

Temperature differences for *trans*-glycosylation and hydrolysis reaction reveal an acceptor binding site in the catalytic mechanism of *Trypanosoma cruzi trans*-sialidase

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Trypanosoma cruzi, the agent of Chagas disease, expresses on its surface a *trans*-sialidase that catalyzes preferentially the transference of α -2,3-linked sialic acid to acceptors containing terminal β -galactosyl residues, instead of the typical hydrolysis reaction, found in most sialidases. The *trans*-sialidase is responsible for the acquisition of the host sialic acid by this protozoan parasite, which does not synthesize sialic acids. Here, we have studied some kinetic properties of a recombinant *trans*-sialidase expressed in *Escherichia coli*. We found that it has sequential-type kinetics for the transferase reaction, as shown for the parasite-derived enzyme. The rates of sialic acid transfer to water (hydrolysis), and to β -galactosyl residues have a unique behavior with respect to the reaction temperature. While the hydrolysis rate of sialyllactose increases continuously up to 35°C, the temperature for the maximal rate of *trans*-glycosylation depends on the acceptor concentration. At low acceptor concentrations the rate of *trans*-glycosylation is maximal at 13°C and independent of the amount of sialic acid donors. With increasing acceptor concentrations, maximal rates of *trans*-glycosylation are shifted to higher temperatures. This finding is explained by an 8-fold increase in the K_m for the acceptor from 13°C to 33°C. Differences in hydrolysis and transfer rates were also obtained by using 4-methylumbelliferyl-N-acetyl-neuraminic acid. However, its hydrolysis rate is much higher than the rate of transference to lactose, suggesting that a long-lived enzyme-sialosyl intermediate is not formed. In addition, lactose does not increase the rate of methyl-umbelliferone release at any temperature, indicating that the rate limiting step is the aglycon release. Based on these results we propose that *trans*-glycosylation in *T. cruzi* sialidase is favored by the existence of a binding site for β -galactosyl residues, which accepts the new glycosidic bond as sialic acid is released from the donor. With increasing temperature the affinity for the acceptor decreases, resulting in a concomitant increase in the rate of transfer to water, which, in turn, can be suppressed by increasing the acceptor concentration.

Key words: *Trypanosoma cruzi*/sialidase/kinetics/reaction mechanism/temperature

Introduction

Most sialidases catalyze preferentially hydrolyzes of terminal sialic acids bound to different glycoconjugates, but have a poor ability to transfer sialic acid to carbohydrates, or other hydroxyl-containing molecules. In contrast, sialidases found in some *Trypanosoma* species catalyze with high efficiency the transference of sialic acids to β -galactosyl acceptor molecules, and are poor hydrolases (Vandekerckhove *et al.*, 1992; Ferrero-Garcia *et al.*, 1993; Scudder *et al.*, 1993). As these protozoan parasites are unable to synthesize sialic acids, such preferential transferase activity allows these parasites to acquire sialic acid from the host (Schenkman *et al.*, 1991, 1993; Engstler *et al.*, 1993; Pontes de Carvalho *et al.*, 1993). In the South American trypanosomes, such as *Trypanosoma cruzi*, the agent of Chagas disease, sialic acids are incorporated into major surface mucin-like molecules (Schenkman *et al.*, 1994b). In African trypanosomes sialic acids are added to major surface glycoproteins of insect forms of the parasite (Engstler *et al.*, 1993; Pontes de Carvalho *et al.*, 1993; Engstler and Schauer, 1994; Frasc, 1994). In both cases, the sialic acid confers a physical barrier and a strong negative charge to the surface of these protozoan parasites.

Similar glycosyl-transferases have been described in several microorganisms. Examples are the enzymes of plaque forming oral bacteria that use sucrose to synthesize glucose and sucrose polymers (Mooser *et al.*, 1987; Mooser and Iwaoka, 1989). These enzymes, like many glycosidases that retain the anomeric configuration of the substrates, can catalyze *trans*-glycosylation reactions and have weak hydrolase activity (Mooser, 1994). Often, stable enzyme-glycosyl intermediates are formed and transfer depends on the relative concentrations of donors and acceptors as well as their association with the enzyme (Sinnott, 1990).

The reasons why the *trans*-sialidase (TS) of trypanosomes has acquired a potent transferase activity are unknown. As all sialidases described so far have some degree of structural similarity, it is possible that selected amino acid modifications resulted in a better transferase ability. In contrast to other sialidases, trypanosome TS might form a more stable sialosyl-enzyme complex, resulting in an activated enzyme complex that mediates the transfer reaction. Alternatively, the enzyme might contain an acceptor binding site close to the sialic acid binding site, which would favor the transfer of sialic acid to the acceptor instead of water.

Among trypanosomes TSs, the most studied is the enzyme of *T. cruzi*. It is encoded by a family of about 80 different genes (Egima *et al.*, 1996), and contains at least two domains with defined functions (Cazzulo and Frasc, 1992; Schenkman *et al.*, 1994a). The amino-terminal domain contains the catalytic site, and five conserved SXXDXGXTW sequences, (Asp box), which are also found at similar positions in viral, bacterial, and mammalian sialidases (Roggentin *et al.*, 1989; Schenkman *et*

al., 1994b). According to the crystal structure of *Salmonella typhimurium* sialidase (Crennell et al., 1993), Asp box are important for the stabilization of the barrel structure formed by these enzymes (Crennell et al., 1993, 1994; Gaskell et al., 1995). The second domain is not required for activity and is formed by variable numbers of 12 amino acid repeats. It enables oligomerization and binding of the enzyme to the parasite surface (Schenkman et al., 1994a; see Figure 1a).

Trypanosoma rangeli is related to *T.cruzi*, and expresses a sialidase that is 70% similar to *T.cruzi* TS at the amino acid level, but has no detectable transferase activity (Buschiazzo et al., 1993; Smith et al., 1996). By using hybrids containing pieces of the *T.cruzi* and *T.rangeli*, Smith and Eichinger (1997) have shown that at least two domains are modified for the acquisition of the transferase activity in *T.cruzi* enzyme. One is probably near the catalytic site, as inferred by analogy with the amino acids in the crystal structure of *S.typhimurium* sialidase. The other domain is toward the carboxy-terminal end in a region presumed to contain an extra-galactose binding site, as was found in the structure of *Micromonospora viridifaciens* sialidase (Gaskell et al., 1995).

To better understand how *T.cruzi* TS has acquired the transferase activity, we have studied some kinetic properties of the

TS expressed in recombinant bacteria. Previous studies have used enzyme isolated from cultured parasites (Scudder et al., 1993). Since many different TS genes can be expressed simultaneously, some of them without enzymatic activity (Uemura et al., 1992; Pollevick et al., 1993; Cremona et al., 1995), these results can be difficult to interpret. Therefore, we constructed a recombinant TS truncated at the first amino acid repeat of the carboxy-terminus followed by a histidine tail. A similar construct has been shown to retain enzymatic activity and expresses the same properties as the parasite-derived enzyme (Campetella et al., 1994; Buschiazzo et al., 1996; Cremona et al., 1996). The recombinant enzyme was expressed in *E.coli* and purified to homogeneity. The kinetic properties of this recombinant TS were compared with the TS purified directly from the parasites, using sialyllactose (SL) as donor and lactose as acceptor. We found no major differences in the kinetic parameters of the enzymes from these two sources. We did find, however, that TS has a unique behavior with respect to the reaction temperature. In the presence of low concentrations of acceptors, transferase activity is maximal at low temperatures whereas hydrolysis is elevated at high temperatures. By increasing the amount of the acceptor, transferase activity predominates at high temperatures. These results, combined with

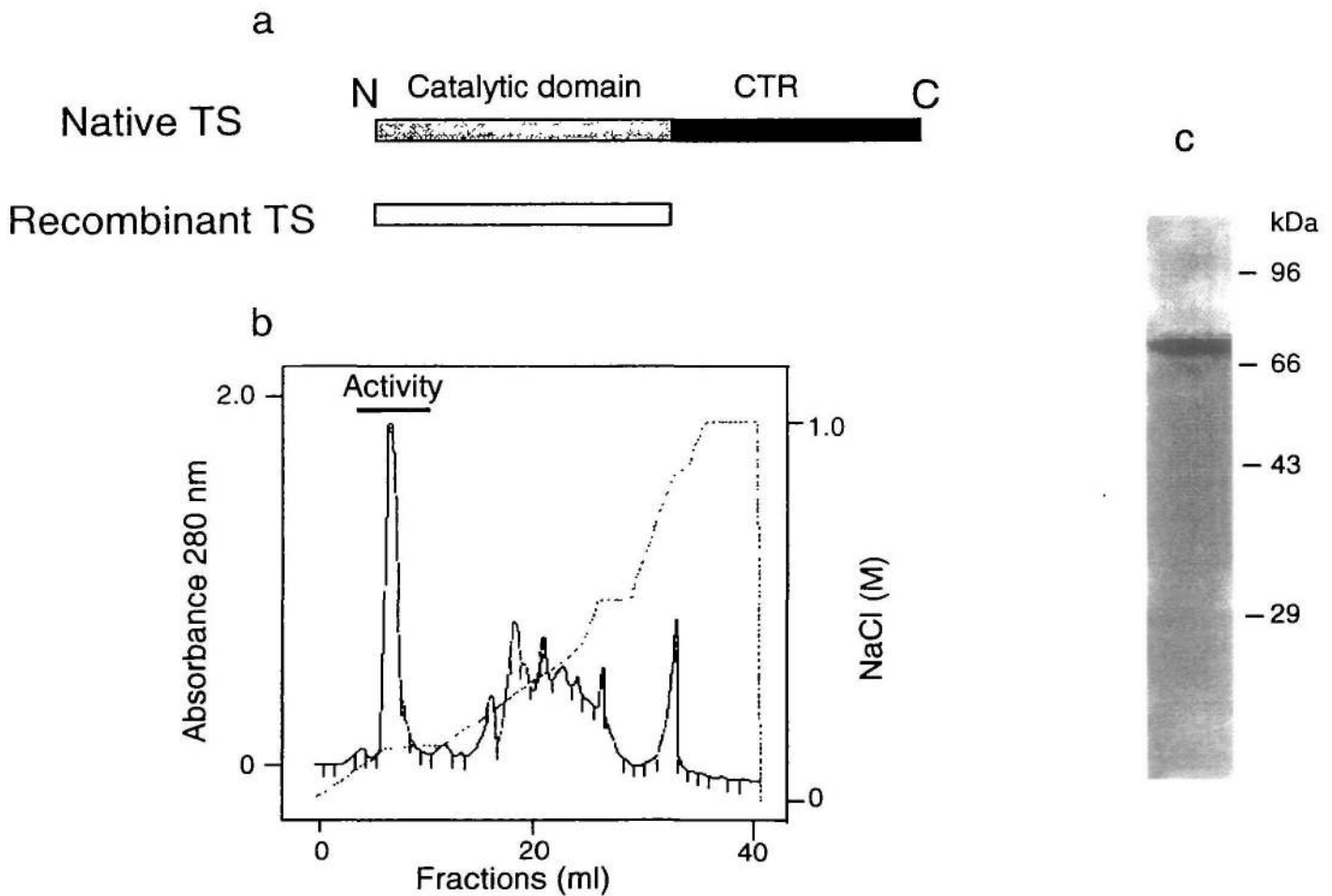


Fig. 1. *T.cruzi* TS structure and purification. (a) Representation of the native and recombinant TS. (b) Profile of recombinant TS elution from the Mono Q column. Bacteria expressing recombinant TS were disrupted by sonication, and purified on the Ni²⁺-agarose column. After elution with 0.5 M imidazol, the enzyme was dialyzed against 20 mM Tris-HCl pH 8.0 and applied into a MonoQ column equilibrated in the same buffer. The enzyme was eluted with a NaCl gradient as indicated (dotted line), and the absorbance at 280 nm (solid line) and the activity followed in all fractions. The fractions containing activity were pooled as shown in the horizontal line. (c) SDS-PAGE of the fractions containing TS activity. A 10% acrylamide gel was loaded with 5 µg of the purified enzyme and after the electrophoresis the gel was stained with Coomassie blue R250. The numbers on the right are MW in kDa.

the analysis of the reaction with 4-methyl-umbelliferyl-N-acetylneuraminic acid (4-MuNana) allow us to define the rate limiting step of the transferase reaction, and to propose a mechanism for the transferase reaction that does not include the formation of a stable sialosyl-enzyme complex.

Results

Double reciprocal plots of the initial rate of *trans*-glycosylation for both recombinant and parasite derived TS show very similar patterns using different concentrations of SL and lactose (Figure 2). The curves intercepted each other at the lower left portion of the Lineweaver-Burk plots. Similar results were found by Scudder *et al.* (1993), using a parasite-derived enzyme. Based on our, and their results, a bisubstrate sequential mechanism likely occurs, but we could not distinguish between a random mechanism and an ordered addition of the donor- and acceptor substrates. The data from Figure 2 were replotted, assuming a sequential bisubstrate mechanism, to calculate the dissociation constants (K_s and K_m) for SL and lactose, V_{max}

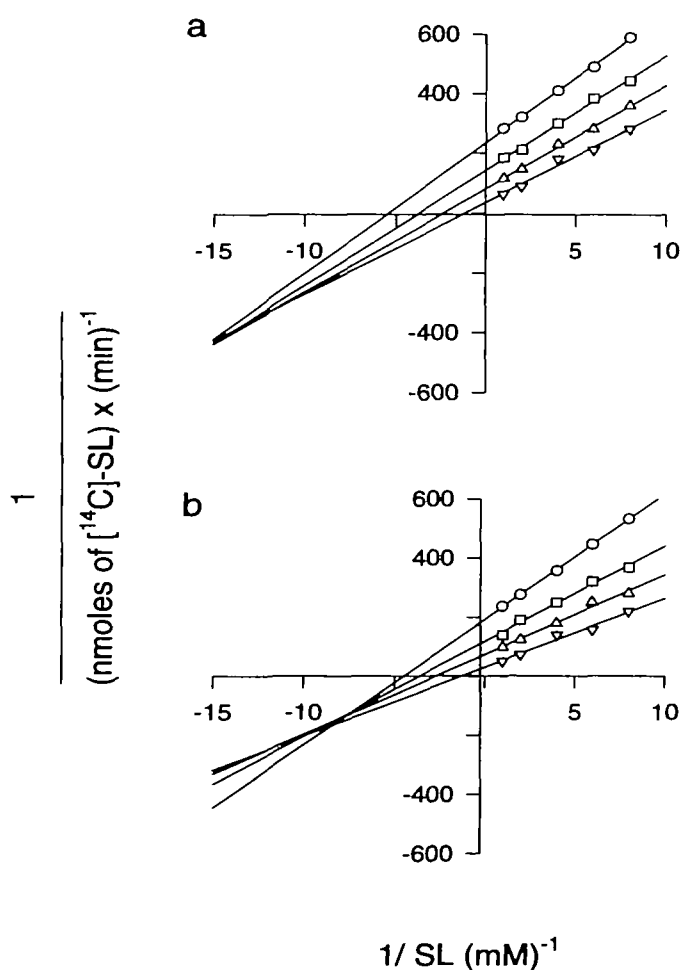


Fig. 2. Kinetic analysis of the *trans*-glycosylation reaction catalyzed by TS. The figure shows the double reciprocal plots of the initial rate (30 min reaction) of sialic acid transfer from SL to [14 C]-lactose at variable SL and lactose concentrations. (a) shows the results obtained with TS isolated from *T. cruzi* supernatants (10 ng per assay), and (b) with recombinant TS (5 ng per assay). Each curve was obtained at 0.0674 mM (circles), 0.0274 mM (squares), 0.0134 mM (triangles), and 0.0074 mM (inverted triangles) of [14 C]-lactose. The reactions were performed at 25°C, and each point represent the mean of duplicate measurements.

and K_{cat} . The values, presented in Table I, were similar for both enzymes, showing that the recombinant enzyme conserved most of the kinetic properties of the products of the gene family expressed by the parasite. K_{cat} values were around 12 s^{-1} , which is larger than the values of 0.7 s^{-1} found by Scudder *et al.* (1993), possibly due to the use of different substrates and experimental conditions. The values of K_{cat}/K_m^{SL} and K_m^{Lac} around $1 \times 10^4 \text{ s}^{-1} \text{ M}^{-1}$ show that *T. cruzi* TS has a moderate catalytic efficiency for the *trans*-glycosylation reaction.

Next, we compared the effect of temperature on the *trans*-glycosylation activity of the recombinant and parasite derived TS. As shown in Figure 3a, we obtained an unusual profile, with a maximal activity around 13°C for both enzyme sources. A similar profile was also found by Scudder *et al.* (1993). Maximal rates for *trans*-glycosylation were between 10°C and 20°C, with 50% of activity around 0°C and 37°C. Above 37°C, the activity dropped until it was undetectable at 56°C. One explanation for this anomalous behavior could be the irreversible thermal denaturation of the enzyme. However, this was not the case, at least up to 42°C, since preincubation of the enzyme at temperatures ranging from 0°C to 42°C did not decrease activity (Figure 3b). Several other experiments were made to check the stability of the parasite and recombinant TS. The most important factor to preserve activity was to prevent enzyme aggregation by using serum albumin in all dilutions and reactions. Under these conditions, even incubations at 37°C for long periods (24–72 h) showed no decrease in activity. Therefore, the decrease in activity for the *trans*-glycosylation reaction at 37°C was not due to enzyme inactivation. The fact that the recombinant TS and the parasite-derived enzyme have similar denaturation curves also shows that absence of N-linked glycosylation and repeats at the carboxy-terminus of the recombinant has no effect on the thermal stability of the enzyme.

The rate of hydrolysis, however, had a different optimal temperature. While *trans*-glycosylation was maximal around 13°C, the hydrolysis rate increased at temperatures up to 35°C (Figure 4a). The hydrolysis rate, however, was much lower than the transfer rate, and we had to use large amounts of enzyme and longer incubation periods to detect free sialic acid. Thus, by expressing both activities as the rate of the product formed per min per microgram of recombinant TS, we noticed that the rate of hydrolysis increased as the rate of *trans*-glycosylation decreased (Figure 4b).

To better understand the reasons why these two reactions had maximal rates at different temperatures, we initially studied the effect of SL and lactose concentrations on the rates of *trans*-glycosylation at 13°C and 33°C. As shown in the double reciprocal plot of the Figure 5a, increasing the lactose concentration, at constant SL concentrations, had a more pronounced effect on the initial rate of *trans*-glycosylation at 33°C than at 13°C. At high acceptor concentrations the maximal rate of transference shifted to higher temperatures. This finding, and the 6- to 8-fold increase in the apparent K_m for lactose at 33°C versus 13°C, suggested that the affinity of the enzyme for lactose decreased at higher temperatures. Yet, we could not detect binding of lactose to the enzyme by equilibrium dialysis, equilibrium columns, or intrinsic fluorescence shifts, possibly because the interaction of lactose was of such a low affinity, with a dissociation constant well above 10^{-4} M^{-1} , which agrees with the K_m^{Lac} (see Table I). Therefore, lactose binding remained undetectable by these conventional methods. Lactose might productively bind only to a form of the enzyme associ-

Table I. Kinetic parameters of *T. cruzi* TS

Trans-glycosylation reaction at 25°C						
Enzyme source	K_m^{SL} (mM)	K_m^{lac} (mM)	K_s^{SL} (mM)	V_{max} (nmoles · min ⁻¹)	K_{cat} (s ⁻¹)	K_{cat}/K_m^{SL} (M ⁻¹ · s ⁻¹)
<i>T. cruzi</i> TS	1.6	0.09	0.07	0.062	12.4	7.8×10^3
Recombinant TS	1.2	0.08	0.13	0.054	12.6	1.1×10^4

Hydrolysis reaction by the recombinant TS						
Substrate	Temperature	K_m (mM)	V_{max} (nmoles · min ⁻¹)	K_{cat} (s ⁻¹)	K_{cat}/K_m (M ⁻¹ · s ⁻¹)	
SL	33°C	0.5	0.06	0.72	1.4×10^3	
SL	13°C	0.25	0.06	0.72	2.8×10^3	
MuNana	33°C	0.5	0.6	7.2	1.4×10^4	
MuNana	13°C	1	0.6	6.0	6×10^3	

K_s^{SL} was calculated by Frienden's method (Dixon and Webb, 1979). V_{max} was calculated from secondary plot of $1/v \times 1/[lac]$ taken from Fig. 2, as the $1/v$ intercept (Dixon and Webb, 1979) for the *trans*-glycosylation reaction. V_{max} and K_m for the hydrolysis reaction were obtained from the intercepts of $1/v \times 1/[SL]$ of the plot shown in Fig. 6. K_m^{Lac} was obtained from the secondary plot of $1/v \times 1/[lac]$ taken from Fig. 2, as the intercept on $1/lac$ axis (Dixon and Webb, 1979). K_m^{SL} was obtained from the secondary plot of $1/v \times 1/[SL]$ taken from Fig. 2 as the intercept on $1/lac$ axis (Dixon and Webb, 1979). K_{cat} was calculated from $V_{max}/[TS]$, assuming the size of the parasite derived enzyme was 120 kDa and the recombinant was 70 kDa.

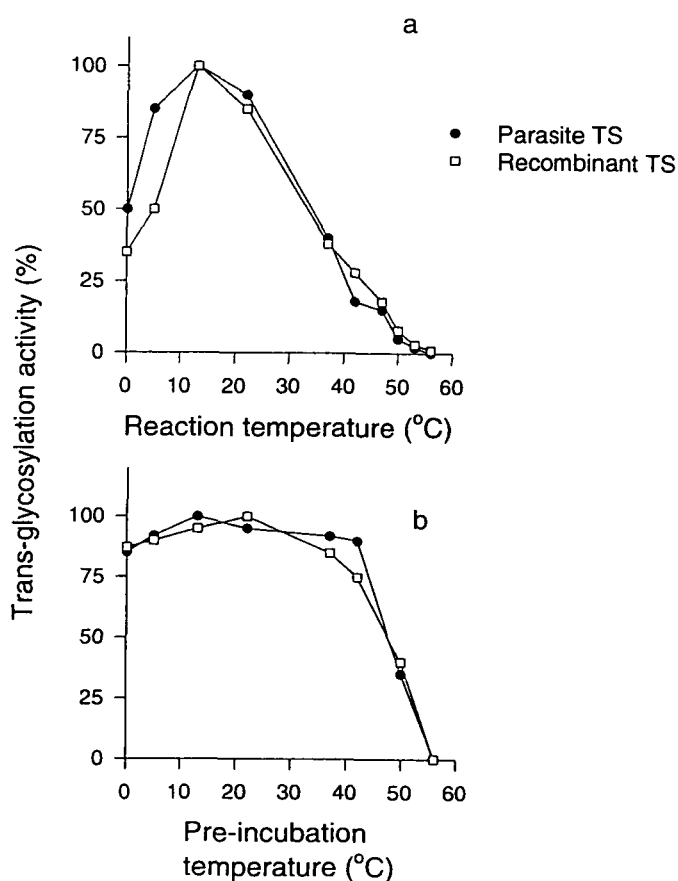


Fig. 3. Effect of temperature and pre-incubation temperature on the TS activity. Parasite (solid circles), or recombinant TS (open squares) were incubated for 30 min at the indicated temperatures (a), or pretreated 15 min at the indicated temperatures followed by incubation for 30 min at 13°C (b) with $7.4 \mu\text{M}$ [¹⁴C]-lactose and 1 mM SL. The amount of [¹⁴C]-SL formed is expressed as the percentage of the maximal activity of duplicate measurements.

ated with the sialic acid donor. In any case, the temperature seems to influence the *trans*-glycosylation rate by changing the binding of lactose to the enzyme. In contrast, the sialic acid donor concentration did not affect the differences in the rates of *trans*-glycosylation and hydrolysis with respect to the temperature. As shown in Figure 5b the rate of *trans*-glycosylation was always higher at lower temperatures at any concentration of SL. This result also provides evidence that SL did not compete with lactose for the presumed acceptor binding site in the enzyme.

Next, we examined the effect of temperature on the hydrolysis and *trans*-glycosylation reactions by using 4-MuNana as sialic acid donor and lactose as acceptor. As shown in the Figure 6, the hydrolysis of 4-MuNana increased up to 33°C, while transferase activity was maximal between 22°C and 28°C. Thus, transfer to a carbohydrate acceptor versus to water were also differentiated by the temperature in the case of 4-MuNana. Interestingly, the rate of transfer to a carbohydrate from 4-MuNana was at least 10 times slower than from SL, suggesting that a long-lived sialosyl-enzyme intermediate was not formed. Otherwise, the transfer rate would be similar for both donor substrates, as found for other enzymes with *trans*-glycosylation ability that form a stable complex with the donor group (Van der Groen *et al.*, 1973; Mooser, 1994). The rate of hydrolysis of 4-MuNana, in contrast, was much higher than hydrolysis of SL. The fact that 4-MuNana was a better substrate for the hydrolysis reaction than SL appeared to result from the increase in the apparent V_{max} for the hydrolysis of the former substrate at both temperatures (Figure 7). There were only small changes in the apparent K_m . In contrast, the *trans*-glycosylation reaction had an increased apparent K_m and a slightly decreased apparent V_{max} for 4-MuNana (Figure 8).

The differences between transfer to lactose and to water using different donors suggested that the nature of the donor substrate influences whether the sialic acid will be transferred to water or to a carbohydrate acceptor molecule. One possible explanation for this finding is that the transfer reaction occurs when the initial sialic acid-donor is broken. Thus, the limiting step for the *trans*-glycosylation reaction would be the initial breakage of the glycosidic bond between the sialic acid and the

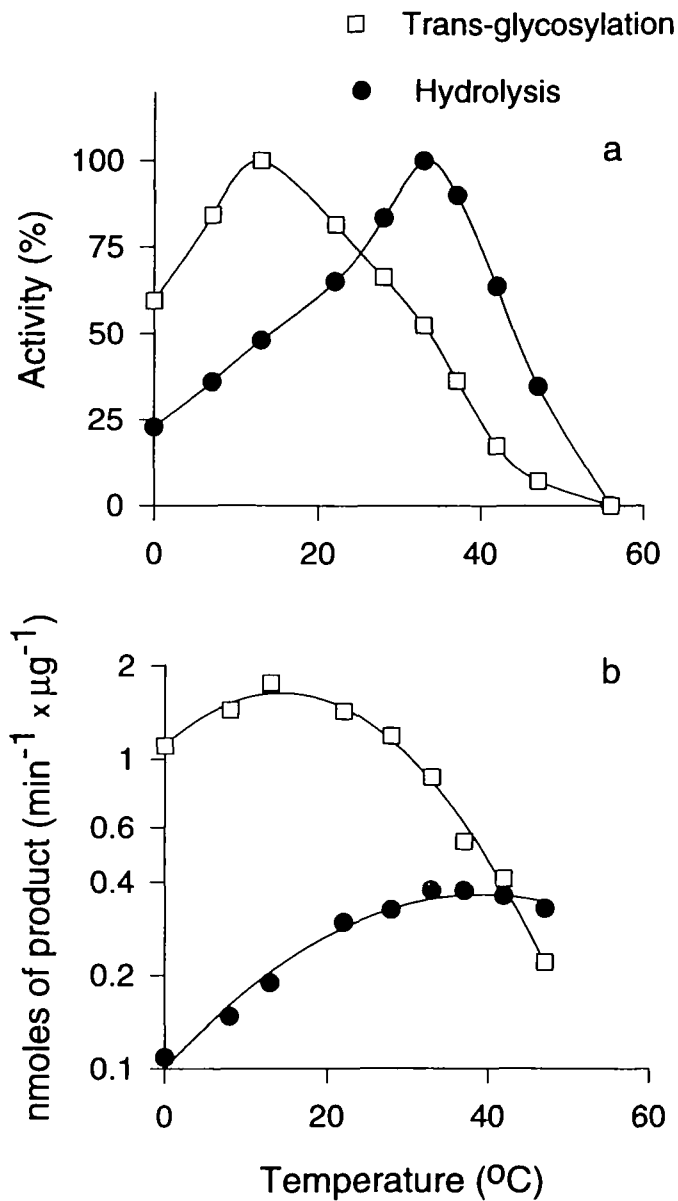


Fig. 4. Effect of temperature on the hydrolysis and *trans*-glycosylation reactions catalyzed by recombinant TS. Recombinant TS was incubated for 30 min at the indicated temperatures. The hydrolysis reactions (solid circles) contained 200 ng of TS and 1 mM SL. Free sialic acid was assayed by the thiobarbituric acid method. The *trans*-glycosylation reactions (open squares) contained 2 ng of TS, 1 mM SL and 7.4 M [¹⁴C]-lactose. In (a), activity is expressed as percentage of maximal activity and in (b), as the product formed per equivalent amounts of TS of triplicate measurements.

aglycone. To test this hypothesis we studied the effect of increasing lactose concentrations on the release of 4-MuNana at different temperatures. As shown in Figure 9, there was no effect on the release of the leaving group, even using very high lactose concentrations, suggesting that at the temperature range examined, the limiting step for the reaction was the initial breakage of the sialic acid bond of the donor.

The fact that the rate of *trans*-glycosylation versus hydrolysis depends on the acceptor concentration at 37°C led us to examine whether parasite sialylation is influenced by the temperature. To detect the amount of sialic acid on the parasite surface we used a monoclonal antibody that only recognizes sialylated epitopes on the parasite surface (mAb 3C9) (Schen-

kman *et al.*, 1991). We found that incubation of nonsialylated trypanomastigote forms with 1 mM SL for 15 min yields the same level of parasite labeling with mAb 3C9 at 4°C (Figure 10b), 20°C (d), and 37°C (c). This result suggests that parasite sialylation occur at all temperatures, which can be explained by the fact that large amounts of acceptors are present on the parasite surface, driven the reaction toward parasite sialylation, even at high temperatures.

Discussion

We have initially shown that a recombinant TS, the product of a single gene, has similar kinetic properties to that of the parasitic enzyme, which is the result of polygenic expression (Uemura *et al.*, 1992; Pollevick *et al.*, 1993; Cremona *et al.*, 1995). Thus, there are no major differences in the catalytic properties among the enzymes encoded by this gene family, or at least, the recombinant enzyme represents an "average" of the multiple activities. The *trans*-glycosylation reaction catalyzed by the recombinant enzyme follows a sequential mechanism as found for the parasite-derived enzyme. As discussed by Scudder *et al.* (1993), we were also unable to distinguish between a random and ordered entrance of the sialic acid donor and acceptors. Nevertheless, the large differences in transferase activities when using SL and 4-MuNana as donors, provided indirect evidence that a sialosyl-enzyme intermediate is not formed. This and the fact that the limiting step for the *trans*-

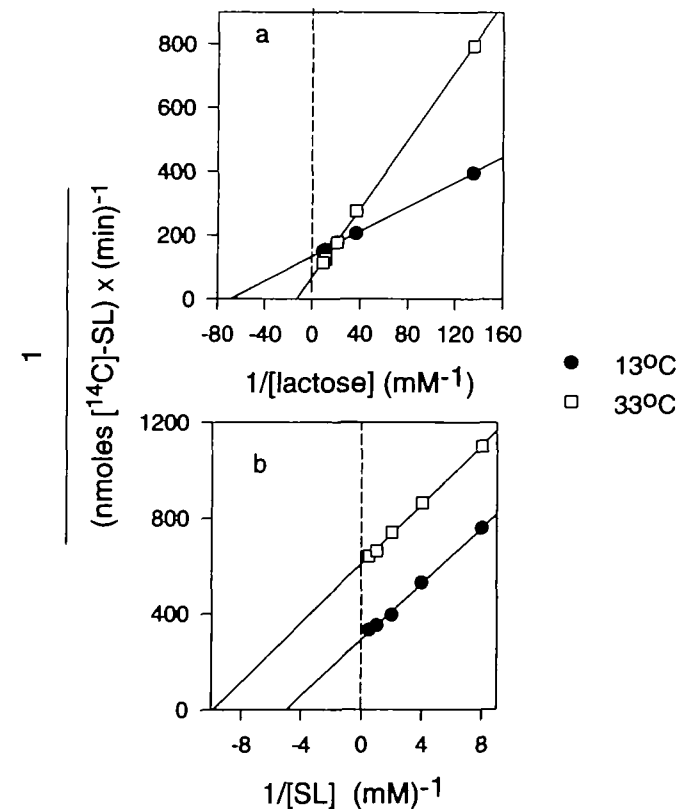


Fig. 5. High lactose concentrations increase the maximal temperature activity for *trans*-glycosylation reaction. Recombinant TS (15 ng) was incubated at 13°C (solid circles) or 33°C (open squares) at different [¹⁴C]-lactose (a) and SL concentrations (b) in 50:1 reaction volume. The amount of [¹⁴C]-SL formed was determined after a period of 30 min. The results are presented as a double reciprocal plot of means of triplicate measurements.

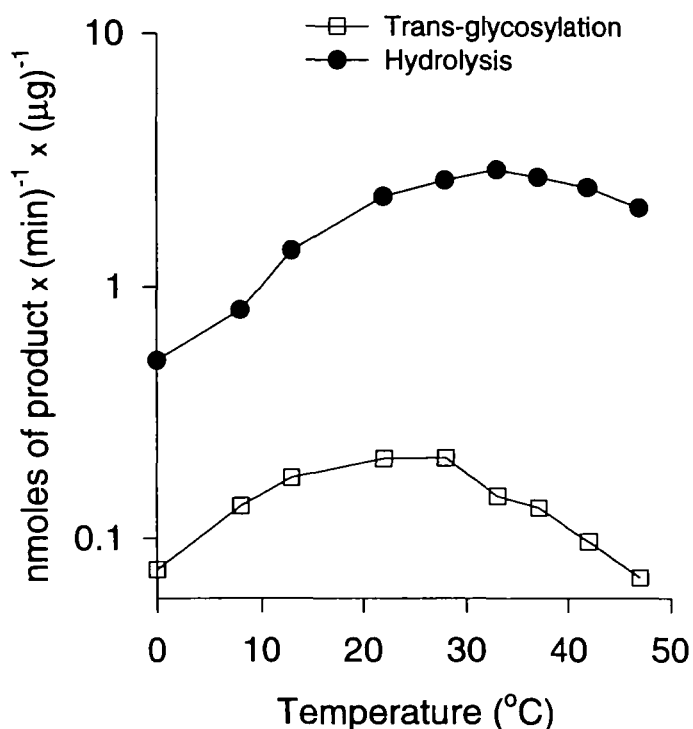


Fig. 6. Effect of temperature on the *trans*-glycosylation and hydrolysis of 4-MuNana. *Trans*-glycosylation reactions were performed using 25 ng of recombinant TS in the presence of 7.4 μM [^{14}C]-lactose and 0.5 mM 4-MuNana. Hydrolysis reactions were also performed using 25 ng of recombinant TS in the presence of 0.5 mM 4-MuNana. After 1 h, [^{14}C]-SL (open squares) and methyl-umbelliferone (solid circles) were measured by the standard assays. The results are means of duplicate measurements.

glycosylation reaction with 4-MuNana is the breakage of the sialosyl-bound in the donor substrate support the idea that the donor and acceptor may coexist in the active site of the *T. cruzi* TS enzyme.

More important, our results show for the first time that *trans*-glycosylation and hydrolysis can be distinguished by using the same donor substrate at different reaction temperatures. While hydrolysis increased up to the temperature that the enzyme undergoes thermal denaturation, *trans*-glycosylation was maximal at lower temperatures. Our results suggest that these differences for the optimal temperatures for hydrolysis and *trans*-glycosylation were due mainly to the differential binding of the acceptor to the enzyme. First, the temperature for the maximal rate of *trans*-glycosylation depended on the concentration of the carbohydrate acceptor, but not on the sialic acid donor. Second, addition of lactose shifted the maximal rates of *trans*-glycosylation toward higher temperatures. It appears that from 13°C to 33°C, the enzyme's affinity for lactose decreased, as shown by the 8-fold increase in the K_m . Third, there was only a small increase in the rate of sialic acid transfer to water when compared with the decrease in the rate of *trans*-glycosylation, showing that hydrolysis is not driven by the decrease of transfer to lactose.

Such a decrease in enzyme affinity might be due local conformational changes, affecting only the binding site for lactose. Unfortunately, we could not determine if the increased temperature directly affected the binding of lactose to the enzyme, or if it affected the mechanism of transfer of sialic acid to lactose that was already bound to the enzyme. We used equilibrium dialysis, equilibrium gel filtration, and intrinsic fluo-

rescence to try to demonstrate lactose binding, without success, most likely because the binding affinity was too low. The TS protein has a region with similarity to a Fn3-type domain, which is required for enzymatic activity (Schenkman *et al.*, 1994a). This region shows some degree of similarity to the putative galactose binding site *M. viridifaciens* sialidase. This region is present between the catalytic domain and the carboxy-terminal repeats of the *T. cruzi* enzyme, and corresponds to the domains that accounts for the difference in size between the 40 kDa and the 60/80 kDa bacterial sialidases (Gaskell *et al.*, 1995). Therefore, this region could represent the lactose binding site in *T. cruzi* TS. Nevertheless, the presence of a lactose binding site would not by itself be sufficient to catalyze efficient *trans*-glycosylation. *Trans*-glycosylation by the *T. cruzi* enzyme might additionally require the close proximity of the acceptor binding site to the catalytic site as found recently for the phosphoglycerate kinase (Bernstein *et al.*, 1997). Thus, an increase in the reaction temperature may also alter the enzyme conformation in a way that changes the distance between the donor and acceptor binding sites.

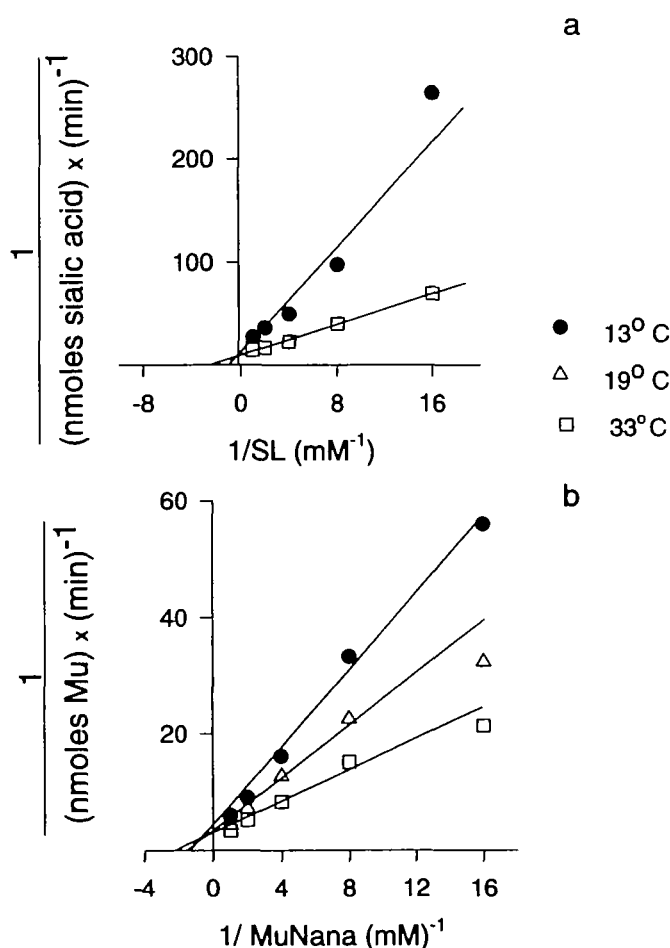


Fig. 7. Kinetics of hydrolysis of SL and 4-MuNana by recombinant TS. Recombinant TS (200 ng) was incubated at 13°C (solid circles), 19°C (open triangles), or 33°C (open squares) at different SL (a), and 4-MuNana concentrations (b), in a volume of 50 μl . Free sialic acid was measured by the thiobarbituric acid method in the case of SL after 2 h incubation. 4-MuNana hydrolysis was measured as fluorescence of 4-methyl-umbelliferone after 1 h incubation (excitation 365 nm, emission 450 nm). The results are means of duplicate measurements and are expressed as a double reciprocal plot. Apparent V_{max} and K_m were estimated by the intercepts on the ordinate and abscissas, respectively.

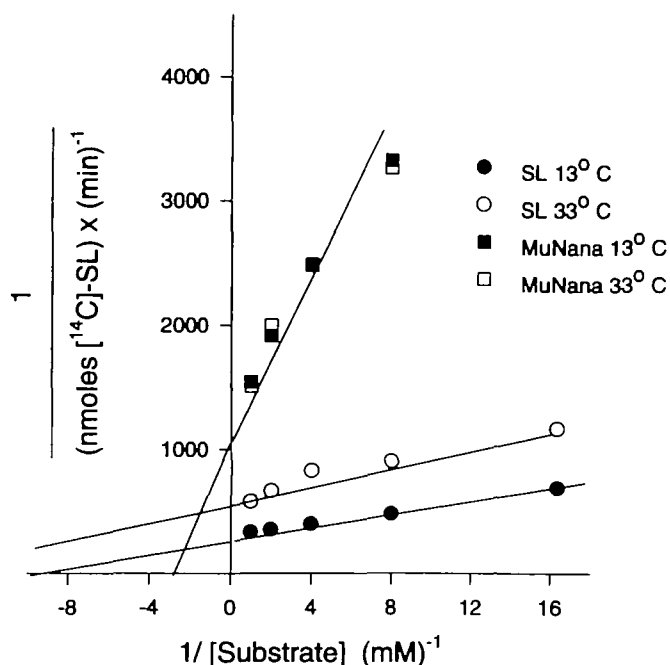


Fig. 8. SL is a better donor for *trans*-glycosylation than 4-MuNana. Recombinant TS (2 ng) was incubated with the indicated amounts of SL (circles), or 4-MuNana (squares) at 13°C (solid symbols) or 33°C (open symbols). After 30 min, the amount of [¹⁴C]-SL formed was measured by the standard assay. The results are means of duplicate measurements and are expressed in double reciprocal plot. Apparent V_{max} and K_m were estimated by the intercepts on the ordinate and abscissas, respectively.

Analysis of the kinetic constants for 4-MuNana as compared to SL showed a decreased V_{max} , an increased K_m for the transfer reaction (see Figure 8), and a large increase in V_{max} for hydrolysis (see Figure 7). The opposite effects in the K_m and V_{max} suggest that the methyl-umbelliferyl group influences the entrance of lactose for the transferase reaction, but not for hydrolysis. The reverse does not seem to occur, since lactose did not affect the hydrolysis of 4-MuNana. In addition, we found that the limiting step for the hydrolysis of 4-MuNana is the initial breakage of the sialosyl-aglycone, in spite of the fact that this reaction proceeds 5–10 times faster than hydrolysis of SL, or even transference to lactose. As discussed previously, the large differences between the rate of transfer of sialic acid from SL versus 4-MuNana indicated that a sialosyl-enzyme intermediate was not formed. Taken together, these data support the idea that the sialic acid donor and the acceptor sites may be close and that both substrates must coexist in the active site of the enzyme for the transferase reaction to occur. It should be noted that the presence of 4-MuNana, although a better substrate for hydrolysis, could eventually prevent entrance of the acceptor in the binding site, which could explain why 4-MuNana is a worse sialic acid donor when compared to SL.

The finding that the limiting step for the reaction was the breakage of the sialic acid bond also differentiates *T. cruzi* TS from the *Trypanosoma brucei* TS. In the latter parasite enzyme, lactose increased the release of 4-methyl-umbelliferone (Engstler *et al.*, 1993). Moreover, *T. brucei* TS had maximal rates of both *trans*-glycosylation and hydrolysis around 35°C (Engstler *et al.*, 1993). These differences suggest that although the enzymes are expressed by related organisms, both of which obtain sialic acid from their hosts, different mechanisms of reac-

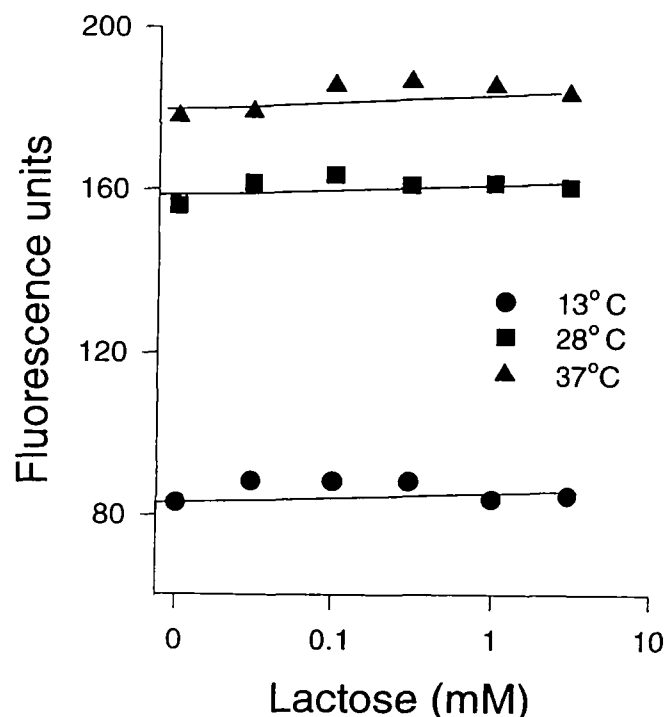


Fig. 9. Lactose does not increase the hydrolysis of 4-MuNana. Recombinant TS (25 ng) was incubated with 0.1 mM 4-MuNana and the indicated amounts of lactose at 13°C (circles), 28°C (squares), and 37°C (triangles). After 1 h, the released methyl-umbelliferone was measured by fluorescence. Each point represent the mean of duplicate measurements. Identical results were obtained in two other sets of experiments.

tions might have evolved. Distinct reaction mechanisms have also been described for sialidases with similar overall structures (Crennell *et al.*, 1993; Guo *et al.*, 1994), in which small amino acid modifications close to the active site seem to alter

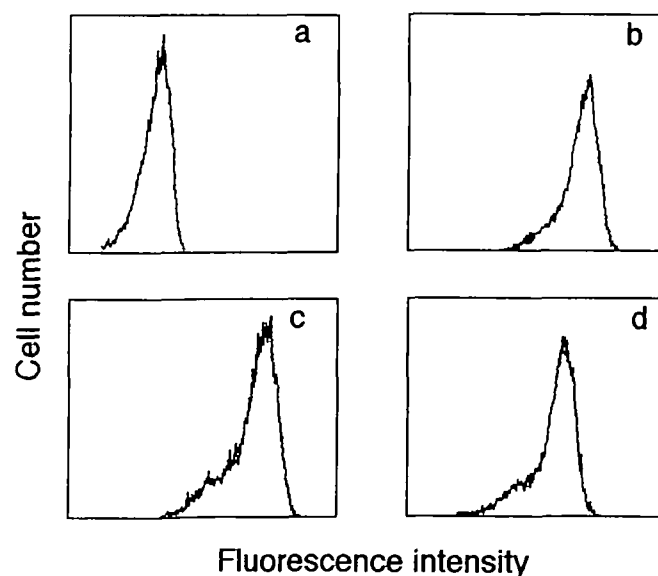


Fig. 10. FACS analysis of *T. cruzi* trypomastigotes incubated 15 min with 1 mM SL and then labeled with mAb 3C9. (a) shows parasites incubated at 37°C and a control antibody. (b) Parasites were incubated at 4°C, (c) at 37°C and (d) at 20°C and bound sialic acid revealed with mAb 3C9. The ordinate axis represent 400 events in each panel.

substrate specificity and reaction mechanism. Other examples include the distinct mechanisms found for the *trans*-glycosylation reactions catalyzed by several hydrolases (Mooser, 1994).

The fact that TS activity is maximal at low temperatures and hydrolysis increases at 37°C initially appears contradictory with the fact that the parasite lives in the mammalian host at 37°C. However, according to our data, transferase versus hydrolysis activity increases with the concentration of acceptors. When the parasite develops in the cytoplasm of mammalian cells, it contains large amounts of nonsialylated mucin-like molecules that cover most of its outer surface. Since these mucins contain multiple β -galactosyl groups, when the parasite disrupt the host cell, the enzyme, also on the parasite surface, will be saturated with acceptors. Thus, all sialic acid donors from the extracellular components of the host will be rapidly incorporated onto the parasite as shown in Figure 10. As the donors are always abundant in the extracellular compartment of the mammalian host, a steady state sialylation level will be attained. Hydrolysis will initially be limited on the parasite surface, but will be more extensive on the host donor proteins that contain a low density of acceptors. This property of the enzyme might be important to fine regulate the parasite–host interactions in different tissues.

It has also been suggested that the TS protein act as a sialic acid lectin (Pereira *et al.*, 1996), due to its poor hydrolytic capacity. Based on the findings presented here, the release of TS bound to sialic acid ligands would occur when an appropriate carbohydrate acceptor is present and the sialyl transfer reaction is completed. This could explain the role of TS during invasion of host cells, the efficiency of which depends on the sialic acid content of the host cells (do Carmo Ciavaglia *et al.*, 1993; Ming *et al.*, 1993; Schenkman *et al.*, 1993). In addition, many *T. cruzi* surface proteins involved in cell invasion have an extensive conserved sequence (Schenkman *et al.*, 1994b), which could represent a binding domain for sialic acid, or other carbohydrates. However, we could never detect binding of purified TS to fetuin-immobilized on agarose, and cell invasion is not affected by adding large amounts of lactose. Whether another type of sialic acid containing molecule could bind TS remains to be investigated.

In conclusion, *trans*-glycosylation is favored in *T. cruzi* sialidase by the fact that the enzyme has a binding site for β -galactosyl residues, which accept the new glycosidic bond as sialic acid is released from the donor. With an increase in the temperature, the enzyme's affinity for the acceptor decreases, with a concomitant increase in the rate of transfer of sialic acid to water. At elevated temperatures this latter reaction can be suppressed by increasing the concentration of the acceptor. As recombinant *T. cruzi* TS can be obtained in a highly homogeneous form (Cremona *et al.*, 1996), and displayed the same activity as expressed by the parasite-derived enzyme, we expect that the combination of mutagenesis, kinetic studies, and the deduction of its tertiary structure will lead to an understanding of the mechanism of *trans*-glycosylation of this unique sialidase.

Materials and methods

Trans-sialidase purification

TS was purified from culture supernatants of *T. cruzi* trypomastigotes by ammonium sulfate precipitation, affinity chromatography through a Tressyl-agarose column with an immobilized monoclonal antibody to TS (mAb 39),

and ion exchange chromatography as described previously (Schenkman *et al.*, 1994a). The recombinant TS was expressed in *E. coli* transformed with the plasmid pTS-cat7. This plasmid contains a 1.9 kb fragment, which encodes the full catalytic domain of TS, inserted into the *NcoI*-*Bam*H1 sites of pQE-60 (Qiagen, Chatsworth, CA). The 1.9 kb fragment was derived by PCR using as template the TS gene of pTS-16 (Schenkman *et al.*, 1994a), and, as primers, the sense oligonucleotide DE15 (5'-GCCCATGGCACCCGGATCGAGCCGAGTT-3') and the antisense oligonucleotide RT154 (5'-CGGGATCCGGGCGTACTTCTTTCAGTCTGCGGCT-3'). The PCR product was initially cloned into pCR-Script vector (Stratagene, La Jolla, CA). As the TS gene contains an internal *Bam*H1 site, the 5' *NcoI*-*Bam*H1 fragment of 340 base pairs was first inserted into pQE-60 followed by addition of the remaining *Bam*H1-*Bam*H1 fragment.

The recombinant TS was purified from *E. coli* (TG1-strain) transformed with pTS-cat 7. Bacteria were grown in SOB medium (Sambrook *et al.*, 1989), containing 50 μ g/ml of ampicillin at 37°C. At OD 600 nm = 0.6–0.7, isopropyl- β -D-thiogalactopyranoside (Life Technologies Gibco BRL, Gaithersburg, MD) was added to a final concentration of 0.1 mM and the culture agitated (150 r.p.m.) overnight at 30°C. Cells were concentrated and washed by centrifugation with 20 mM Tris-HCl, pH 8.0, resuspended in 50 mM sodium phosphate, 0.3 M NaCl, 2 mM MgCl₂, 1 mM PMSF, pH 8.0, and disrupted by 10 \times 1 min pulses of ultrasound. The bacterial lysates were cleared by ultracentrifugation at 100,000 \times g for 60 min and the resulting supernatant was incubated with Ni-NTA-agarose (Qiagen). After 60 min, the resin was washed with 50 mM sodium phosphate, 0.3 M NaCl, 2 mM MgCl₂, 1 mM PMSF, pH 8.0, followed by a second wash with 50 mM sodium phosphate, 0.3 M NaCl, 10% glycerol, pH 6.0. TS was eluted with 0.5 M imidazole in 50 mM sodium phosphate, 0.3 M NaCl, 10% glycerol, pH 6.0, and dialyzed against 20 mM Tris-HCl, pH 8.0. The enzyme was further purified by ion exchange chromatography through a Mono Q column equilibrated in 20 mM Tris-HCl, pH 8.0 and eluted with a linear gradient of 0 to 1 M NaCl in the same buffer. The purity of each batch of TS was checked by 10% SDS-polyacrylamide gel electrophoresis, and the protein concentration was estimated by the Bradford procedure (Bio-Rad, Hercules, CA). Figure 1b shows the elution pattern of a representative batch of rTS of the Mono Q column, and Figure 1c the SDS gel stained with Coomassie blue of the activity containing fraction. From a 2 l initial culture we obtained from 2 to 4 mg of purified enzyme, which was stored in aliquots at -20°C.

Activity measurements

TS activity was measured by following transfer of sialic acid from SL to D-glucose-1-[¹⁴C]-lactose (Amersham, Buckinghamshire, UK). The reactions were made in 50 μ l of a solution containing 20 mM HEPES pH 7.0 buffer, 0.2% ultrapure bovine serum albumin (Boehringer, Mannheim, Germany), the indicated concentrations of the sialic acid donor, and 0.36 nmol (7.2 μ M) of radioactive lactose (60 mCi/mmol). Unlabeled lactose was added when required to adjust to the desired final concentration of acceptor. The reactions were initiated by addition of enzyme. Incubations proceeded at the indicated temperatures and were stopped by addition of 1 ml of water. The radioactive sialylated products were purified by chromatography in 0.5 ml columns of QAE-Sephadex A-25, equilibrated with water. Unreacted [¹⁴C]-lactose was washed out with 8 ml of water and bound [¹⁴C]-SL was eluted with 1 ml of 1 M ammonium formate and quantified by liquid scintillation. On these conditions, complete sialyl transfer reaction yields about 30,000 cpm. However, as the sialic acid exchange reaction is not linear above 25% of total transfer, all measurements were made by diluting the enzymes to obtain no more than 7500 cpm.

Hydrolysis of SL was measured by thiobarbituric assay. The assays contained 20 mM HEPES pH 7.0 buffer, 0.002% serum albumin, in 40 μ l and the indicated amounts of substrates. At the end of incubations, released sialic acid was determined by the assay described previously (Powell and Hart, 1986), followed by light absorption at 549 nm. To obtain detectable amounts of free sialic acid, the hydrolysis reactions were incubated longer than the isotopic exchange reactions.

Hydrolysis and sialic acid transfer to radiolabeled lactose were also performed by using 4-methyl-umbelliferyl-N-acetylneuraminic acid (4-MuNana) as donor substrate. In these cases both reactions could be performed simultaneously in a 100 μ l volume containing 20 mM HEPES pH 7.0, 0.2% BSA, and the indicated amounts of 4-MuNana and radiolabeled lactose. At the end of the incubation period, half of the mixture was diluted with 0.1 M Tris-HCl pH 9.5 and the fluorescence emission at 450 nm of methyl-umbelliferone with excitation at 365 nm measured directly in a Perkin-Elmer spectrofluorimeter. The concentration of dye was determined by measuring the fluorescence of a standard of methyl-umbelliferone in the same conditions. The other half of the sample was diluted in 1 ml of water and the radiolabeled SL measured as described above. All measurements were made in triplicate and represent at

least two independent experiments. The results are expressed as nanomoles of the product formed per minute.

Immunofluorescence experiments and FACS analysis

Trypomastigotes from Y strain, were obtained from culture supernatants of LLCMK₂ cells grown in low glucose Dulbecco's modified Eagle's medium with penicillin, streptomycin, and 10% fetal bovine serum at 37°C, 5% CO₂ as described previously (Schenkman *et al.*, 1994a). At the third day after infection the medium was replaced by DMEM containing 0.2% BSA. Parasites were collected at the fifth day, washed, and incubated in the presence of 1 mM of SL at the indicated temperatures at 2×10^7 parasites/ml in DMEM, 0.2% BSA. After 15 min, the parasites were centrifuged and resuspended in DMEM-0.2% BSA and 0.02% NaN₃ at 4°C and incubated 30 min with 3 µg/ml of mAb 3C9, which reacts with a sialic acid dependent epitope (Schenkman *et al.*, 1991). Parasites were washed by centrifugation, resuspended in 0.1 ml of DMEM-0.2% BSA, and then fixed with 10 volumes of 4% paraformaldehyde in PBS. The fixative was removed by centrifugation. Parasites were washed with PBS and treated with 1% BSA in PBS followed by incubation with a fluorescein isothiocyanate conjugated goat anti-mouse IgG (Boehringer, Einheiten, Germany). Parasites were washed again, resuspended in 0.1 ml of DMEM-0.2% BSA, fixed with 4% paraformaldehyde, and analyzed by FACS as in reference (Schenkman *et al.*, 1991).

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Abbreviations

TS, *trans*-sialidase; 4-MuNana, 4-methyl-umbelliferyl-N-acetyl-neuraminic acid; SL, sialyllactose.

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