

NMR spectroscopic and molecular modeling investigations of the *trans*-sialidase from *Trypanosoma cruzi*

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Nuclear magnetic resonance (NMR) spectroscopy was used to investigate the transfer of sialic acid from a range of sialic acid donor compounds to acceptor molecules, catalyzed by *Trypanosoma cruzi trans*-sialidase (TcTS). We demonstrate here that NMR spectroscopy is a powerful tool to monitor the *trans*-sialidase enzyme reaction for a variety of donor and acceptor molecules. The hydrolysis or transfer reactions that are catalyzed by TcTS were also investigated using a range of *N*-acetylneuraminosyl-based donor substrates and asialo acceptor molecules. These studies showed that the synthetic *N*-acetylneuraminosyl donor 4-methylumbelliferyl α -D-*N*-acetylneuraminide (MUN) is hydrolyzed by the enzyme ~3–5 times faster than either the disaccharide Neu5Ac α (2,3)-Gal β 1Me or the trisaccharide Neu5Ac α (2,3)Lac β 1Me. In the transfer reaction, we show that Neu5Ac α (2,3)Lac β 1Me is the most favorable substrate for TcTS and is a better substrate than the naturally-occurring *N*-acetylneuraminosyl donor α 1-acid glycoprotein. In the case of MUN as the donor molecule, the transfer of Neu5Ac to different acceptors is significantly slower than when other *N*-acetylneuraminosyl donors are used. We hypothesize that when MUN is bound by the enzyme, the orientation and steric bulk of the umbelliferyl aglycon moiety may restrict the access for the correct positioning of an acceptor molecule. AutoDock studies support our hypothesis and show that the umbelliferyl aglycon moiety undergoes a strong *pi*-stacking interaction with Trp-312. The binding properties of TcTS towards acceptor (lactose) and donor substrate (Neu5Ac) molecules have also been investigated using saturation transfer difference (STD) NMR experiments. These experiments, taken together with other published data, have clearly demonstrated that lactose in the absence of other coligands does not bind to the TcTS active site or other binding domains. However, in the presence of the sialic acid donor, lactose (an asialo acceptor) was observed by NMR spectroscopy to interact with the enzyme's active site. The association of the asialo acceptor with the active site is an absolute requirement for the transfer reaction to proceed.

Key words: Chagas' disease/NMR spectroscopy/sialic acid/*Trypanosoma cruzi*

Introduction

Chagas' disease is an incurable condition that afflicts millions of individuals in Latin America. The intracellular protozoal parasite *Trypanosoma cruzi* is responsible for this debilitating condition (Schenkman *et al.*, 1991). As part of the infection process, the parasite sheds a developmentally regulated *trans*-sialidase that is believed to play a role in parasite–host cell interactions (Schenkman *et al.*, 1994), but a population of TcTS also remains linked via a glycosylphosphatidylinositol anchor to the parasite surface (Previato *et al.*, 1995).

Trypanosomes are unable to synthesize sialic acids and use the *trans*-sialidase to scavenge sialic acids from exogenous cell surface glycoconjugates. Acquisition of the Neu5Ac moieties results in the *T. cruzi* parasite acquiring a negatively charged glycopeptide coat that allows it to survive in the bloodstream (Engstler and Schauer, 1993; Schauer *et al.*, 1995). The trypanosomes transfer sialic acids to mucin-like acceptors present in their plasma membrane (Ferrero-Garcia *et al.*, 1993; Schenkman *et al.*, 1991; Scudder *et al.*, 1993) and these mucins have been implicated in cell adhesion and invasion (Pollevick *et al.*, 2000; Schenkman *et al.*, 1991).

Interestingly, the capacity of *T. cruzi* to acquire sialic acids from host cells is a feature shared by a restricted set of protozoa, including *Trypanosoma brucei* and *Trypanosoma congolense* (Engstler *et al.*, 1993, 1995; Medina-Acosta *et al.*, 1994a, b; Schenkman *et al.*, 1991). The regiospecificity of the *trans*-sialidase appears to be absolute, with the enzyme only transferring α (2,3)-linked sialic acids (Ferrero-Garcia *et al.*, 1993; Schenkman *et al.*, 1991; Vandekerckhove *et al.*, 1992) from the host to terminal β -linked galactoside residues in the mucin-like glycoproteins of the parasite. This enzyme is, however, considered to be a modified sialidase rather than a sialyltransferase, because it does not utilize CMP-*N*-acetylneuraminic acid as the sialosyl donor (Paulson and Colley, 1989). Interestingly, no other sialidase is as efficient at transferring *N*-acetylneuraminic acid; in fact, very few glycosidases are more efficient in glycotransfer rather than glycohydrolysis of a terminal sugar residue.

Presently there is a paucity of therapeutic agents able to adequately arrest the infection by the *T. cruzi* parasite and the progression of Chagas' disease (Buschiazzo *et al.*, 2002; Croft and Karbwang, 2000; Morello, 1988; Urbina, 2000). The *T. cruzi trans*-sialidase (TcTS) is considered a valid target for drug design because it appears to play a pivotal

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role in the successful life cycle of the parasite that has had such a significant health impact on the human population.

Recently the X-ray structure of TcTS and TcTS in complex with substrates and sialidase inhibitors has been published (Buschiazzo *et al.*, 2002). A significant number of amino acid residues are conserved within the active site of TcTS that are common to all known sialidases, reflecting a strong evolutionary link to other microorganisms. However, critical amino acid residue differences between well-known viral, bacterial, and mammalian sialidases and the parasite *trans*-sialidase provide a basis for an explanation of the interesting glycotransfer enzymatic activity of TcTS. Interestingly, these structural studies revealed that *N*-acetylneuraminic acid binding triggers a conformational switch that further activates TcTS and modulates the binding affinity of the acceptor substrate (Buschiazzo *et al.*, 2002).

For native TcTS, the side chain of Tyr-119 is found to adopt a conformation where the aromatic ring is directed toward the floor of the catalytic pocket and is stabilized by water-mediated hydrogen bonds, occupying some of the region where *N*-acetylneuraminic acid would be expected to reside. In effect, this side chain is observed to block substrate binding in the active site. Tyr-119 is only one of a number of residues contributing to a hydrophobic environment located within the catalytic site. On the occupation of the catalytic site by substrates or inhibitors, the aromatic ring of Tyr-119 is found to adopt two alternative positions. In one orientation, the side chain moves to participate in a hydrogen-bond interaction with the glycerol sidechain of *N*-acetylneuraminic acid ligand, and in so doing is able to orient itself more favorably to exploit the hydrophobic environment defined by a number of hydrophobic residues. In the second orientation, the aromatic side chain resides completely outside the *N*-acetylneuraminic acid-binding cleft. In the absence of *N*-acetylneuraminic acid, no lactose association to the enzyme had been detected (Buschiazzo *et al.*, 2002). However when *N*-acetylneuraminic acid binds the active site residue Tyr-119 side chain rotates to the outside location. This switching mechanism allows an asialo acceptor to enter the active site (Buschiazzo *et al.*, 2002).

Our long-standing interest in the *trans*-sialidase, in particular the use of nuclear magnetic resonance (NMR) spectroscopic techniques to study this enzyme (Wilson *et al.*, 2000) has led us to perform an extensive NMR-based investigation of TcTS with a variety of substrate and acceptor molecules. We felt that this study may provide new insight into the preference of the enzyme for various donor substrates as well as possibly shedding light on how these donor substrates and other ligands interact with the enzyme at the molecular level.

Docking studies of the sialic acid donor molecule 4-methylumbelliferyl α -D-*N*-acetylneuraminide (MUN) into the binding site of TcTS using the AutoDock program were also performed to provide an explanation of our determined NMR-based relative rates. Additionally, ^1H saturation transfer difference (STD) NMR experiments of fully active TcTS in complex with lactose and lactose in the presence with *N*-acetylneuraminic acid provide direct evidence that lactose in the absence of other ligands does not bind TcTS.

Results

^1H NMR spectroscopy was used to monitor the TcTS catalyzed reaction for a variety of diverse sialic acid donor and acceptor molecules. In undertaking these experiments, we attempted to quantify the data by acquiring time-coursed NMR spectra. Our method shows that NMR spectroscopy is an excellent tool for monitoring enzyme reactions and has several advantages over typical biochemical assays. The advantage of using NMR for monitoring enzyme reactions is that this noninvasive method allows the build-up of products and consumption of substrates to be monitored simultaneously. An essential requirement for investigating enzyme-mediated reactions by NMR is that the substrates and products must show at least one resolved signal (Wilson *et al.*, 2000).

In this study, an extensive NMR-based enzyme reaction analysis using a broad variety of *N*-acetylneuraminosyl donor and acceptor molecules is presented. As stated earlier and as reported by others, TcTS is far more efficient at transferring *N*-acetylneuraminic acid residues to acceptor molecules than to water (Parodi *et al.*, 1992; Schenkman *et al.*, 1992). To determine relative rates, ^1H NMR spectra were acquired for each of the *N*-acetylneuraminic acid-based substrates in the presence of *N*-acetylneuraminosyl acceptor molecules and TcTS over time. Figure 1 shows the evolution of reaction **i** using MUN as *N*-acetylneuraminic acid donor and Gal β 1Me as *N*-acetylneuraminic acid acceptor molecule over time. It can be clearly seen that after a 35-min incubation period, the product Neu5Ac(2,3)-Gal β 1Me has been formed and the H1Gal proton signal at 4.39 ppm is visible. With increasing incubation times, this signal shows a clearly greater intensity, whereas the signal intensity of the *N*-acetylneuraminic acid donor molecule MUN (e.g., H3eq at 2.87 ppm, $-\text{CH}_3$ at 2.47 ppm) noticeably decreases.

Integral values for key resonances of the substrate and catalysis products were monitored during the catalytic reaction. From these data the relative rate of the reaction was calculated. The published K_m value for sialyllactose is 1.2 mM for the recombinant TcTS (Ribeirão *et al.*, 1997). NMR experiments were conducted with substrate concentrations at least three times higher than the K_m value to ensure that the enzyme was completely saturated and therefore the reaction kinetics were independent of substrate concentration. Under these conditions the determination of initial slopes in the linear regime of the time-based function was performed and then calculated into relative rates. Our aim was not to perform a full NMR-based kinetic investigation, but rather an NMR-based comparative rate analysis that will provide information concerning preferred *N*-acetylneuraminosyl donor/acceptor specificity. Absolute rates have been determined for selected compounds.

The TcTS catalyzed hydrolysis of N-acetylneuraminosyl donors

The primary catalytic function of TcTS in the life cycle of *T. cruzi* is the transfer of Neu5Ac from host cell sialylglycoconjugates to terminal β -Gal residues of mucin-like molecules on the parasite surface. In the absence of suitable Neu5Ac acceptor molecules, TcTS catalyzes sialoside

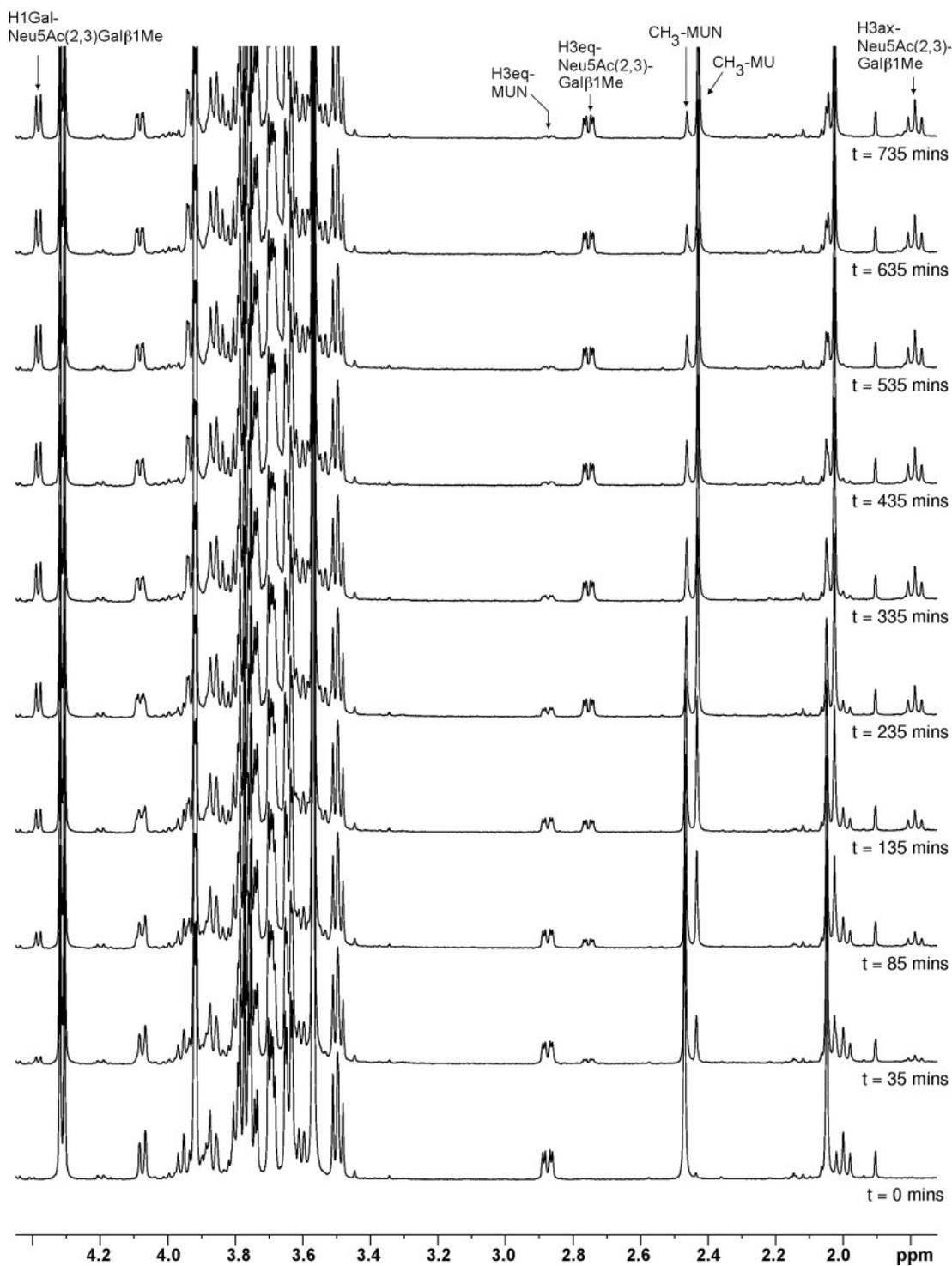


Fig. 1. ¹H NMR spectra of reaction **i** at different times using MUN as the neuraminic acid donor and Galβ1Me as neuraminic acid acceptor molecule at 600 MHz and 298 K. The formation of the product Neu5Ac(2,3)Galβ1Me can be clearly seen by the increasing signal intensity of the protons H1-Gal and H3eq. In addition, the decreasing intensity of the neuraminic acid donor molecule MUN can be detected by following the signal intensities at 2.87 ppm (H3eq) and 2.47 ppm (–CH₃).

hydrolysis, cleaving the Neu5Ac residue from sialyl donors to liberate Neu5Ac and asialoglyco conjugates. It has previously been shown (Todeschini *et al.*, 2000) that the hydrolysis of substrates such as MUN and α(2,3)-sialyllactose by

TcTS proceed with retention of anomeric configuration. In an attempt to better understand this reaction, the present study investigates the capacity of TcTS to act as a glycohydrolase on a range of synthetic and naturally occurring

Table I. Hydrolysis of substrates by TcTS (decay of *N*-acetylneuraminosyl donor signals and build up of β -Neu5Ac).

	<i>N</i> -acetylneuraminosyl donor	Product	Build up of β -Neu5Ac signal	Decay of Neu5Ac donor signals
a	MUN	4-Methylumbelliferyl	1.00	1.00
b	Neu5Ac α (2,3)Gal β 1Me	Gal β 1Me	0.37	0.28
c	Neu5Ac α (2,3)Lac β 1Me	Lac β 1Me	0.26	0.20
d	α 1-acid glycoprotein	Asialo α 1-acid glycoprotein	0.63	—
e	Neu5Ac α (2,6)Lac β 1Me	No hydrolysis	No hydrolysis	No hydrolysis

Relative rates were obtained by normalizing the initial slope of the integrals versus time function of reaction **a** to 1.00.

substrates (Table I). ^1H NMR spectra were acquired for each of the *N*-acetylneuraminic acid-based substrates in the presence of TcTS over time. Integral values for key resonances of the substrate (such as methyl group of the methylumbelliferyl aglycon in MUN) and catalysis products (the H-3eq proton of β -Neu5Ac, δ 2.22 ppm) were monitored during the catalytic reaction. The use of the integral value for the thermodynamically more stable β -Neu5Ac anomer is an indirect approach to the quantification of relative rates, because the TcTS acts with retention of configuration and releases the α -Neu5Ac anomer that mutarotates to the β -Neu5Ac anomer. However it is not unreasonable to assume that the mutarotation rate is similar for all investigated reactions, and this is further supported by the fact that the relative rates (as determined by use of the integral value for the methyl group of the methylumbelliferyl aglycon in MUN) are in very good agreement with the rates determined from the H-3eq proton of β -Neu5Ac (Table I). From these data the relative rate of hydrolysis compared to MUN was calculated (Table I).

The synthetic substrate MUN (**a**, Table I) is hydrolyzed more rapidly ($13 \mu\text{M min}^{-1}$) than the sialosides Neu5Ac α (2,3)Gal β 1Me (entry **b**, 3.6-fold slower) and Neu5Ac α (2,3)Lac β 1Me (**c**, 5-fold slower) when considering the sialoside signal decay. These observations are in good agreement with relative rates determined by indirect standard biochemical and radioisotope-labeled assays (Ribeirão *et al.*, 1997). It proved more difficult to directly monitor the signal decay of the Neu5Ac-containing glycoprotein α 1-acid glycoprotein (**d**, Table I) as a result of the complexity of the spectra. For this reason the progression of the hydrolysis of α 1-acid glycoprotein was measured by indirect means. Accordingly, the build-up of β -Neu5Ac, the thermodynamically more stable anomer of Neu5Ac in solution, was monitored by the increasing intensity of the diagnostic H-3eq proton of β -Neu5Ac. In this way it was possible to estimate the hydrolysis rate of α 1-acid glycoprotein and compare this to the rate of MUN hydrolysis. As shown, α 1-acid glycoprotein is hydrolyzed 1.6 times slower than MUN (**d**, Table I). As expected, no hydrolysis of Neu5Ac α (2,6)Lac β 1Me (**e**, Table I) was observed, because TcTS does not recognize α (2,6)-linked sialic acids (Scudder *et al.*, 1993; Vandekerckhove *et al.*, 1992). Interestingly, similar rates of hydrolysis for the α (2,3)-linked substrates, Neu5Ac α (2,3)Gal β 1Me and Neu5Ac α (2,3)Lac β 1Me, were observed.

The TcTS catalyzed transfer of N-acetylneuraminic acid from N-acetylneuraminosyl donors

The transferase activity of TcTS catalyzes the transfer of α (2,3)-linked *N*-acetylneuraminic acid to terminal β -D-galactose-based acceptors. This reaction is the most important function of the TcTS enzyme because the enzyme is more efficient in the transfer of *N*-acetylneuraminic acid from donor to acceptor molecules than in the hydrolysis of *N*-acetylneuraminic acid-containing molecules. In a recent study (Buschiazzo *et al.*, 2002), the structural basis for the transferase reaction has been elucidated and an explanation offered as to why TcTS is more efficient in the glycotransfer reaction. In the present study we compare the *N*-acetylneuraminosyl-based donor capability of seven substrates for TcTS with a range of asialo acceptor molecules. The results from this study are presented in Table II and were obtained by analysis of the ^1H NMR spectra for each of the *N*-acetylneuraminosyl-based substrates in the presence of TcTS and different asialo acceptors over time. Integral values for key resonances of the donor, acceptor, and transfer products were monitored during the course of the reaction. From these data the relative rates were calculated and are presented in Table II.

N-acetylneuraminosyl acceptor substrate specificity

To evaluate the importance of the nature of the acceptor molecule, the relative rates of transfer of Neu5Ac to varying acceptors were compared using the same Neu5Ac donor molecule (Table II). Previously it has been suggested (Scudder *et al.*, 1993) that the nature of the acceptor molecule may influence Neu5Ac transfer rates; through the employment of NMR spectroscopic methods, we believed that we could provide definitive and direct evidence for this hypothesis.

In an initial set of experiments (**f** and **g**, Table II), it was clearly established that Neu5Ac itself, even in the presence of excellent asialo acceptors such as Lac β 1Me and *N*-acetylglucosamine, is unable to act as an *N*-acetylneuraminosyl donor, because no transfer products were observed. It is perhaps not surprising that Neu5Ac is not transferred by TcTS, given that a hydroxyl group is not a particularly good leaving group. The capacity of the α -methyl glycoside of Neu5Ac (**h**, Table II) to be hydrolyzed or used as a donor in the transfer reaction by TcTS was also investigated, and it was established that neither hydrolysis nor transfer

Table II. Transfer of Neu5Ac from *N*-acetylneuraminosyl donor to asialo acceptors by TcTS (*N*-acetylneuraminosyl acceptor substrate specificity).

	<i>N</i> -acetylneuraminosyl donor	Asialo acceptor	Relative rates
f	Neu5Ac	Lacβ1Me	No transfer
g	Neu5Ac	<i>N</i> -acetyllactosamine	No transfer
h	Neu5Acα2Me	Lacβ1Me	No transfer
i	MUN	Galβ1Me	1.00
j	MUN	Lacβ1Me	2.43
k	MUN	<i>N</i> -acetyllactosamine	1.08
l	MUN	Asialofetuin	1.29
m	Neu5Acα(2,3)Galβ1Me	Lacβ1Me	1.00
n	Neu5Acα(2,3)Galβ1Me	<i>N</i> -acetyllactosamine	0.99
o	Neu5Acα(2,3)Galβ1Me	Galβ- <i>S</i> -1Me	0.53
p	Neu5Acα(2,3)Galβ1Me	Asialofetuin	0.08
q	Neu5Acα(2,3)Lacβ1Me	Galβ1Me	1.00
r	Neu5Acα(2,3)Lacβ1Me	<i>N</i> -acetyllactosamine	—*
s	Neu5Acα(2,3)Lacβ1Me	Asialofetuin	0.02
t	Neu5Acα(2,6)Lacβ1Me	Lacβ1Me	No transfer
u	α1-acid glycoprotein	Galβ1Me	1.00
v	α1-acid glycoprotein	Lacβ1Me	0.92
w	α1-acid glycoprotein	<i>N</i> -acetyllactosamine	—

Relative rates were obtained by normalizing the initial slope of the integrals versus time function of each given *N*-acetylneuraminosyl donor molecule reaction **a**, **i**, **m**, **q**, and **u** to 1.00. Some reaction relative rates could not be obtained due to signal overlap or line broadening of signals denoted by asterisks.

occurred with this sialoside. The observed lack of transfer reaction with the α-methyl glycoside of Neu5Ac is possibly due to either the poor leaving group properties of the aglycon unit, lack of recognition of the substrate or a combination of both.

A number of donor substrates have been used to investigate trypanosomal trans-sialidase hydrolase and transferase activities (Engstler *et al.*, 1993, 1995; Harrison *et al.*, 2001; Ribeiro *et al.*, 1997). For our own NMR purposes, we decided to examine the transfer capabilities of TcTS with the synthetic α-*N*-acetylneuraminide, MUN, together with a variety of acceptors. As can be seen in Table II, the relative rate of transfer of Neu5Ac from MUN to different acceptors was indeed influenced by the nature of the acceptor. Thus the transfer of the Neu5Ac moiety from MUN to Lacβ1Me (**j**, Table II) was found to be significantly faster (2.4 times) than to Galβ1Me (**i**, Table II) or to *N*-acetyllactosamine (**k**, Table II). Indeed, the latter two acceptors (Galβ1Me and *N*-acetyllactosamine) showed similar transfer rates. Furthermore, when the naturally occurring Neu5Ac acceptor asialofetuin was investigated, similar rates to Galβ1Me were also observed (**l**, Table II). The observations with MUN as the source of Neu5Ac suggest that Lacβ1Me is clearly the best acceptor molecule.

The transfer of Neu5Ac from the *N*-acetylneuraminosyl donor Neu5Acα(2,3)Galβ1Me to a variety of acceptors was

also investigated. This series of experiments showed that Lacβ1Me (**m**) (36 μM min⁻¹) and *N*-acetyllactosamine (**n**) were the most competent Neu5Ac acceptor molecules, with relative transfer rates of 1.00 and 0.99, respectively (Table II). Interestingly, the transfer from Neu5Acα(2,3)Galβ1Me to Galβ-*S*-1Me (**o**, Table II) exhibited a 1.8-fold slower transfer rate as compared to Lacβ1Me. Asialofetuin (**p**, Table II) showed the poorest Neu5Ac acceptor properties, with an approximately 12.5-fold slower transfer rate when compared with Lacβ1Me.

Similar trends in Neu5Ac acceptor abilities were observed when Neu5Acα(2,3)Lacβ1Me was used as the *N*-acetylneuraminosyl donor molecule (**q**, **r**, and **s**, Table II). In this series of experiments, Galβ1Me (**q**) exhibited the fastest Neu5Ac acceptor rate, with asialofetuin (**s**) ~50-fold slower.

In an attempt to more closely simulate conditions that the TcTS enzyme would encounter on the host cell surface, the ability of the naturally occurring α1-acid glycoprotein to act as *N*-acetylneuraminosyl donor was investigated. Interestingly, similar rates of Neu5Ac transfer were observed to the acceptors Galβ1Me (**u**) (1.00) and Lacβ1Me (**v**) (0.92). In the case of MUN as the donor a significant (almost 2.5-fold) difference in the acceptor capabilities of Lacβ1Me (**j**) versus Galβ1Me (**i**) was found. The observation that there is essentially no difference in the acceptor capabilities of these sugars when a glycoprotein is used as the source of Neu5Ac is supportive of the notion that the nature of the acceptor molecule could be less important in the natural environment of the enzyme.

N-acetylneuraminosyl donor substrate specificity

Having investigated the effect of the acceptor for a given *N*-acetylneuraminic acid donor, we felt it also appropriate to compare the different donors for a given acceptor molecule. The results from this investigation are summarized in Table III, with the *N*-acetylneuraminic acid donor MUN being set at a rate of 1.0 for each acceptor. As can be seen, for the asialo acceptor Galβ1Me/Galβ*S*-1Me, the most efficient transfer rate was observed with Neu5Acα(2,3)Lacβ1Me as the source of *N*-acetylneuraminic acid (6.79-fold better than MUN, **q**, Table III). Neu5Acα(2,3)Lacβ1Me (**q**) was over three times better as a donor than Neu5Acα(2,3)Galβ1Me (**o**, Table III) in donating Neu5Ac, which is consistent with data determined by radiolabeled-based standard biochemical assays previously reported in the literature (Scudder *et al.*, 1993). Strikingly, the naturally occurring *N*-acetylneuraminosyl donor, α1-acid glycoprotein, was the least efficient donor (**u**, Table III), exhibiting a 4.5-fold slower transfer of Neu5Ac compared with MUN. Similar trends were noted for the transfer rates of Neu5Ac to Lacβ1Me or *N*-acetyllactosamine acceptors (compare **j**, **m**, **v**, **k**, **n**, **r**, **w**, Table III).

Hydrolysis versus transfer reaction

As stated earlier and as reported by others, TcTS is far more efficient at transferring *N*-acetylneuraminic acid residues to acceptor molecules than to water (Parodi *et al.*, 1992; Schenkman *et al.*, 1992). In an attempt to quantify the comparative rates of transfer to hydrolysis, we present our findings in graphical form (Figure 2). As can be clearly seen

Table III. Transfer of Neu5Ac from *N*-acetylneuraminosyl donor to asialo acceptors by TcTS (*N*-Acetylneuraminosyl donor substrate specificity).

	<i>N</i> -acetylneuraminosyl donor	Asialo acceptor	Relative rates
i	MUN	Gal β 1Me	1.00
o	Neu5Ac α (2,3)Gal β 1Me	Gal β -S-1Me	2.08
q	Neu5Ac α (2,3)Lac β 1Me	Gal β 1Me	6.79
u	α 1-acid glycoprotein	Gal β 1Me	0.22
j	MUN	Lac β 1Me	1.00
m	Neu5Ac α (2,3)Gal β 1Me	Lac β 1Me	1.67
v	α 1-acid glycoprotein	Lac β 1Me	0.15
k	MUN	<i>N</i> -acetylglucosamine	1.00
n	Neu5Ac α (2,3)Gal β 1Me	<i>N</i> -acetylglucosamine	3.38
r	Neu5Ac α (2,3)Lac β 1Me	<i>N</i> -acetylglucosamine	—*
w	α 1-acid glycoprotein	<i>N</i> -acetylglucosamine	—
l	MUN	Asialofetuin	1.00
p	Neu5Ac α (2,3)Gal β 1Me	Asialofetuin	0.12
s	Neu5Ac α (2,3)Lac β 1Me	Asialofetuin	0.12

Relative rates were obtained by normalizing the initial slope of the integrals versus time function of each given asialo acceptor molecule reaction i, j, k, and l to 1.00. For some reactions, relative rates could not be obtained due to signal overlap or line broadening of signals, denoted by asterisks.

with MUN (Figure 2), a substrate that does not contain a carbohydrate aglycon, there is little difference in the observed hydrolysis and transfer rates, indicating that the rate-limiting step is the release of the umbelliferyl group (Ribeirão *et al.*, 1997). Stark differences were, however, observed in the hydrolysis versus transfer profile of Neu5Ac α (2,3)Gal β 1Me as donor compared with Neu5Ac α (2,3)Lac β 1Me. The relative rates of transfer of Neu5Ac from Neu5Ac α (2,3)Gal β 1Me to Lac β 1Me is 7.6-fold faster than the corresponding hydrolysis reaction. Similarly, the transfer of Neu5Ac from Neu5Ac α (2,3)-Lac β 1Me to Gal β 1Me is 31-fold faster than the corresponding hydrolysis of Neu5Ac α (2,3)Lac β 1Me (Figure 2). These results support the finding that for carbohydrate-based aglycon containing Neu5Ac donor molecules the transfer reaction is the most dominant reaction performed by TcTS. These results are not surprising because the major role of this enzyme *in vivo* is to sequester *N*-acetylneuraminic acid from host cells and transfer them to asialoglycoconjugates on the parasite surface. Interestingly, the observed rates of the hydrolysis and transfer reactions for α 1-acid glycoprotein are similar, and the transfer reaction is much reduced in rate compared to the synthetic donors Neu5Ac α (2,3)-Gal β 1Me and Neu5Ac α (2,3)Lac β 1Me. This observation may be the result in part of the previously mentioned difficulties that were experienced in determining product integral values from the NMR spectrum for the reactions using the donor substrate α 1-acid glycoprotein.

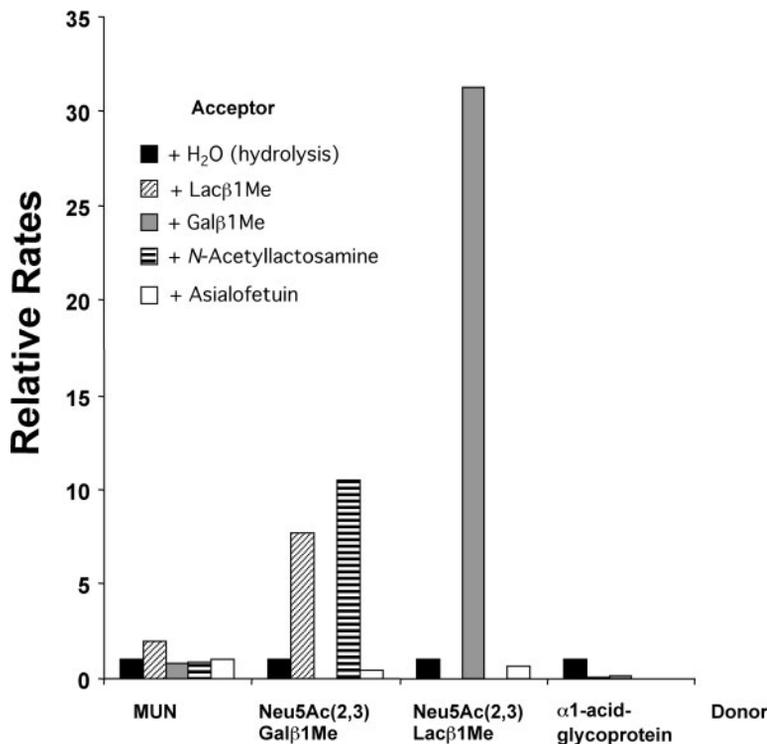


Fig. 2. Graphical representation of the relative rates, determined by integration of the NMR signals over time and linear regression of the integral values within the linear regime. The relative rates for each Neu5Ac donor molecule (MUN, Neu5Ac(2,3)Gal β 1Me, Neu5Ac(2,3)Lac β 1Me, and α 1-acid glycoprotein) for the hydrolysis reaction was set to 1.00. The transfer reactions were calculated relative to the hydrolysis reaction. Most strikingly, the rates of the hydrolysis and transfer reaction using MUN as a Neu5Ac donor are similar, whereas the rates of hydrolysis versus transfer of Neu5Ac(2,3)Lac β 1Me indicate a 31-fold increase.

Docking studies of MUN into the binding site of TcTS

The transfer of *N*-acetylneuraminic acid from the donor molecule Neu5Ac α (2,3)Lac β 1Me to the acceptor Gal β occurs 7.7 times faster than when using MUN as the donor molecule. When *N*-acetylneuraminosyl donors bind to TcTS, there is a resulting conformational rearrangement of Tyr-119 allowing the acceptor to enter the active site (Buschiazzo *et al.*, 2002). However, in the case of MUN as the donor molecule the transfer of Neu5Ac to different acceptors is significantly slower than when other *N*-acetylneuraminosyl donors are used (Figure 2). This may simply be a result of the fact that when MUN is bound by the enzyme, the orientation and steric bulk of the umbelliferol aglycon moiety may restrict access for the positioning of an acceptor molecule. This notion prompted us to evaluate this hypothesis through an investigation of the binding properties of TcTS toward MUN using the program AutoDock 3.0 (Morris *et al.*, 1998).

In this study we used the pdb code 1MS0 with Neu5Ac2en (DANA) and lactose bound to the active site (Buschiazzo *et al.*, 2002). We felt that with bound Neu5Ac2en the conformational rearrangement of Tyr-119 should have already occurred, and it is likely that the same rearrangement will happen when MUN binds to the enzyme. Our docking study shows that the *N*-acetylneuraminic acid component of MUN occupies the same position in the active site as Neu5Ac2en. However, the umbelliferol aglycon of MUN occupies the same region of the binding site where the acceptor lactose is positioned in the X-ray structure. It is important to note that the torsion angles of MUN were kept flexible during the docking experiment. Figure 3 shows the active site of TcTS and the docked MUN molecule superimposed with bound lactose molecules derived from the X-ray structure.

NMR study of TcTS binding to acceptor-like molecules in the presence of *N*-acetylneuraminic acid

STD NMR spectroscopy is a powerful tool for probing protein–ligand interactions (Meyer and Mayer, 1999; Meyer and Peters, 2003). In the present study, we used STD NMR spectroscopy to further investigate the solution binding behavior of TcTS toward acceptor-like molecules. In these experiments, only those ligands that have binding affinity toward the biomolecule under investigation will be detected in the STD spectrum. Figure 4 summarizes the results of our STD investigation of TcTS using Lac β 1Me as the acceptor molecule in the absence and presence of a sialyl donor molecule. Figure 4a shows the ^1H NMR spectrum of Lac β 1Me. The initial STD NMR experiments were carried out on the acceptor Lac β 1Me in the presence of TcTS but the absence of a *N*-acetylneuraminic acid donor (Figure 4b). Interestingly, no signals attributable to Lac β 1Me were detected in the STD NMR spectrum in accordance with the assumption that in the absence of a donor molecule, Lac β 1Me does not display any binding affinity toward TcTS (Buschiazzo *et al.*, 2002). Because it is well established that lactose is an acceptor for the TcTS catalyzed transfer of *N*-acetylneuraminic acid, it is reasonable to assume that the lactose moiety must bind to the enzyme at some stage during the

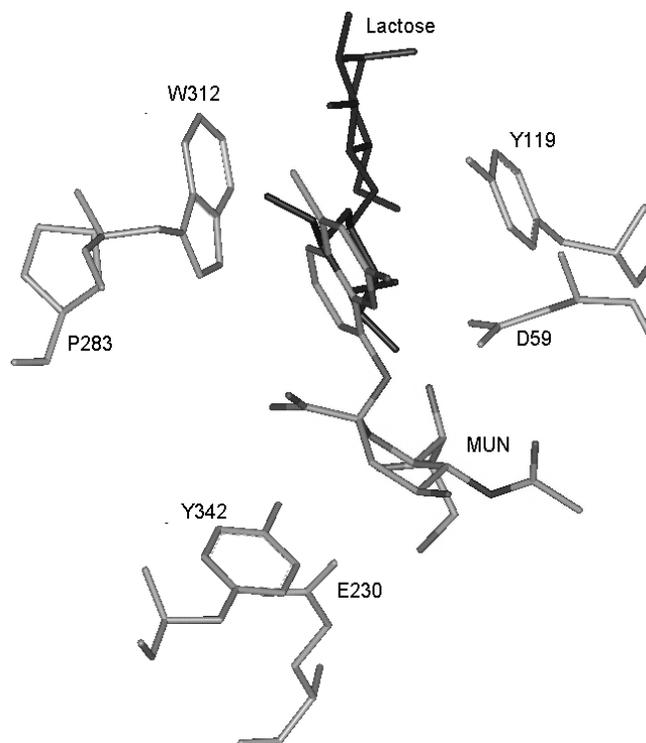


Fig. 3. A molecular model derived from the crystal structure of TcTS (Buschiazzo *et al.*, 2002) and the docking of the *N*-acetylneuraminosyl donor MUN. This figure shows the two key active site aromatic residues (Tyr-119 and Trp-342) in light gray that appear to stabilize the aromatic aglycon unit (4'-methylumbelliferone). Significant *pi*-stacking is predicted between the aglycon and these two aromatic residues.

catalytic cycle. For this reason, the STD NMR experiments were repeated with Lac β 1Me and TcTS in the presence of Neu5Ac as shown in Figure 4d. Figure 4c shows the ^1H NMR spectrum of Lac β 1Me and Neu5Ac for comparison. In Figure 4d the anomeric protons of Lac β 1Me are observed at $\sim\delta$ 4.3 ppm and demonstrates that in the presence of Neu5Ac, the lactoside attains sufficient binding affinity for TcTS. Furthermore, the appearance of the NHAc methyl protons at δ 2.0 ppm in the STD NMR spectrum (Figure 4d) clearly demonstrates that Neu5Ac is also bound to TcTS.

The 3D structure of TcTS establishes that the enzyme is made up of two domains, an *N*-terminal catalytic domain connected through a long α -helix to a *C*-terminal-lectin domain (Buschiazzo *et al.*, 2002). Todeschini and co-workers have investigated a mutant form of TcTS with a non-functioning catalytic site to explore the impact on *N*-acetylneuraminic acid binding (Todeschini *et al.*, 2002). In this study NMR and antibody experiments were utilised to probe the binding capabilities of *N*-acetylneuraminic acid-containing probes to this modified trans-sialidase (Todeschini *et al.*, 2002). From this study it was noted that the modified enzyme was still able to bind to *N*-acetylneuraminic acid-containing entities, in particular those entities with an α -2,3 linkage (Todeschini *et al.*, 2002). It was therefore concluded that the inactive form of TcTs, effectively acting as a lectin, may have a role in binding Neu5Ac-containing entities

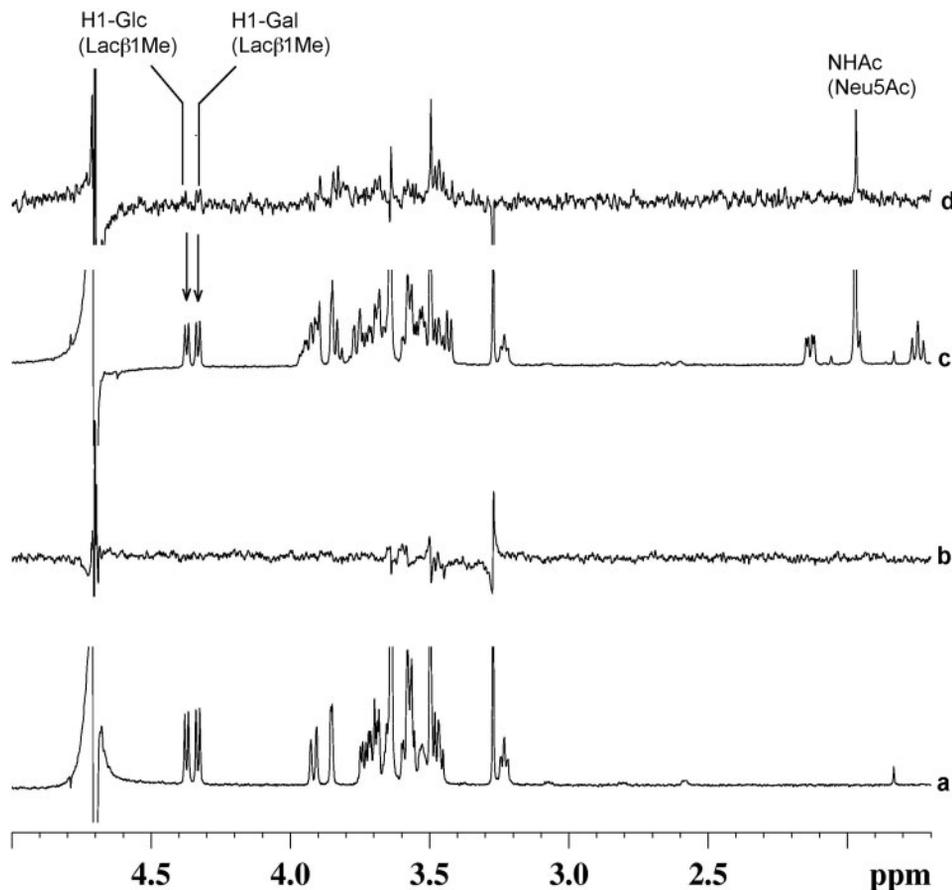


Fig. 4. (a) ^1H NMR and (b) STD NMR spectra of lactose in complex with TcTS. (c) ^1H NMR and (d) STD NMR spectra of a mixture of lactose and *N*-acetylneuraminic acid in complex with TcTS. For the STD spectra the protein was saturated with 40 selective Gaussian-shaped pulses (50 ms each) resulting in a total saturation time of ~ 2 s (on resonance at 7.5 ppm and off resonance at 40 ppm). The residual water signal was reduced using the WATERGATE sequence. The galactose moiety of lactose is close in contact with the protein only when *N*-acetylneuraminic acid is present. All spectra were recorded at 600 MHz, 298 K, and in a deuterated phosphate buffer (50 mM, pH 7.0).

(Todeschini *et al.*, 2002). This finding could have important implications in the life cycle of the parasite since the inactive form of the enzyme may play some role in *T. cruzi*-host cell interactions. Very recently Todeschini and colleagues (2004) showed that enzymatically inactive TcTS binds sialyl and β -galactopyranosyl residues in a sequential order-like mechanism that supports the finding from Buschiazzo *et al.* (2002). The authors have used the inactive mutant of TcTS to prevent *N*-acetylneuraminic acid transfer reaction during the STD NMR experiment and showed for the first time that a lectin recognizes two distinct ligands in a sequential order-like mechanism.

In the present study we investigated by STD NMR spectroscopic methods the binding modes of lactose and Neu5Ac to wild-type fully active TcTS. We believe that STD NMR binding experiments using the fully active enzyme instead of an inactive mutant form (Todeschini *et al.*, 2004) may provide very useful information about how the wild-type enzyme interacts with various ligands. Thus we have investigated the binding capability of TcTS and lactose in the absence and in the presence of *N*-acetylneuraminic acid. The present study clearly shows that wild-type TcTS does not bind lactose in the absence of

N-acetylneuraminic acid, but the protein accommodates the acceptor molecule when *N*-acetylneuraminic acid is present.

Discussion

Comparison of the TcTS catalyzed hydrolysis and transfer reactions

A number of biochemical studies on protozoal *trans*-sialidases have been reported (Engstler *et al.*, 1993, 1995; Medina-Acosta *et al.*, 1994a,b; Schenkman *et al.*, 1991). Several of these nonspectroscopic-based studies have investigated a range of synthetic and natural *N*-acetylneuraminosyl donors in the presence of relevant acceptors (Engstler *et al.*, 1995; Medina-Acosta *et al.*, 1994a,b; Schenkman *et al.*, 1991) and have provided a valuable data set of relative hydrolysis and transfer rates for these donors. Additionally, in some of these previous studies that employed the sialoside and *N*-acetylneuraminosyl donor MUN (Harrison *et al.*, 2001; Todeschini *et al.*, 2000), it was concluded that aglycon release is a rate-limiting step because the type of acceptor did not appear to influence the

rate of MU aglycon release. Interestingly, in a mixed donor aglycon study (Harrison *et al.*, 2001) the rate of release the aglycon Gal β 1 \textit{para} -nitrophenol, containing both a carbohydrate and noncarbohydrate moiety, did appear to be influenced in the presence of the acceptor Gal β (1,3)GlcNAc β 1Octyl.

In the present study, the observation that the rate of decay of donor signals compares well with the rate of aglycon unit release for all of the *N*-acetylneuraminosyl donors investigated, whether the donor has a carbohydrate or noncarbohydrate aglycon, lends further weight to the general conclusion that aglycon release is indeed the rate-limiting step. The fact that there is no difference between these transfer rates for any of these donors confirms that at least on the NMR time scale, a long-lived sialosyl-enzyme intermediate is not formed in the usual catalytic cycle of TcTS—a conclusion that strongly supports earlier work by the Schenkman group (Ribeirão *et al.*, 1997). Of course it does not rule out the possibility of a shorter-lived intermediate that is not discernible by NMR methods. Elegant kinetic isotope effect studies by the group of Horenstein (Yang *et al.*, 2000) have clearly demonstrated that although a long-lived sialosyl-enzyme intermediate may not be accumulated (Ribeirão *et al.*, 1997) in the catalytic process, nucleophilic participation in the development of the transition state is present. Nucleophilic participation could be the result of either an intramolecular event or nucleophilic attack by a suitably positioned amino acid residue within the active site. Very recently the existence of a covalent sialosyl-enzyme intermediate has been suggested (Watts *et al.*, 2003). In an attempt to slow the rate of Neu5Ac transfer and therefore extend the life span of any *N*-acetylneuraminosyl-enzyme intermediates, these workers used the donor substrate 3 β -fluoro-*N*-acetylneuraminosyl fluoride resulting in the detection by mass spectrometric techniques of a transient covalently linked *N*-acetylneuraminosyl-enzyme intermediate. From the TcTS X-ray crystal structure, the likely active site residue nucleophile is Tyr-342, given its proximity to the anomeric center. Taken together, these findings suggest a more sequential mechanism, implying that the binding of the substrate is the first step, followed by cleavage of the sialosidic bond via an active site amino acid residue-catalyzed (nominally Tyr-342) nucleophilic displacement reaction leading to the formation of a transient covalently linked sialosyl-enzyme intermediate. This intermediate then undergoes a second nucleophilic displacement event by attack of water (hydrolysis) or by the hydroxyl group of an appropriate asialo acceptor (such as lactose or *N*-acetylglucosamine).

Our present study alongside earlier work (Ribeirão *et al.*, 1997; Yang *et al.*, 2000) and the evidence for the existence of a *N*-acetylneuraminosyl-enzyme covalent intermediate (Watts *et al.*, 2003; Yang *et al.*, 2000) suggests that although the rate of decay of donor signals compared to the rate of aglycon unit release are similar for all of *N*-acetylneuraminosyl donors studied to date, irrespective of the aglycon moiety, the life span of the intermediate is relatively short and cannot be absolutely discerned by traditional kinetic methods, including NMR spectroscopy, for rate comparisons.

Relative rates of transfer

All relative rates of transfer of *N*-acetylneuraminic acid discussed hereafter are expressed as the rate of donor decay. An analysis of MUN as the *N*-acetylneuraminosyl donor, comparing the relative rate of transfer to water (hydrolysis) versus transfer to asialo carbohydrate acceptors is shown in Figure 2. From this NMR study the rate of transfer to water (hydrolysis) was in general similar to the rate of transfer to asialo carbohydrate acceptors, irrespective of the acceptor that was employed. This is entirely consistent with the conclusions drawn from classical kinetic data (Ribeirão *et al.*, 1997). It has been reported that the preferential cleavage (hydrolysis) of MUN over *N*-acetylneuraminylactose may be attributed to the fact that the 4-methylumbelliferyl aglycon is a better leaving group than the carbohydrate-based aglycon units of natural substrates (Todeschini *et al.*, 2000). Comparison of the rate of transfer of *N*-acetylneuraminic acid from Neu5Ac α (2,3)Gal β 1Me to water (hydrolysis) with the rate of transfer of Neu5Ac to Lac β 1Me or *N*-acetylglucosamine is shown in Figure 2. The rate of transfer of Neu5Ac α (2,3)Gal β 1Me to water (hydrolysis) is markedly reduced compared to the transfer reactions to either of the lactose-containing asialo acceptors Lac β 1Me (7.7 times faster) and *N*-acetylglucosamine (10.5 times faster). Most striking was the observation (Figure 2) that the rate of transfer of Neu5Ac from Neu5Ac α (2,3)Lac β 1Me to water (hydrolysis) as compared to Gal β 1Me as the asialo acceptor was in the ratio of 1:31. These findings suggest that the aglycon unit of the *N*-acetylneuraminosyl donor does have an important influence on the overall rate of transfer, as exemplified by the fact that a lactose-containing *N*-acetylneuraminosyl donor is more effective than the corresponding galactose-containing donor and presumably reflects the preferred specificity of the trans-sialidase (Schenkman *et al.*, 1992; Vandekerckhove *et al.*, 1992). An explanation for the improved affinity and preferred specificity for disaccharide aglycon units is evident from an inspection of the active site region (Buschiazzo *et al.*, 2002) in which it has been concluded that additional active site interactions, in particular stacking interactions with two key aromatic residues Tyr-119 and Trp-312, may occur with the reducing-end sugar moiety (glucose in the case of the aglycon lactose).

Docking of MUN into the active site of TcTS using AutoDock

Our docking study clearly shows that the 4-methylumbelliferyl aglycon unit is stabilized through a *pi*-stacking event to other aromatic groups of neighboring amino acid residues, in particular Tyr-119 and Trp-312 (Figure 3). As a result of the aromatic nature of the aglycon, this would lead to a significantly improved stacking arrangement over the usual carbohydrate-based aglycon already discussed. Restricted access of acceptor molecules translates into observed slower rate of transfer. In our view this also offers a reasonable explanation as to why the mixed aglycon donor (Harrison *et al.*, 2001), in terms of a relative transfer rates, falls in between *N*-acetylneuraminosyl donors that have carbohydrate aglycons and those that do not. In the mixed aglycon donor instance, the benefits of the

carbohydrate aglycon moiety improve the transfer rate over an aromatic aglycon donor like MUN, whereas the aromatic moiety, through *pi*-stacking interactions, slows the departure of the aglycon residue after the first nucleophilic displacement event, resulting in a poorer transfer rate when compared with carbohydrate-only aglycon donors (e.g., *N*-acetylneuraminylactose). Alternatively or in combination, the positioning of the umbelliferyl aglycon of MUN may simply be slowing the reorientation of the Tyr-119 residue, thus causing a reduction in the transfer rates due to a longer time before the acceptor can bind. Others have also professed this alternative hypothesis (Ribeirão *et al.*, 1997).

The relative transfer rates that we have obtained by NMR methods for TcTS appear to be entirely consistent with rates determined by other indirect methods for *trans*-sialidases from other trypanosomes (Engstler *et al.*, 1993, 1995). For example the Schauer group have determined, by standard fluorometric or radiolabeled acceptor-based assays, transfer rates of various sialosyl donors and asialo acceptors with *trans*-sialidases from *T. brucei* and *T. congolense* (Engstler *et al.*, 1993, 1995). In these studies it was found that *N*-acetylneuraminic acid from *N*-acetylneuraminosyl donors that have carbohydrate aglycons is more readily transferred to asialo acceptors than from donors that have noncarbohydrate aglycons (e.g., MUN).

STD NMR investigations of TcTS

In the recent X-ray crystallographic structure determination of TcTS complexed with Neu5Ac2en and lactose, it was observed that the galactose moiety of lactose interacts with Asp-59 and that the glucose moiety resides in an orientation pointing out of the active site cavity (Buschiazzo *et al.*, 2002). The STD NMR shows evidence of lactose binding to TcTS, but because of the signal-to-noise level it is not possible to determine whether the galactose or the glucose moiety is in close proximity to TcTS. Nevertheless the NMR findings (Figure 4) provide for the first time direct evidence in solution that an acceptor molecule is only recognized and bound by the fully active enzyme in the presence of a *N*-acetylneuraminic acid donor. Others have shown in a surface plasmon resonance study of an enzymatically inactive TcTS mutant (Asp-59 to Asn) (Buschiazzo *et al.*, 2002) that the asialo acceptor lactose by itself does not bind to the inactive enzyme; however, on addition of *N*-acetylneuraminic acid or *N*-acetylneuraminylactose lactose was clearly observed to associate with TcTS. Our findings are in complete agreement with those observations.

Conclusion

This study has demonstrated that NMR spectroscopy is a valuable tool for providing a direct method for the investigation of the association and rate comparison of TcTS donors and acceptors. From this study we have shown that Neu5Ac α (2,3)Lac β 1Me as the *N*-acetylneuraminosyl donor and Gal β 1Me as the asialo acceptor are clearly the most efficient combination for the transfer of *N*-acetylneuraminic acid. Whereas for most *N*-acetylneuraminosyl donors studied the transfer reaction is significantly faster than the hydrolysis reaction, this is not the case when MUN was used as the *N*-acetylneuraminic acid donor. Molecular

modeling studies have provided evidence that suggests a strong *pi*-stacking interaction of the aromatic umbelliferyl aglycon with Trp-312 that may be the reason for the slow transfer of Neu5Ac from MUN. Significantly, we have been able to provide direct evidence by STD NMR experiments that in the absence of a sialosyl donor the asialo acceptor lactose does not bind.

Materials and methods

Asialofetuin, *N*-acetylglucosamine, Gal β 1Me, and α 1-acid glycoprotein were purchased from Sigma Aldrich (Sydney, Australia) *N*-acetylneuraminic acid was obtained as a gift from GlaxoSmithKline. Neu5Ac α (2,3)Lac was obtained from Neose Technologies and subjected to high-pressure liquid chromatography purification. MUN (Myers *et al.*, 1980), Lac β 1Me (Fazli *et al.*, 2001), Neu5Ac α 2Me (Kononov *et al.*, 1998), Gal β -S-1Me (McGeary *et al.*, 2001), Neu5Ac α (2,3)Gal β 1Me (Wilson *et al.*, 1999), Neu5Ac α (2,3)Lac β 1Me (Wilson *et al.*, 2000) and Neu5Ac α (2,6)Lac β 1Me (Wilson *et al.*, 1999) were prepared according to published procedures.

TcTS was kindly provided by Professor Carlos Frasch (Unité de Biochimie Structurale, Département d'Immunologie, Paris). The enzyme was delivered in 85% saturated ammonium sulfate solution. The suspension was centrifuged at 10,000 rpm for 30 min at 4°C, and the pellet was resuspended in 20 mM Tris buffer. After dialysis (overnight) against 20 mM Tris buffer (pH 8), the enzyme was then exchanged into a deuterated 50 mM phosphate buffer using Centricon filters (excluding molecular mass of 10 kDa; Millipore). The enzyme migrated as a homogenous species with a molecular weight of ~83 kDa on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. The final protein concentration was determined by the standard Bradford test (purchased from Sigma) using bovine serum albumin as a standard.

Hydrolysis reactions

For the hydrolysis reactions the following solutions (a to e) were prepared, with the sialyl donor dissolved in deuterated 50 mM phosphate buffer (600 μ l, pH 7.0): **a**: 1.125 mg (2.41 μ mol) MUN (3.89 mM); **b**: 2 mg (4.12 μ mol) Neu5Ac(2,3)Gal β 1Me (6.65 mM); **c**: 2.0 mg (3.09 μ mol) Neu5Ac(2,3)Lac β 1Me (4.98 mM); **d**: 3.66 mg (0.08 μ mol) α 1-acid glycoprotein (0.13 mM); **e**: 1.65 mg (2.55 μ mol) Neu5Ac(2,6)Lac β 1Me (4.11 mM); **f**: 3.4 mg (10.49 μ mol) Neu5Ac α 1Me (16.92 mM). In a typical experiment, a spectrum of the sialyl donor dissolved in 600 μ l deuterated phosphate buffer (50 mM) was recorded ($t = 0$ min). Subsequently, to each sample 20 μ l protein solution containing 12 μ g TcTS was then added. After addition of enzyme, 1 H NMR experiments were recorded at regular time intervals (every 10 min over a time period of ~2000 min).

Transfer reactions

For transfer reactions the following solutions (f to w) were prepared with the sialyl donor and sialyl acceptor dissolved in deuterated 50 mM phosphate buffer (600 μ l, pH 7.0): **f**: 1.24 mg (4.01 μ mol) Neu5Ac (donor) (6.47 mM) and

2.14 mg (6.01 μ mol) Lac β 1Me (acceptor) (9.61 mM); **g**: 0.74 mg (2.39 μ mol) Neu5Ac (donor) (3.85 mM) and 1.47 mg (3.70 μ mol) *N*-acetylactosamine (acceptor) (5.97 mM); **h**: 3.4 mg (10.52 μ mol) Neu5Ac α 2Me (donor) (16.97 mM) and 7.49 mg (21.03 μ mol) Lac β 1Me (acceptor) (33.92 mM); **i**: 1.125 mg (2.41 μ mol) MUN (3.89 mM) (donor) and 0.93 mg (4.79 μ mol) of Gal β 1Me (7.73 mM) (acceptor); **j**: 1.125 mg (2.41 μ mol) MUN (3.89 mM) (donor) and 1.7 mg (4.77 μ mol) of Lac β 1Me (7.69 mM) (acceptor); **k**: 1.125 mg (2.41 μ mol) MUN (3.89 mM) (donor) and 1.47 mg (3.70 μ mol) *N*-acetylactosamine (5.97 mM) (acceptor); **l**: 2.25 mg (4.82 μ mol) MUN (7.77 mM) (donor) and 2.6 mg (0.06 μ mol) asialofetuin (0.1 mM) (acceptor); **m**: 1.41 mg (2.91 μ mol) Neu5Ac(2,3)Gal β 1Me (4.69 mM) (donor) and 1.33 mg (3.73 μ mol) of Lac β 1Me (6.02 mM) (acceptor); **n**: 1.163 (2.4 μ mol) of Neu5Ac(2,3)Gal β 1Me (3.87 mM) (donor) and 1.47 mg (3.70 μ mol) *N*-acetylactosamine (5.97 mM) (acceptor); **o**: 2.56 mg (5.27 μ mol) Neu5Ac(2,3)Gal β 1Me (8.50 mM) (donor) and 7.07 mg (33.6 μ mol) Gal β -S-1Me (54.19 mM) (acceptor); **p**: 4.0 mg (8.24 μ mol) Neu5Ac(2,3)-Gal β 1Me (13.29 mM) (donor) and 3.66 mg (0.08 μ mol) asialofetuin (0.13 mM) (acceptor); **q**: 2.0 mg (3.09 μ mol) Neu5Ac(2,3)Lac β 1Me (4.98 mM) (donor) and 1.2 mg (6.18 μ mol) Gal β 1Me (9.97 mM) (acceptor); **r**: 2.5 mg (3.94 μ mol) Neu5Ac(2,3)Lac β 1Me (6.35 mM) (donor) and 2.9 mg (7.30 μ mol) *N*-acetylactosamine (11.80 mM) (acceptor); **s**: 5.8 mg (8.13 μ mol) Neu5Ac(2,3)Lac β 1Me (13.1 mM) (donor) and 3.66 mg (0.08 μ mol) asialofetuin (0.13 mM) (acceptor); **t**: 1.25 mg (2.52 μ mol) Neu5Ac(2,6)Lac β 1Me (4.06 mM) (donor) and 1.32 mg (3.70 μ mol) Lac β 1Me (5.97 mM) (acceptor); **u**: 2.9 mg (0.07 μ mol) α 1-acid glycoprotein (0.13 mM) (donor) and 0.77 mg (4.00 μ mol) Gal β 1Me (6.45 mM); **v**: 2.90 mg (0.07 μ mol) α 1-acid glycoprotein (0.13 mM) (donor) and 1.27 mg (3.56 μ mol) Lac β 1Me (5.74 mM); **w**: 2.14 mg (0.05 μ mol) α 1-acid glycoprotein (0.08 mM) (donor) and 2.43 mg (6.11 μ mol) *N*-acetylactosamine (9.85 mM).

In a typical experiment, a mixture of sialyl donor and acceptor was dissolved in 600 μ l deuterated phosphate buffer (50 mM) and the 1 H NMR spectra were recorded at $t=0$ min. Subsequently, to each 600- μ l NMR sample 20 μ l protein solution containing 12 μ g TcTS was then added to give a total volume of 620 μ l (23.32 nM protein). After addition of enzyme, 1 H NMR experiments were recorded at regular time intervals (every 5 min over a time period of ~1500 min).

Molecular docking of MUN using AutoDock

For docking studies, the molecular structure of MUN was built with INSIGHTII software. The Neu5Ac moiety was used in a boat chair conformation as observed in the crystal structure of the complex between sialic acid and influenza virus sialidase (1MWE). For the docking procedure, the program AutoDock 3.0 (Morris *et al.*, 1998) was used to explore the binding conformation of MUN. The pdb code 1MS0 (Buschiazzi *et al.*, 2002) was used and all water molecules, bound Neu5Ac2en (DANA), and lactose were removed. Twelve active torsions have been selected to be fully flexible during the docking experiment. For the docking a grid spacing of 0.375 Å and 60 \times 60 \times 60 numbers of

points were used. The Lamarckian genetic algorithm was adopted using the default settings, except for the number of energy evaluation, which was set to 1.25 Mio. Amber united atoms were assigned to the protein using the program AutoDock Tools. AutoDock generated 10 possible binding conformations for MUN that show all similar binding conformations. The bound conformation of MUN was then superimposed with the lactose and DANA bound to TcTS (Figure 3). To validate the use of the AutoDock program, the docking studies were performed on the reference compound, DANA. AutoDock successfully reproduced the experimental binding conformation of the reference ligand.

NMR spectroscopy

All NMR experiments were performed on a Bruker Avance DRX 600 MHz spectrometer at 298 K. Suppression of the residual HDO signal was achieved by presaturation with a weak rf field for 2 s during the relaxation delay. Data acquisition and processing were performed with XWINNMR software (Bruker) run on a Silicon Graphics O2 workstation. Fourier transformation and base line correction was performed for the 1 H NMR experiment at $t=0$ min. Automatic processing and baseline correction was then applied using the same parameters for all 1 H NMR experiments. Relative integration was performed manually for the 1 H NMR spectrum at $t=0$ min using the XWINNMR integration submenu. Integration for all 1 H NMR spectra was achieved by applying the same integration range using the automation program multi_int eg.

An AWK script was applied to interchange columns to rows and the data were then transferred to Excel (Microsoft) running on a Macintosh computer. Relative integrals were normalized to 1.0 for the maximum integral value observed. Graphs were plotted with proFit. Initial slopes were obtained by linear regression analysis of the curves in the linear region of the function and were then calculated to relative rates.

STD NMR experiments

The protein was saturated on resonance at 7.5 ppm and off resonance at 40 ppm with a cascade of 40 selective Gaussian-shaped pulses, of a 50-ms duration with a 100- μ s delay between each pulse in all STD experiments. The total duration of the saturation time was 2 s. The reaction cocktail for the STD NMR experiments was comprised of 30 μ g TcTS in 600 μ l deuterated phosphate buffer (50 mM, pH 7.0). Addition of 350 μ g (0.98 μ mol) Lac β 1Me gave a molecular ratio of TcTS:Lac β Me of 1:2750. A further experiment was performed after addition of 300 μ g (0.97 μ mol) Neu5Ac to give a molecular ratio of TcTS : Lac β 1Me : Neu5Ac of 1:2750:2686. A total of 512 scans per STD NMR experiment was acquired in conjunction with a WATERGATE sequence to suppress the residual HDO signal.

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Abbreviations

MUN, 4-methylumbelliferyl α -D-N-acetylneuraminide, NMR, nuclear magnetic resonance; STD, saturation transfer difference; TcTS, *Trypanosoma cruzi* trans-sialidase.

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