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Note

The *trans*-sialidase from *Trypanosoma cruzi* efficiently transfers α-(2→3)-linked *N*-glycolylneuraminic acid to terminal β-galactosyl units

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Abstract—The *trans*-sialidase from *Trypanosoma cruzi* (TcTS), the agent of Chagas' disease, is a unique enzyme involved in mammalian host-cell invasion. Since *T. cruzi* is unable to synthesize sialic acids de novo, TcTS catalyzes the transfer of α-(2→3)-sialyl residues from the glycoconjugates of the host to terminal β-galactopyranosyl units present on the surface of the parasite. TcTS also plays a key role in the immunomodulation of the infected host. Chronic Chagas' disease patients elicit TcTS-neutralizing antibodies that are able to inhibit the enzyme. *N*-Glycolylneuraminic acid has been detected in *T. cruzi*, and the *trans*-sialidase was pointed out as the enzyme involved in its incorporation from host glycoconjugates. However, *N*-glycolylneuraminic acid α-(2→3)-linked-containing oligosaccharides have not been analyzed as donors in the *T. cruzi trans*-sialidase reaction. In this paper we studied the ability of TcTS to transfer *N*-glycolylneuraminic acid from Neu5Gc(α2→3)Gal(β1→4)GlcβOCH$_2$CH$_2$N$_3$ (**1**) and Neu5Gc(α2→3)Gal(β1→3)GlcNAcβOCH$_2$CH$_2$N$_3$ (**2**) to lactitol, *N*-acetyllactosamine and lactose as acceptor substrates. Transfer from **1** was more efficient (50–65%) than from **2** (20–30%) for the three acceptors. The reactions were inhibited when the enzyme was preincubated with a neutralizing antibody. K_m values were calculated for **1** and **2** and compared with 3′-sialyllactose using lactitol as acceptor substrate. Analysis was performed by high-performance anion-exchange (HPAEC) chromatography. A competitive transfer reaction of compound **1** in the presence of 3′-sialyllactose and *N*-acetyllactosamine showed a better transfer of Neu5Gc than of Neu5Ac.

Keywords: HPAEC; Neu5Gc transfer; *trans*-Sialidase; *Trypanosoma cruzi*

The *trans*-sialidase from *Trypanosoma cruzi* (TcTS), the agent of Chagas' disease, is a unique enzyme important for the survival of the parasite in the mammalian or insect host.[1,2] This process involves the transfer of α-(2→3)-linked sialic acid from host glycoconjugates to terminal β-galactopyranosyl units of parasite mucins.[3] This is the pathway that *T. cruzi* utilizes to incorporate sialic acid, since it is unable to synthesize it. The TcTS not only acts on the surface of the parasite, but it is also shed into the blood during infection.[4] Animals that survive infection, as well as chronic Chagas' disease patients, elicit TS-neutralizing antibodies that are able to inhibit TS enzymatic activity, thus limiting the period in which the TS can systemically operate to the early steps of the infection.[5]

In strains belonging to the less virulent lineage 1, terminal β-D-galactofuranosyl units are also present in the oligosaccharides.[6–9] We have recently proved that the presence of the furanosyl units does not hamper sialylation of the terminal galactopyranose in the oligosaccharides.[10] We have also described that lactose derivatives could act as inhibitors of the enzyme, proving that the sugar β-linked to galactopyranose is not critical for the acceptor properties. In fact, lactitol and lactobionic acid, which are open-chain derivatives of lactose, are very good acceptors of sialic acid. Lactitol, which was the best of the ones tested, effectively inhibited the

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transfer of sialic acid to *N*-acetyllactosamine and inhibited parasite resialylation.[11] Lactitol also prevented the apoptosis caused by the TcTS.[12]

Recombinant TS has also been used for the preparation of sialylated oligosaccharides using a phenolic sialoside[13–16] or sialyllactose obtained from bovine colostrum[17,18] as donors.

N-Glycolylneuraminic acid (Neu5Gc) occurs frequently in the animal kingdom, but is presumed absent from normal human tissues.[19] Its presence in human tumor cells has been described.[20] The authors attributed the enrichment of Neu5Gc in carcinomas and fetuses to a higher uptake by these fast-growing tissues. Traces of Neu5Gc could be incorporated from dietary

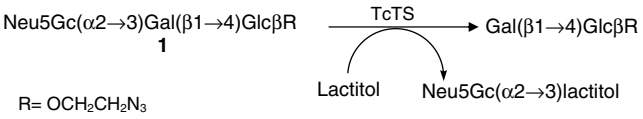

Figure 1. *N*-Glycolylneuraminic acid transfer by *Trypanosoma cruzi trans*-sialidase from donor **1** to lactitol.

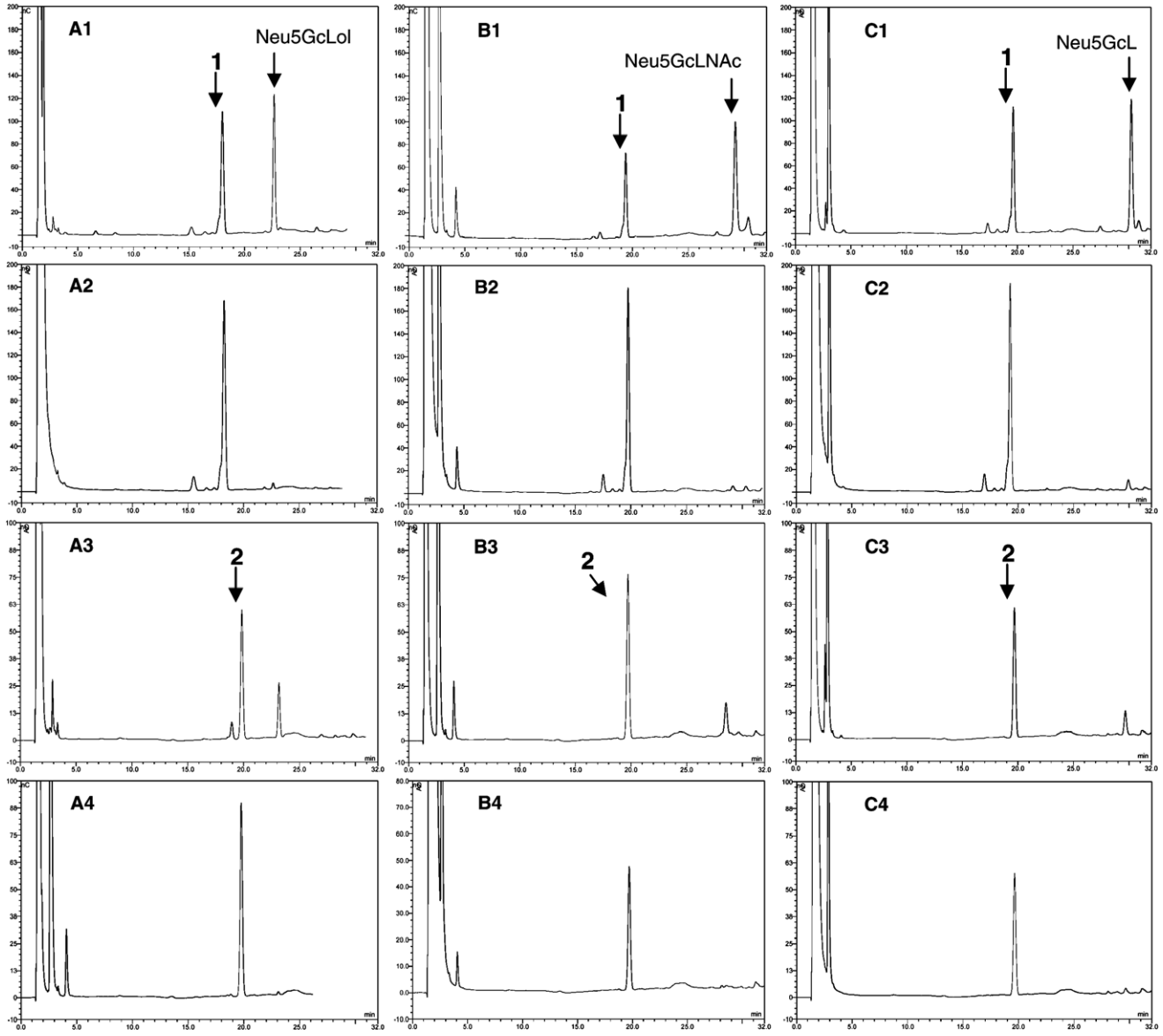

Figure 2. Transfer analysis of Neu5Gc from Neu5Gc-oligosaccharides to acceptor substrates by the *trans*-sialidase from *Trypanosoma cruzi*. **A**(**1**–**4**), acceptor substrate lactitol; **B**(**1**–**4**), acceptor substrate *N*-acetyllactosamine; **C**(**1**–**4**), acceptor substrate lactose. **A1**–**C1** donor **1** was incubated with the acceptor and *trans*-sialidase for 15 min at 25 °C, and the reaction mixtures were analyzed by HPAEC-PAD as described under Section 1. **A2**–**C2** is the same as above but the *trans*-sialidase was preincubated with a neutralizing antibody. **A3**–**C3** donor **2** was used and incubated as for donor **1**. **A4**–**C4** donor **2** and TcTS preincubated with the antibody were used. Neu5GcLol, *N*-glycolylneuraminyllactitol; Neu5GcLNAc, *N*-glycolylneuraminyl *N*-acetyllactosamine; Neu5GcL, *N*-glycolylneuraminyllactose; **1**, Neu5Gcα2→3Galβ1→4GlcβOCH$_2$CH$_2$N$_3$; **2**, Neu5Gcα2→3Galβ1→3GlcNAc-βOCH$_2$CH$_2$N$_3$.

sources,[21] as humans are genetically unable to produce it, and circulating *anti*-Neu5Gc antibodies are present in most normal humans.

N-Glycolylneuraminic acid has been detected in *T. cruzi* by selective acid hydrolysis of epimastigote cells. It was rightly suggested by the authors that the Neu5Ac/Neu5Gc ratios obtained from different strains reflected the composition of the culture medium.[22] Accordingly, it was reported that blood-stream trypomastigote forms obtained from infected mice contain mainly Neu5Gc, and a *trans*-sialidase was suggested as responsible for its incorporation.[23] *N*-Glycolylneuraminic acid is found in 98% of the total sialic acids in mouse serum.[24] However, as far as we know, Neu5Gcα(2→3)-linked oligosaccharides have only been tested as donors in the *trans*-sialidase reaction of *Trypanosoma congolense*. In this case, a culture supernatant of procyclic cells was used as a source for the enzyme, with a labeled substrate as acceptor.[25]

In the present work, we studied for the first time the ability of recombinant TcTS to transfer Neu5Gc from Neu5Gcα(2→3)Galβ1-R trisaccharides to *N*-acetyllactosamine, lactose or lactitol, which are all good acceptor substrates for the enzyme.

The transfer to terminal β-galactopyranosyl units was studied using Neu5Gcα2→3Galβ1→4GlcβOCH_2-CH_2N_3 (**1**) and Neu5Gcα2→3Galβ1→3GlcNAcβO-$CH_2CH_2N_3$ (**2**) as donor substrates.

As shown in Figure 1 the reaction is illustrated for donor **1** and lactitol as acceptor. Analysis was performed using high-performance anion-exchange chromatography with pulse amperometric detection (HPAEC-PAD). Under the conditions used (see Section 1), the retention times for compounds **1** (t_R 18.00 min) and **2** (t_R 19.70 min) were similar but well separated from the substrates and products in each case. For the three acceptor substrates, the transfer from trisaccharide **1** was more efficient (transfer values 50–65%) than from trisaccharide **2** (20–30%) (Fig. 2). As expected, the presence of an additional OH, in the sialic acid N-substituent, increases the retention time of the glycolylated derivatives with respect to the acetylated ones. For example, Neu5GcLactitol (t_R 22.6 min) elutes more than three times later than Neu5AcLactitol (t_R 6.1 min) (not shown). Free Neu5Gc was eluted at 26.1 min and was not detected, excluding hydrolase activity for the TcTS. To compare the affinity of the *N*-glycolylneuraminic acid oligosaccharides to the enzyme, K_m values were calculated for **1**, **2** and 3′-sialyllactose using lactitol as the acceptor substrate, under the same conditions, showing that both Neu5Gc-disaccharides were better substrates than 3′-sialyllactose having **1** ($K_m = 0.84$ mM, $V_m = 0.48$ pmol/min ng) higher affinity than **2** ($K_m = 1.53$ mM, $V_m = 0.33$ pmol/min ng). In agreement with our results, in a comparative study using Neu5Acα(2→3) linked oligosaccharides, it was shown that the saccharides in which Gal is β-(1→4)-linked to GlcNAc were better donors of Neu5Ac than those in which Gal is β-(1→3)-linked.[26] The K_m and V_m values we obtained for 3′-sialyllactose were 3.21 mM and 1.70 pmol/min ng, respectively. Hydroxylation of the *N*-acyl moiety in the sialic acid offers the possibility of another interaction with a conveniently located amino acid of the TcTS.

It has been determined that the neutralization of TcTS circulating activity by monoclonal neutralizing antibodies (mNtAb) reduced the sialic acid content in platelets[27]

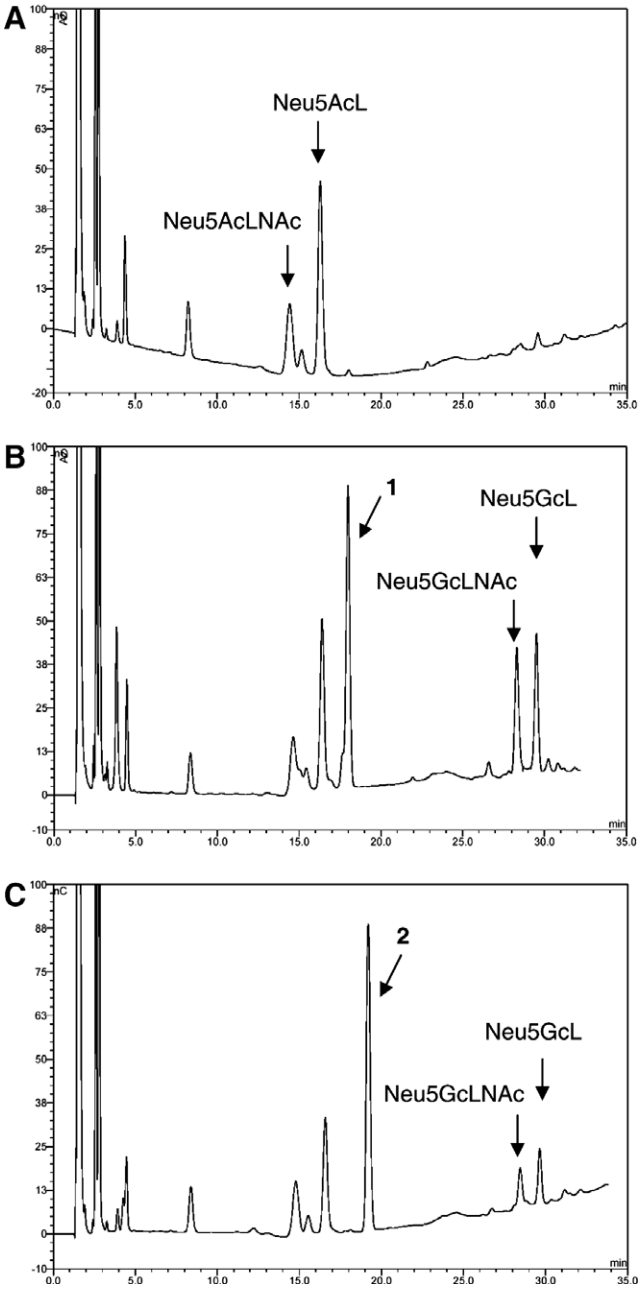

Figure 3. Competitive transfer of *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid by TcTS. 3′-Sialyllactose was incubated with *N*-acetyllactosamine as acceptor substrate, the TcTS (panel A) and in the presence of donors **1** (panel B) or **2** (panel C) and analyzed by HPAEC-PAD. Abbreviations and conditions as for Figure 2.

and prevented the immune system damage, even in extremely virulent models.[5] In our case, transfer of Neu5Gc was almost completely inhibited when the enzyme was incubated with the monoclonal antibody previous to incubation with the acceptor substrates (Fig. 2). This result confirms that the same active site is being used for the transfer of sialic acid from Neu5Gc and Neu5Ac-containing glycoconjugates.

Competitive transfer of Neu5Gc with respect to Neu5Ac was studied for oligosaccharides **1** and **2** by incubating each with sialyllactose, TcTS and *N*-acetyllactosamine as acceptor substrate, with all substrates in 1 mM concentrations (Fig. 3). The peak at 8.40 min corresponds to free sialic acid present in our sample of Neu5AcL, but does not interfere with the experiment. Comparison with the incubation in the absence of the Neu5Gc oligosaccharides (Fig. 3A) showed that, apparently, Neu5GcLNAc was formed in a larger amount than Neu5AcLNAc in the presence of compound **1** and sialyllactose as donors. Moreover, the lactose obtained when Neu5Ac is transferred from Neu5Aclactose to *N*-acetyllactosamine is readily used by TcTS as an acceptor substrate forming Neu5Gclactose (Fig. 3, panels B and C). Neu5GcL is co-chromatographed with a sample obtained from bovine colostrum (not shown).

In conclusion, we describe for the first time that *N*-glycolylneuraminic acid is efficiently transferred by a recombinant TcTS from synthetic Neu5Gc-containing trisaccharides to the common *N*-acetyllactosamine unit of glycoconjugates. The reaction could be used for preparative purposes of Neu5Gc-containing glycoconjugates.

1. Experimental

1.1. Oligosaccharide substrates

The donor substrates, Neu5Gcα2→3Galβ1→3GlcNAcβ$OCH_2CH_2N_3$ and Neu5Gcα2→3Galβ1→4Glcβ $OCH_2CH_2N_3$. were kindly provided by the Consortium for Functional Glycomics. Lactose was from Calbiochem (San Diego, CA). Lactitol and *N*-acetyllactosamine were purchased from Sigma Chemical Co. Neu5AcLactose (3′-Sialyllactose) was obtained from bovine colostrum by an adaptation of a reported method.[28]

1.2. General procedures

For the sialylation experiments a recombinant TcTS expressed in *Escherichia coli* was kindly provided by A. C. C. Frasch, and a TS-neutralizing mouse monoclonal antibody (immunoglobulin G2a) directed to the active site of the enzyme was from O. Campetella (UNSAM, Universidad Nacional de General San Martin, Buenos Aires, Argentina).

Analysis by HPAEC-PAD was performed using a Dionex ICS-3000 HPLC system equipped with a pulse amperometric detector (PAD). A CarboPac PA-100 ion-exchange analytical column (4 × 250 mm) equipped with a guard column PA-100 (4 × 50 mm) was eluted with a linear gradient over 60 min from 50 to 300 mM NaAcO in 100 mM NaOH at a flow rate of 1.0 mL/min at room temperature.

1.3. Enzyme kinetics

Reaction mixtures of 20 μL containing 20 mM Tris buffer, pH 7, 30 mM NaCl, 1 mM Neu5Gcα2→3Galβ1→4GlcβO$CH_2CH_2N_3$ (**1**) or Neu5Gcα2→3Galβ1→3GlcNAcβO$CH_2CH_2N_3$ (**2**) as donors and 1 mM lactitol, *N*-acetyllactosamine or lactose as acceptor substrates were incubated with 300 ng purified *trans*-sialidase. After incubation, reaction mixtures were diluted with 40 μL deionized water and frozen. Sialylation of acceptors was calculated as the percentage of the sialylated product obtained over the total amount of sialic acid (free or linked to a saccharide).

For K_m calculations, 1 mM lactitol and different concentrations of the donor substrates were incubated for 15 min at 25 °C as before. Samples were then diluted 5 times, and 20 μL of each were analyzed by HPAEC. The extent of sialylation of substrates was calculated from the decrease in the concentration of the donor substrate using galacturonic acid as internal standard, in comparison with the corresponding control without enzyme. The K_m values were obtained graphically by the Lineweaver–Burk method.[29]

1.4. Inhibition of TcTS by neutralizing monoclonal antibody

Reaction mixtures of 20 μL containing 20 mM Tris buffer, pH 7, 30 mM NaCl, 1 mM Neu5Gcα2→3Galβ1→4GlcβO$CH_2CH_2N_3$ (**1**) or Neu5Gcα2→3Galβ1→3GlcNAcβO$CH_2CH_2N_3$ (**2**) as donors and 1 mM lactitol, *N*-acetyllactosamine or lactose as acceptor substrates were incubated with 300 ng purified *trans*-sialidase previously incubated for 15 min with the neutralizing monoclonal antibody. After incubation, reaction mixtures were diluted with 40 μL of deionized water and frozen.

1.5. Competitive transfer of Neu5Gc and Neu5Ac to *N*-acetyllactosamine

Reaction mixtures of 20 μL containing 20 mM Tris buffer, pH 7, 30 mM NaCl, 1 mM 3′-sialyllactose, 1 mM *N*-acetyllactosamine, and 1 mM disaccharides **1** or **2** were incubated with purified *trans*-sialidase for 15 min at room temperature. Samples were then diluted 5 times with deionized water and analyzed by HPAEC.

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