. Interestingly, the SA activity of TS-form 2 was not inhibited by these
compounds at all concentrations tested.

In contrast, the synthetic SA inhibitor N-(4-nitrophenyl)oxamic acid inhibited SA activity by
25% at a concentration of 2 mM, but the transfer activity by only 5% and 10% for TS-form 1 and 2, respectively. Furthermore, the anti-malaria drug suramin which has previously been shown to be a strong inhibitor of ganglioside SA from human brain tissue (IC50 7 µg/ml) (46), exhibited a slight (17%) inhibitory effect on the SA activity of TS-form 2 at a concentration of 25 µg/ml, whereas the transfer activity of the two TS forms was not effected.

Anti-T.con. TS mAb was found to only slightly inhibit (20% at 100 µg/ml) the transfer activity of both enzyme forms and it had no effect on the SA activity of TS-form 2. Glycosaminoglycans, like heparan sulfate and chondroitin sulfate A, as well as dextran sulfate are known inhibitors of mammalian sialidases (47), but they neither inhibited the transfer activity of TS-form 1 and 2, nor the SA activity of TS-form 2 at a concentration of 25 mg/ml. The addition of 1 M NaCl or 1 M KCl resulted in the reduction of SA and transfer activities by greater than 50%, however, full activity could be restored after desalting. Moreover, the addition of 5 mM Co2+, Zn2+ and Fe2+ inhibited SA and transfer activities of both TS forms by 20-40%, whereas the addition of 20 mM Ca2+, Mg2+ and Mn2+ had no effect on SA and transfer activities of both TS forms. This confirms earlier findings that TS are not activated by Ca2+ (5;18) as opposed to most viral and bacterial SA that require Ca2+ for full activity (48).

DISCUSSION

Apart from TS expressed in trypanosomes, TS activity has also been reported in
Endotrypanum species (49), in Corynebacterium diphtheriae (50), and in human plasma (51).
However, only trypanosomal TS from the American trypanosome *T.cr.* and the African trypanosome *T.b.br.*, have been studied in detail. In order to expand our knowledge concerning this peculiar enzyme, we investigated the TS expressed from the African trypanosome *T.con.*. The fact that *T.con.* produces a soluble TS simplified the purification, since the usage of detergents and other substances or enzymes enhancing solubilization, which often decrease enzyme activity, could be avoided. Nevertheless, different cultivation conditions were tried in order to reduce the content of contaminating protein in the culture supernatant.

Cultivation in FCS-containing media resulted in the majority of the enzyme activity being detected in the culture supernatant, whereas when cultivated in FCS-free media *T.con.* TS activity was found to be mainly membrane-associated. It is unclear if *T.con.* produces a membrane-bound TS, which is GPI-anchored and released into the medium due to the action of the parasites’ own proteases and phospholipases, or if two different *T.con.* TS species exist, one soluble and one membrane-bound, which are expressed depending on cultivation conditions. Since the GPI anchor has no influence on the enzymatic activity (52) and soluble proteins are easier to purify, we isolated *T.con.* TS from concentrated culture supernatant using FCS-free media because the specific activity of TS was markedly increased.

Employing different purification techniques two major peaks of TS activity were detected, both possessing SA and transfer activity, but differing in their transfer to SA activity ratio, as well as molecular weights and isoelectric points. Following SDS-PAGE, both purified *T.con.* TS forms appeared as a single 90 kDa protein band, indicating that they may be aggregates of the same monomer. TS-form 2, as observed by gel filtration, seems to form homodimers (~180 kDa), whereas TS-form 1 probably exists as oligomers (tetramer or higher), resulting in the high molecular weight observed by gel filtration (~350-600 kDa).

Similar findings have been reported for *T.cr.* and *T.b.br.* TS. That is, TS from *T.cr.* trypomastigotes generates multimeric aggregates (15;16), the monomers varying from 120 to...
180 kDa in the cell-derived, bloodstream forms and 90 kDa in the insect forms (53). In *T.b.br.* the TS was also found to be multimeric, with two major broad peaks of approximately 180 and approximately 660 kDa. SDS-PAGE under reduced conditions of those peaks revealed major bands between 60 and 80 kDa (6). Other studies reported the purification of a 67 kDa monomeric surface TS from *T.b.br.* (7;18). However, so far, the reason for the generation of multimeric aggregates of TS, as well as their composition, has not been studied.

In the case of *T.con.* we were able to prove, using micro-sequencing, that the isolated 90 kDa protein of TS-form 2 is indeed a TS. Interestingly, in both *T.con.*TS forms a 90 kDa protein band was detected using the anti-*T.con.*TS mAb 7/23, indicating that TS-form 2, which was not micro-sequenced, could also be identified as TS. However, further investigations are necessary to determine whether the 90 kDa proteins from both forms represent monomers with the same primary structure and perhaps different folding, or are products of different TS genes, since the anti-*T.con.*TS mAb 7/23 might recognize a common epitope or tertiary structure of both TS forms.

The finding that under mild conditions GARP is co-purified and co-immunoprecipitated with TS-form 1 seems plausible for a number of reasons. Firstly, GARP is the natural substrate of *T.con.*TS. Secondly, protein complexes concerning various SA (54) and protein-protein interactions involving other glutamic acid rich proteins, also referred to as GARP’s, have been reported (55;56). Moreover, the possibility that the trypanosomal GARP can mediate or facilitate the formation of oligomers of TS-form 1 is supported by the finding that the interaction between the cGMP-gated channel and peripherin-2 proteins of the rod photoreceptor outer segment of vertebrates are mediated by a glutamic acid rich protein (56).

At this stage, it is unclear whether an association between trypanosomal GARP and TS-form 1 would have a stabilizing effect on TS-form 1 that could account for not only the differences in transfer and SA activities, but also the differences in kinetic properties observed for TS-form 1 and 2. However, Poetsch *et al.* (2001) also observed an interaction between
mammalian phosphodiesterase and mammalian GARP, which led the authors to speculate that this association may play a role in modulating phosphodiesterase activity (56).

The fact that only TS-form 1 is co-purified and co-immunoprecipitated with GARP may be due to the possibility that only one of the two TS forms, TS-form 1, can interact with GARP. Considering that TS-form 1 has been shown to utilize various acceptor substrates with a higher catalytic efficiency, it is possible that GARP stabilizes TS-form 1 or that the 90 kDa protein bands of TS-form 1 and 2 actually represent monomers of identical molecular weight, but are encoded by two different genes, from which one codes for a protein with a slightly enhanced acceptor binding capacity (TS-form 1). This concept is sustained by recent findings identifying two different T.con.TS gene sequences, which share only 50% identity with each other3.

The isolated T.con.TS forms were found to have the same donor and acceptor substrate preferences, however, interestingly TS-form 1 utilized the acceptor substrates more efficiently than TS-form 2. On the other hand, the various donor substrates tested were utilized with similar efficiencies by both TS forms. Furthermore, SA activity was predominately found in TS-form 2, whereas TS-form 1 possesses significantly less SA activity and higher transfer activity. Taken together, these results suggest that the activity associated with TS-form 2 mainly represents SA, in which transfer activity is decreased, possibly due to reduced acceptor binding capacity. This is further substantiated by the finding that some known SA inhibitors, such as suramin inhibited SA activity of T.con.TS, whereas the transfer activity was unchanged. In contrast, Neu2en5Ac, a known SA inhibitor, inhibited transfer activity but not SA activity.

At this stage a potent TS inhibitor is not available. This may reflect the complexity of the SA and transfer mechanism of TS. It might be possible that donor substrate analogues as TS inhibitors may only be effective in the presence of an appropriate acceptor substrate analogue.
That is, the synthesis of multivalent inhibitors possessing both a donor and acceptor substrate analogue may provide very specific TS inhibitors for combating trypanosomiasis.

In conclusion, via micro-sequencing we have identified a single protein of approximately 90 kDa as T.con.TS. We were able to produce anti-T.con.TS mAb (mAb 7/23) which could, because of its specificity, be a valuable diagnostic tool for distinguishing between procyclic forms of T.b.br.TS and T.con.TS. We also show for the first time that TS forms exist that differ remarkably in their transfer to SA activity ratios, with these differences possibly being due to different acceptor substrate binding capacities. Moreover, we demonstrated another intriguing difference between both TS forms, the ability of TS-form 1 to associate with GARP. Therefore one can speculate that not only do active and inactive TS forms exist, but also TS forms with different transfer efficiencies, perhaps due to variations in their primary structure and/or protein folding, or due to the possible stabilization of TS through an interaction with GARP.

Studies on the enzymatic transfer of sialic acid catalyzed by TS, particular substrate specificities, will enhance its biotechnological applications. For example, TS could potentially be utilized for the sialylation of the T- and T\textsubscript{N}- antigens, as has been shown using bacterial SA (57) and human and mouse recombinant sialyltransferases (58). The sialylation of recombinant glycoproteins, as well as human milk compounds like LNT and LNnT, could also conceivably be carried out utilizing TS.

Given that trypanosomiasis has reached epidemic magnitude in some countries, one should consider methods to control not only the disease, but also its transmission stage inside the vector (59). Efforts have been made to establish bush-free belts, in order to reduce the spread of the tsetse fly. Taking this into consideration, the development of various TS inhibitors could not only serve in combating trypanosomes inside the host, in the case of T.cr., but also inside the vector, in the case of T.b.br. and T.con.
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FOOTNOTES

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1 FCS, fetal calf serum; GARP, glutamic acid-alanine-rich protein; IEF, isoelectric focusing; mAb, monoclonal antibody; MU, 4-methylumbelliferone; MUGal, 2′(4-methylumbelliferyl)galactoside; MULac, 2′(4-methylumbelliferyl)lactoside; MUNeu5Ac, 2′(4-methylumbelliferyl)-\(\alpha\)-D-\(\alpha\)-N-acetylneuraminic acid; Neu5Ac, \(\alpha\)-N-acetylneuraminic acid; Neu2en5Ac, 5-\(\alpha\)-acetyl-2-deoxy-2,3-didehydro-neuraminic acid; PARP, procyclic acidic repetitive protein; SA, sialidase; \(\alpha\)2,3-SL, sialyllactose (Neu5Ac\(\alpha\)2,3-lactose); \textit{T.b.br.}, \textit{Trypanosoma brucei brucei}; \textit{T.con.}, \textit{Trypanosoma congolense}; \textit{T.cr.}, \textit{Trypanosoma cruzi}; \textit{T.r.}, \textit{Trypanosoma rangeli}; TS, trans-sialidase

2 Silke Schrader, Evelin Tiralongo, Alberto C.C. Frasch, Teruo Yoshino and Roland Schauer, A non-radioactive 96-well-plate assay for screening trans-sialidase activity, manuscript in preparation

3 Evelin Tiralongo, Ilka Martensen, Joachim Grötzinger, Joe Tiralongo and Roland Schauer, Trans-sialidase conserves most of the critical active site residues found in other trans-sialidases, submitted to the Journal “Molecular and Biochemical Parasitology”
FIGURE LEGENDS

Fig. 1: Elution profiles of the various chromatography steps used during the purification of *T.con.* TS forms. Details of conditions for sample application and elution are given under Experimental Procedures. (A) Ion exchange chromatography on Q-Sepharose FF of concentrated *T.con.* culture supernatant. Elution was performed using a linear gradient from 0 to 0.8 M NaCl. (B) Elution profile of gel filtration chromatography on Sephadex-G150 SF, following Q-Sepharose FF, performed for TS-form 1 (fractions 35-65, figure 1A) resulting in a molecular weight of 350-600 kDa. The Sephadex-G150 SF column was calibrated with the following protein standards: thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), albumin (67 kDa) and chymotrypsinogen A (25 kDa). (C) IEF chromatogram performed at a pH range 4-6 using TS-form 2 (fractions 20-35, figure 1A) as sample. The small activity peak between pH 4-5 represents residual TS-form 1 activity, which was not completely separated from TS-form 2 following the first purification step. TS-form 2, represented by the large activity peak, possesses an pI of pH 5-6.5. (D) Elution profile of gel filtration chromatography on Sephadex-G150 SF, following IEF, performed for TS-form 2 (pH 5-6.5, figure 1C) resulting in a molecular weight of 130-180 kDa.

Fig. 2: Silver-stained gel following SDS-PAGE showing the various stages in the purification of *T.con.* TS form 1 and 2. Two to four µg of protein was applied to each well. Lane 1: concentrated culture supernatant, lane 2: TS-form 1 following Q-Sepharose FF, lane 3: TS-form 1 following gel filtration, lane 4: TS-form 1 following immunoaffinity chromatography, lane 5: TS-form 2 following Q-Sepharose FF, lane 6: TS-form 2 following IEF, lane 7: TS-form 2 following gel filtration, lane 8: TS-form 2 following immunoaffinity chromatography. The arrow indicates the protein band which was used for micro-sequencing.
Fig. 3: Immunoblots of *T.con.*TS forms with anti-*T.con.*TS mAb (mAb 7/23) and anti-*T.cr.*TS antiserum following SDS-PAGE. A: Immunostaining of *T.con.*TS with anti-*T.con.*TS mAb 7/23. Lane 1: Concentrated culture supernatant, lane 2: TS-form 1, lane 3: TS-form 2. B: Immunostaining of recombinant *T.cr.*TS (lane 1), recombinant *T.b.br.*TS (lane 2) and, *T.con.*TS (lane 3) with *T.con.*TS mAb 7/23. C: Immunostaining of recombinant *T.cr.*TS (lane 1) and *T.con.*TS (lane 2) with anti-*T.cr.*TS antiserum.

Fig. 4: Immunoblots of *T.con.*TS following SDS-PAGE. Lane 1: Concentrated culture supernatant, lane 2: TS-form 2 following Q-Sepharose FF, lane 3: TS-form 1 following Q-Sepharose FF, lane 4: TS-form 2 following gel filtration, lane 5: TS-form 1 following gel filtration, lane 6: TS-form 2 following immunoaffinity chromatography, lane 7: TS-form 1 following immunoaffinity chromatography.

Fig. 5: Immunoblots following immunoprecipitation of TS and GARP from concentrated culture supernatant with anti-*T.con.*TS mAb (mAb 7/23). A: Immunostaining with anti-*T.con.*TS mAb 7/23 (IgG2b) as primary antibody and anti-mouse IgG as secondary antibody. Lane 1: Immunoprecipitation in the absence of anti-*T.con.*TS mAb 7/23 (control), lane 2: Immunoprecipitation in the presence of anti-*T.con.*TS mAb 7/23. B: Immunostaining with anti-*T.con.* GARP mAb (IgG3) as primary antibody and biotinylated anti-mouse IgG3 as secondary antibody. Lane 1: Immunoprecipitation in the absence of anti-*T.con.*TS mAb 7/23 (control), lane 2: Immunoprecipitation in the presence of anti-*T.con.*TS mAb 7/23.
Table 1: Summary of the purification of both TS forms of *T. con.*

| Purification step | Protein (mg) | Total Activity (mU) \(^a\) | Specific Activity (mU/mg) | Enrichment (x-fold) | Recovery (%) |
|-------------------|-------------|-----------------|-----------------|-----------------|--------------|
|                   |             | SA activity     | Transfer activity | SA activity     | Transfer activity | SA activity | Transfer activity |
| TS-form 1         |             |                 |                  |                 |              |             |                   |
| Conc. Cult S/N \(^b\) | 3750 | 6000            | 15000            | 1.6            | 4             | 1           | 1                | 100  | 100 |
| Q-Sepharose FF    | 180 | 300             | 5000             | 1.6            | 28            | 1           | 7                | 5    | 33  |
| Sephadex-G150 SF  | 20 | 0.3             | 2400             | 0.015          | 120           | 0.01        | 30               | 0.005 | 16 |
| mAb 7/23 – rProtein A-Sepharose | 0.175 | 0.015          | 750              | 0.09           | 4285          | 0           | 1071             | 0    | 5   |
| TS-form 2         |             |                 |                  |                 |              |             |                   |
| Conc. Cult S/N    | 3750 | 6000            | 15000            | 1.6            | 4             | 1           | 1                | 100  | 100 |
| Q-Sepharose FF    | 1900 | 4400            | 10500            | 2.3            | 5.5           | 1.4         | 1.4              | 73    | 70 |
| IEF pH 4-6        | 43 | 65              | 750              | 2.3            | 17.6          | 1.4         | 4.4              | 1.1   | 5  |
| Sephadex-G150SF   | 0.3 | 4               | 180              | 13             | 600           | 8.1         | 150              | 0.07  | 1.2 |
| mAb 7/23 – rProtein A-Sepharose | 0.003 | 0.11           | 42               | 44             | 16800         | 27.5        | 4200             | 0.002 | 0.28 |

\(^a\) One unit of activity equals one µmol of MU released per minute, which is equivalent to 1 µmol of sialic acid released or transferred per minute.

\(^b\) Conc. Cult S/N: concentrated culture supernatant
Table 2: Comparison of the peptide sequence VVDPTVVAK derived from a 90 kDa protein representing TS-form 1 following immunoaffinity purification with the sequences of known trypanosomal SA and TS including the newly identified *T.con.*TS sequences. The italicized sequences show 100% identity with the found peptide. The GenBank accession numbers of the sequences are stated in parentheses.

| Enzymes          | Peptide sequence |
|------------------|------------------|
| *T.r.*SA (U83180)| VMDATVIVK        |
| *T.cr.*TS (D50685)| VVDPTVIVK       |
| *T.b.br.*TS (AF310232) | VVDPTVVAK      |
| *T.con.*TS1 (AJ535487) | VVDPTVVAK      |
| *T.con.*TS2 (AJ535488) | VVDPTVVVK      |
Table 3: Kinetic parameters for both *T.con.*TS forms. Apparent $V_{\text{max}}$ and $K_m$ values were calculated from the Michaelis-Menten curve. Apparent $V_{\text{max}}/K_m$ values represent the catalytic efficiency.

| Substrates | TS-form 1 |  |  | TS-form 2 |  |  |
|------------|-----------|---|---|-----------|---|---|
|            | $V_{\text{max}}$ (mU/mg) | $K_m$ (mM) | $V_{\text{max}}/K_m$ | $V_{\text{max}}$ (mU/mg) | $K_m$ (mM) | $V_{\text{max}}/K_m$ |
| MUGal      | 27        | 0.5 | 54 | 10        | 0.7 | 14 |
| MULac      | 120       | 0.9 | 133 | 26        | 1.0 | 26 |
| α2,3-SL    | 22        | 0.3 | 73 | 6         | 0.2 | 30 |
| MUNeu5Ac   | n.d.¹     | n.d. | n.d. | 11        | 0.09 | 122 |

¹ n.d.: not determined
Table 4: Substrate specificity of both *T.con*.TS forms.

| Substrates | Concentration a | Form 1 | Form 2 |
|------------|-----------------|--------|--------|
| **A) Donor** | Relative transfer activity (\%) b | Form 1 | Form 2 |
| Sialyl-\(\alpha\)2,3-\(N\)-acetyllactosamine; Neu5Ac\(\alpha\)2,3Gal\(\beta\)1,4GlcNAc | 1 mM | 111 | 115 |
| Sialyl-\(\alpha\)2,3-lactose; Neu5Ac\(\alpha\)2,3Gal\(\beta\)1,4Glc | 1 mM | 100 | 100 |
|  | 0.5 mM | 90 | 90 |
|  | 0.25 mM | 70 | 70 |
| Sialyl-\(\alpha\)2,6-lactose; Neu5Ac\(\alpha\)2,6Gal\(\beta\)1,4Glc | 1 mM | 31 | 26 |
| Sialyl-Lewis \(\alpha\); Neu5Ac\(\alpha\)2,3[Gal\(\beta\)1,3]GlcNAc\(\beta\)1,3Gal\(\beta\)1,4Glc | 0.25 mM | 11 | 11 |
| Fetuin | 0.5 mM | 78 | 78 |
| Sialyloligosaccharides, bovine milk | 0.5 mM | 79 | 70 |
| Sialyloligosaccharides, human milk | 0.5 mM | 70 | 66 |
| Glycomacropeptide | 0.5 mM | 77 | 71 |
| Apolactoferrin | 0.5 mM | 45 | 20 |
| **B) Acceptor** | Relative transfer activity (decrease in %) | Form 1 | Form 2 |
| \(N\)-acetyllactosamine; Gal\(\beta\)1,4GlcNAc | 1 mM | 49 | 24 |
| Lacto-\(N\)-biose I; Gal\(\beta\)1,3GlcNAc | 1 mM | 40 | 13 |
| Lacto-\(N\)-neotetraose; Gal\(\beta\)1,4GlcNAc\(\beta\)1,3Gal\(\beta\)1,4 Glc | 1 mM | 56 | 39 |
| Lacto-\(N\)-tetraose; Gal\(\beta\)1,3GlcNAc\(\beta\)1,3Gal\(\beta\)1,4 Glc | 1 mM | 31 | 12 |
| Lactose; Gal\(\beta\)1,4Glc | 1 mM | 45 | 24 |
| Lactitol | 1 mM | 61 | 35 |
| Galactose-\(\beta\)1,4-galactose; Gal\(\beta\)1,4Gal | 1 mM | 37 | 15 |
| Galactose | 5 mM | 14 | 7 |
| Glucose | 5 mM | 0 | 0 |
| Mannose | 5 mM | 0 | 0 |
| Maltose | 5 mM | 0 | 0 |

A) Relative transfer rates from sialic acid-containing compounds onto 0.5 mM MUGal. B) Relative transfer rates of sialic acid from \(\alpha\)2,3-SL given as percent reduction in the synthesis of MUGalNeu5Ac. For details see “Experimental Procedures”. \(^a\) The concentration of potential donors is stated as the concentration of bound sialic acid. \(^b\) The same total transfer activity was used for both TS forms in all assays.
Figure 1

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Figure 2

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Two trans-sialidase forms with different sialic acid transfer and sialidase activities from Trypanosoma congolense
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