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Biochemical characterisation of trans-sialidases from *Trypanosoma congolense*

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I. Acknowledgment

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II. Structure of Thesis

The thesis herein is structured into two major parts: preliminary pages and substantive chapters. The preliminary pages are made up of the title page, declaration of originality of work, Table of contents, and finally, a summary of findings. The summary comes in English and Deutsch.
There are 5 substantive chapters divided into one introductory chapter called **Introduction**, three r**esult** chapters, numbered consecutively as Chapters 2 through 4 and made up of two publications and a manuscript. Chapter 5 is the discussion chapter. The reader should note that because each publication/manuscript has a detailed discussion, the discussion as presented in Chapter 5 is rather an overview of findings, possible implications and suggestions for further research.
Chapter 1, the introductory chapter is a synopsis of current state of the art and chronicles a wide variety of aspects considered relevant to the current study. It introduces the reader to the problem of Animal African Trypanosomosis (AAT) and Human African Trypanosomosis (HAT), the involvement of sialic acid (Sia) and trans-sialidases (TS) in trypanosomiasis. More emphasis is placed on trans-sialidases from African trypanosomes but due to the inescapable fact that more is known about the American *T. cruzi* TS (TcTS), a large number of references are made to TcTS.
Chapter 2/Publication 1 is on *T. congolense* trans-sialidase 1 (TconTS1) variants, the group with the largest gene members in this organism. Cloning, sequencing and biochemical attributes of two variants have been reported and compared with TcTS.
Chapter 3/Publication 2 deals with the remaining TconTS genes of TconTS2, TconTS3 and TconTS4. The model blood glycoprotein fetuin has been used as donor in presence and absence of acceptor disaccharide lactose to further asses the sialidase and trans-sialidase activities of these genes. While this chapter reports also on the cloning and sequences of TS gens from *T. brucei* (TbTS), no enzymatic activities of the TbTS are reported here. Instead, data is mined for another African AAT-causing parasite, *T. vivax* and phylogenetic analysis employed using full lengths (open reading frames, ORFs), catalytic and lectin domains separately to delineate TS orthologues among these group of organisms.
Chapter 4/Manuscript characterises the activities of TconTS genes on a complex and biologically relevant substrate, mammalian serum glycoconjugates.

III. Summary

The work compiled here aimed at biochemically characterising trans-sialidase (TS) genes from *Trypanosoma congolense*, the most prevalent causative agent of animal African trypanosomiasis, also called nagana. Using published partial TS sequences as starting queries, database at the Welcome Trust Sanger Institute (WTSI) was queried and TS1 genes identified. This is a multi-copy gene group comprising 11 members. Members were cloned and recombinant protein expressed in fibroblasts and purified proteins assayed for enzyme activities (Publication 1/Chapter 1). All TS1 variants were found to be active TS enzymes, transferring Sia from donor fetuin to the lactose acceptor substrate and could resialylate asialofetuin up to 50%. Use of synthetic substrates revealed poor specific activities.

Further searches on WTSI using TconTS1 gene variants as queries revealed 3 other TS genes, TconTS2, TconTS3 and TconTS4, all conserving the critical amino acids required in activity. A further 3 distantly related genes, this latter group is presumed inactive due to lack of conservation of critical amino acids (Publication 2/Chapter 3). TS/sialidase ratios confirmed the former to be active trans-sialidases but with differing specific activities. A mouse monoclonal antibody raised from native proteins probably containing other TconTS and not only TconTS1 reacted with both TconTS1 and TconTS2, two of the proteins with the highest specific activities. Incidentally, the epitope for the antibody is localised on the lectin domain (LD) in both enzymes. Phylogenetic analysis using the LD grouped TconTS1 and TconTS2 together while the use of catalytic domain (CD) grouped them separately. This suggests a possible role for the LD in enzyme activities and hence pathology of nagana.

To gain knowledge on the role of TS in pathogenesis of nagana, blood glycoconjugates were employed as substrates and activities of TS gene characterised (Manuscript/Chapter 4). All TconTS proteins transferred Sia from serum-bound glycoconjugates to lactose. In absence of lactose, TconTS1 and TconTS2 released free Sia from the serum-glycoconjugates, possibly explaining the high amounts of free Sia observed in blood and serum of animals suffering nagana. TconTS3 showed an unidentified product peak in presence of serum-bound Sia and lactose, while 3 unidentified signals probably representing serum-glycoconjugates inherent in serum were altered by TconTS2. Collectively, the above indicate the possibility of acceptor and donor preferences and show that expressing more than one TS gene at a time could be beneficial to the parasite.

Since TS genes are expressed in two different hosts systems; the *Glossina* insect vector and the mammalian hosts with different pH systems, pH optima for the enzymes was studied. TconTS2 exhibited a wide pH optima that would make it active in both host systems.

IV. Zusammenfassung

Die hier vorgelegte Arbeit befasst sich mit der biochemischen Charakterisierung von Trans-sialidase (TS)-Genen aus *Trypanosoma congolense*, dem Hauptverursacher der Afrikanischen Trypanosimiasis, auch bekannt als Nagana. Unter Verwendung von bereits veröffentlichten Teilsequenzen der TS, konnten TS1-Gene aus der Datenbank des Welcome Trust Sanger Institute (WTSI) identifiziert werden. Dabei handelt es sich um eine „Multi-Copy"-Genefamilie, die 11 Mitglieder umfasst, die alle durch Klonierung als rekombinante Proteine in Fibroblasten exprimiert und deren spezifische Enzymaktivitäten gemessen wurden (Veröffentlichung 1/ Kapitel 1). Es konnte gezeigt werden, dass alle TS1 Varianten Enzymaktivität besitzen und in der Lage sind sowohl Sialinsäure vom Akzeptorsubstrat Fetuin auf das Donorsubstrat Laktose zu übertragen, als auch desialyliertes Fetuin wieder bis zu 50% zu resialylieren. Der Einsatz von synthetischen Substraten ergab nur geringe spezifische Enzymaktivitäten.
Zusätzliche Recherchen in der WTSI Datenbank mit Hilfe der TconTS1 Genvarianten als Grundlage, führten zu 3 weiteren nahen TS verwandten Genen, die hier als TconTS2, TconTS3 und TconTS4 bezeichnet werden und 3 entfernter verwandten Genen. Letztere scheinen inaktive TS Formen zu sein, da sie die für einen Transfer/Hydrolyse konservierten, essentiellen Aminosäuren nicht enthalten (Veröffentlichung 2/ Kapitel 3). Das Verhältnis von Transfer- zu Sialidase-Aktivität der drei nahe verwandten TS Gene bestätigt diese als aktive Trans-sialidasen, jedoch mit unterschiedlichen spezifischen Enzymaktivitäten. Ein gegen TconTS gerichteter, monoklonaler Antikörper zeigt deutliche Kreuzreaktivität mit TconTS1 und TconTS2, die beiden Proteine mit den höchsten spezifischen Aktivitäten. Interessanterweise ist das Epitop dieses Antikörpers jeweils in der Lektin-Domäne (LD) der TSs lokalisiert. Phylogenetische Analysen über die LD zeigen die gemeinsame Gruppierung von TconTS1 und TconTS2, wo hingegen die der katalytischen Domäne (CD) beide voneinander separiert. Diese Befunde könnten auf eine mögliche Rolle der LD in Hinsicht auf die spezifische Enzymaktivität und damit auch auf deren Rolle in der Pathologie der Nagana hinweisen.
Um weitere Erkenntnisse bezüglich der Rolle der TS in Krankheitsverlauf der Nagana zu gewinnen, wurden Glykokonjugate aus dem Blut als potentielle Substrate eingesetzt und die Aktivitäten der TS Gene charakterisiert (Manuskript/ Kapitel 4). Alle TconTS Proteine transferieren Sia von Glykokonjugaten aus dem Blutserum auf Laktose. In Abwesenheit von Laktose konnte eine erhöhte Freisetzung von Sialinsäure aus den Glykokonjugaten durch TconTS1 und TconTS2 nachgewiesen werden, die möglicherweise eine Erklärung für die hohen Konzentrationen an freier Sialinsäure im Blut und Serum von mit Nagana infizierten Tieren ist. Mit TconTS3 erhält man ein bisher noch nicht identifiziertes Produkt in Anwesenheit von serumgebundener Sialinsäure und Laktose, während 3 bisher nicht identifizierte Signale in Serum-Proben, die möglicherweise Serum-Glycokonjugate repräsentieren, von TconTS2 verändert werden. Zusammenfassend zeigen die

genannten Ergebnisse eine potentielle Akzeptor- sowie Donor-Substrat Präferenz und deuten darauf hin, dass die gleichzeitige Expression verschiedener TS Gene ein entscheidender Vorteil für den Parasit sein könnte.

Da Trans-sialidasen in zwei verschiedenen Wirtsystemen exprimiert werden; im Insektenvektor *Glossina* und in Säugern als Wirt, welche sich durch unterschiedliche pH-,Systeme auszeichnen, wurden auch Untersuchungen zum pH-Optimum der Trans-sialidasen realisiert. Dabei zeigte TconTS2 ein Optimum über einem breiten pH-Bereich, was ein Hinweis auf dessen Aktivität im Wirtsystem darstellt.

V. Abbreviations

AAT	Animal African Trypanosomosis
ABC	ATP-binding cassette transporter
a-HT	atypical infections of humans
AQPs	Aquaglyceroporins
ATP	Adenosine triphosphate
Å	angstrom
BSF	Blood stream forms
CD	catalytic domain
CMAH	cytidine mononucleotide actyl hydrolase
CMPH	Cytidine 5'-momphosphate-*N*-acetylneuaminic acid hydrolase
DA	Diamidine
DANA	2-deoy-2,3-didehygro-*N*-acatylneuraminic acid
DNA	Deoxyribonucleic acid
EMBL	European Molecular Biology Laboratory
EST	Expressed sequence tags
EtBr	Ethidium bromide
Gal	Galactose
G2	gap 2 (a stage in cell division cycle)
GARP	Glycine-Alanine Rich protein
HAT	Human African Trypanosomiasis
ISM	isomethimidium chloride
KDN	3-deoxy-D-glycero-D-galacto-2-nonulosonic acid
LD	lectin domain
ManNAc	*N*-acetyl-D-mannosamine
mRNA	messenger RNA
MUNANA	methylumbelliferyl *N*-acetylneuraminic acid
Neu	neuraminic acid
NeuB	sialic acid synthetase neuB
NeuC	sialic acid synthetase neuC
Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
NMR	Nuclear magnetic resonance
MRPs	multi-drug resistance proteins
ORF	Open reading frame
RBC	red blood cells

SAPA	shed acute phase antigen
Sia	Sialic acid
Sias	Sialic acids (a set of sialic acid molecules)
SPR	surface plasmon resonance
STD	Saturation Transfer Difference
TconTS	*T. congolense* trans-sialidase
TbTS	*T. brucei* trans-sialidase
TcTS	*T. cruzi* trans-sialidase
TevTS	*T. evansi* trans-sialidase
TLF	Trypanolytic lysis factor
TrSA	*T. rangeli* sialidase
TS	trans-sialidase
TvivTS	*T. vivax* trans-sialidase
UDP-GlcNAc	Uridine 5'-diphosphate-*N*-acetylglucosamine
USD	United States dollars
VSG	variant surface glycoprotein
WHO/TDR	World Health Organisation/Tropical Disease Research
WSTI	Welcome Sanger Trust Institute

1.0.

Introduction

1.1 Trypanosomes

1.2 Life cycle of *T. congolense*

1.3 Sialic acids and trans-sialidase in nagana

1.3.1 Sialic acids

1.3.1.1 Sia synthesis and/or acquisition by organisms

1.3.2 Trans-sialidases

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1.3.2.2 Trans-sialidase structure

1.3.2.3 Conserved amino acids in TS catalytic pocket of African Trypanosomes

1.3.2.4 Mechanistic basis of trans-sialylation and hydrolysis

1.3.2.5 Substrates for trans-sialidase

1.3.2.6 Inhibitors of trans-sialidase

1.3.2.7 Drug transport and resistance in *Trypanosoma* spp

1.4 Aims

1.5 References

1.1.Trypanosomes

Trypanosomes are protozoans of the order kinetoplastida. This group of organisms infects millions of humans and animals and includes two genera, *Leishmania* and the digenetic flagellates, *Trypanosoma*. Members of the genus *Trypanosoma* are responsible for several neglected tropical diseases, both in man and his animals. However, only two cause significant human diseases on the African continent. These two, both subspecies of *Trypanosoma brucei*, are *T. b. gambiense* and *T. b. rhodesiense* responsible for the Human African Trypanosomiasis (HAT), also known as sleeping sickness. Another human infective *Trypanosoma*, *T. cruzi*, the etiological agent of Chagas' disease is responsible for the American trypanosomiasis. This condition afflicts millions of people in central and southern America and kills a reported 15, 000 yearly (Murcia et al. 2013).

The causative agent of HAT, *T. brucei* is further divided into two subspecies based on molecular typing. These are *T. brucei gambiense*, which causes a chronic form of sleeping sickness in West and Central Africa and *T. brucei rhodesiense* responsible for the acute form of the disease in Eastern and Southern Africa. The human-infective sub-species are considered to have arisen from the animal pathogen *T. brucei* that have acquired the ability to infect humans by resisting the trypanolytic lysis factor, TLF (Pays and Vanhollebeke 2008). A subspecies of *T. brucei*, *T. brucei brucei*, is not pathogenic to humans but instead infects ruminants and other animals, in addition to t other species, *Trypanosoma congolense* and *T. vivax*. Together, these *Trypanosoma* parasites are the causative agents of the Animal African Trypanosomiasis (AAT), widely known as nagana. Clinical signs of nagana are weight loss, anaemia and immunosuppression. Another pathogenic species e.g. *T. simiae*, is also present on the African continent. In common with the human parasites, the animal parasites are cyclically transmitted by haematophagous dipteran tsetse flies, *Glossina* species. Though humans have an innate protection against the animals infective species conferred on them by the trypanolytic lysis factor Apolipoprotein L-1 (apoL-1), present in human serum (Vanhamme et al. 2003), cases of a-typical infections of humans (a-HT) by the animal trypanosomes have been reported (Truc et al. 2013).

Traditionally, trypanosomes have been classified on the basis of site of development in the tsetse vector. This has given rise to four subgenera comprising (i). *Trypanozoon* e.g. *T. brucei* with midgut and salivary glands as sites of development, (ii). *Nannomonas* e.g. *Trypanosoma congolense* with midgut and proboscis as sites for development (iii). *Duttonella* where members develop in the proboscis and cibarium, an example include *T. vivax* (iv). *Pcynomonas* where members develop in the midgut, salivary glands and the proboscis. An example here is *T. suis*. This classification is not without problems and suggestions have been made on resolving them (Gibson 2007). Advances in molecular identification tools and phylogenetic analysis (Majiwa et al. 1993, McNamara, Mohammed and Gibson 1994, Solano et al. 1999, Desquesnes et al. 2001, Desquesnes, Ravel and Cuny 2002, Geysen, Delespaux and Geerts 2003, Malele et al. 2003, Hamilton et al. 2008,

Adams et al. 2010b, Silbermayr et al. 2013) have lead to discovery of new trypanosomes. Advances in these methods portends great impact on understanding the diversity, distribution and biology, e.g host ranges of these trypanosomes, genetic exchange (Duffy et al. 2009, Morrison et al. 2009, Holzmuller et al. 2010) and species delineation for example, *T. godfreyi* (reviewed in Adams, Hamilton and Gibson 2010).

Tsetse flies are exclusive to Africa in a distribution that stretches between 14° North and 29° South of the equator (Krafsur 2009). About 30 extant taxa of these insects have been reported (Leak 1998). Apart from *T. evansi*, with a much wider distribution found in Africa, the Americas, Asia, Middle and Far East and even Europe (Gutierrez et al. 2010), the exclusiveness of tsetse to Africa explains the restriction of diseases caused by *T. brucei brucei*, and *T. congolense* within sub-Sahara Africa, resulting in losses of several billion dollars annually (Steverding 2008). *T. evansi* is the causative agent of the animal trypanosomiasis known as *surra*. In contrast to the other animal trypanosomes, this species has different vectors and is mechanically transmitted by biting flies of the genera *Tabanus* and *Stomoxys*. Its wider occurrence is attributed to the total adaptation to mechanical mode of transmission (Stephen, 1986) and to the transport of cattle from Africa in the 19th century (Jones and Davila 2001).

On the other hand, *T. cruzi*, the etiological agent of Chagas' disease is transmitted by bugs of the family Reduvidae (order Hemiptera). Although molecular typing of *T. cruzi* has identified two lineages (Fernandes et al. 1998), only one lineage, the type 2 is responsible for Chagas' disease. *T. cruzi* has been studied more than the African counterparts (reviewed in Dc-Rubin and Schenkman 2012). The prospect of several common aspects of biology between these parasites (transmitted by insect vectors, common aspects of basic biochemistry) would have occasioned that knowledge gained on one species could be applied in the understanding of the other. Contrary to these expectations, profound differences at the level of host-parasite interactions have greatly reduced this prospect.

1.2. Life cycle of *T. congolense*

It was assumed that the major differential attribute in the life cycles of *T. congolense* (Nanomonas) and its relative *T. brucei* (Trypanozoon) in *Glossina* vector was in the final destination of the metacyclics (reviewed in Hoare 1972). The complex life cycle of this organism was understood in terms of bloodstream form (BSF) parasites proliferating in the mammalian blood and getting ingested by tsetse while feeding. This follows establishment of infection in the midgut of vector accompanied by several changes in form and migrating anteriorly through the foregut to the proboscis for transmission into the mammalian host (see an illustration of the classical life cycle of *T. congolense* in Figure 1). While *T. congolense* metacyclics are found in the proboscis, those of *T. brucei* are located in the salivary glands, giving rise to the subgenera classification of these two

tsetse transmitted species. *T. brucei* is more studied among the African trypanosomes and the assumption that *T. congolense* shared similar aspects of the vector stage life with *T. brucei* led to a wide gap between knowledge of life cycles of these species as that of *T. brucei* continuously increased over the years from studies on the differentiation of bloodstream forms (Roditi, Carrington and Turner 1987, Tetley et al. 1987, Acosta-Serrano et al. 2001, Urwyler et al. 2007), cell cycle studies (Hutchinson et al. 2007) and genetic exchange and meiosis (Zampetti-Bosseler et al. 1986, Peacock et al. 2012). Generally, both species develop from blood stream forms into metacyclics by establishing infection in the ectoperitrophic space of the midgut where they subsequently invade the proventiculus in the mid gut, accompanied by loss of variant surface glycoprotein (VSG) forms.

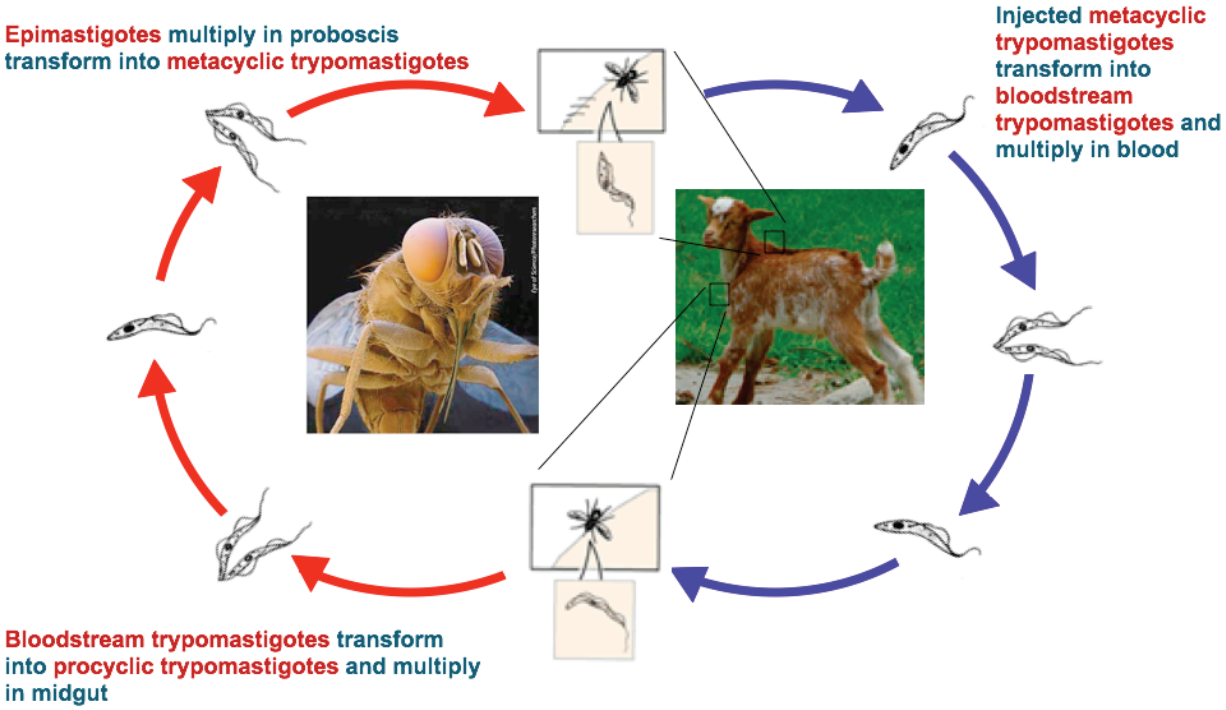

Figure 1. Life cycle of *Trypanosoma congolense*. Note that differences exist in the tsetse as regards sites of development when compared to other nagana-causing African trypanosomes as *T. brucei* develops in the salivary glands after midgut establishment while *T. vivax* does not require a midgut establishment (modified version from http://www.dpd.cdc.gov/dpdx by Koliwer-Brandl, PhD thesis, 2011).

The VSG is replaced by procyclins, a collection of GPI-anchored glycoproteins (Pearson 2001) encoded by a small number of genes and hence limited potential for variation as the major surface protein in *T. brucei* (Butikofer et al. 1997, Acosta-Serrano et al. 1999), but the major surface coat in *T. congolense* is carbohydrate (Utz et al. 2006).

Recently, Peacock et al. (2012) using a combination of cell biology and microscopy clearly elucidated significant differences existing between transitional stages in the foregut and the mouthparts. In *T. congolense*, the proventricular trypomastigotes constitute a morphologically

discrete population that passes through the proventricular membrane to the foregut lumen before migrating to the proboscis. In the proboscis, it develops into epimastigotes and attaches via the flagella to the chitinous lining of the proboscis and cibarium as was previously described by Jefferies, Helfrich and Molyneux (1987). According to Thevenaz and Hecker (1980), the epimastigotes finally proliferates in the cibarium into the infective metacyclics that are already preadapted with VSG replacing the carbohydrate coat for life in the mammalian blood stream.

In *T. brucei*, an arrest occurs during G2 life cycle stage with a 4 N DNA content. The arrested stage divides asymmetrically into short and long epimastigotes. Both of these forms are found in the foregut (Gluenz et al. 2008) with the short form finally migrating anteriorly to colonise the salivary glands. Peacock et al. (2012) showed that there is a contrast between *T. brucei* and *T. congolense*. The asymmetric division in the former is achieved by the kinetoplasts migrating round the nucleus towards the anterior, resulting in the formation of 2 daughter epimastigote cells when the nucleus divides. In *T. congolense*, while there is migration of the kinetoplast around the nucleus to the anterior, an elongation of the posterior end occurs. This process in *T. congolense* could be accomplished with or without cell division. The resultant long epimastigotes (about 30 μm) becomes reduced in size (ca. 13 μm) forming the infective metacyclics. Successful completion of this life cycle depends on several factors mostly inherent in the biology of fly vector, trypanosome and blood meal and have been reviewed by Dyer et al. (2013).

1.3. Sialic acids and Trans-sialidase in nagana

1.3.1. Sialic acids

Sialic acids (Sia) sugars and the multi gene protein family trans-sialidases play key roles in nagana. Sia is a monosaccharide family of 9-carbon backbone that incorporate a carboxy functional group. The carboxyl group confers upon them the negative charge and acidity. There are presently over 60 known members of this special class of compounds (Kamerling and Gerwig 2006). Sia are restricted in their distribution as they occur in the deuterostomes and some bacteria. Except for the report by (Shah et al. 2003) that provides evidence for the occurrence of Sia in plant cells, plants are generally considered lacking Sia (Zeleny et al. 2006). Certain features make these compounds unique; in vertebrates, the glycan chains of vertebrates' glycoconjugates are composed mostly of 5-carbon and 6-carbon sugars. Others include their being subject to a remarkable number of modifications as well as occupation of the terminal end of glycan chains. Their synthesis is by condensation of a neutral 6C unit with the 3C molecule, pyruvate (see **Section 1.3.1.1** for the *de novo* pathway of Sia biosynthesis). Finally, while all other vertebrates' monosaccharides are activated in form of uridine or guanidine dinucleotides, Sia are activated as cytidine mononucleotides, a high-energy donor sugar (Angata and Varki 2002).

Originally defined as derivatives of neuraminic acid (5-amino-3, 5-dideoxy-D-glycero-D-galacto-2-

nonulosonic acid, (Neu), the definition of Sia was expanded upon discovery of 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN). KDN has a hydroxyl group at position 5 of the 9-carbon backbone instead of the amino group. These two compounds are very similar in structure, occurrence and biosynthetic pathways (Angata & Varki, 2002). All other currently recognised Sia are biosynthetic derivatives of either *N*-acetylneuraminic acid (Neu5Ac) or KDN. Though highly diversified, the most common Sia in mammals are Neu5Ac and Neu5Gc, the latter resulting from an addition of oxygen atom to the *N*-acetyl group at C5 of the precursor CMP-Neu5Ac, forming *N*-glycolylneuraminic acid (Neu5Gc). This reaction that forms Neu5Gc is catalysed by CMP-Neu5Ac hydroxylase (CMPH). Although among the most common Sia, second only to Neu5Ac in mammals, birds, reptiles, amphibians and fish, and the predominant Sia in man's closest relatives, the great Apes, it is hardly found in humans, except in foetuses, brain and tumour cells, thus indicating a possible dietary uptake (Varki 2001, Sprenger and Duncan 2012). The structural gene, cytidine mononucleotide acetyl hydroxylase gene, *CMAH* responsible for modifying Neu5Ac is inactive in humans due to a mutation that results in deletion of exon 6 of the *CMAH* (Varki, 2001).

In addition to the *N*-acetyl and *N*-glycolyl classes of Sia and and leading to the large diversity in this group of compounds are common modifications of the the ring or exocyclichydroxyls. These include one to three acetyl groups that could be added to the hydroxyl of C4 in the sugar ring, or to C7, C8 and C9 hydroxyls of the glycerol side chain. Sia may also be modified with lactyl, sulfhydryl, or methyl groups, although this is less usual a modification rather than acetylation (Vimr et al. 2004). Further variety of Sia is increased by intra or intermolecular lactamization involving the carboxylate at C1 and intermolecular lactamization between C1 and C5 in the case of Neu (Angata & Varki, 2002).

More than 20% of Sia bound to glycoproteins of human RBC may be forms other than Neu5Ac, the predominant Sia in humans, including Neu (Bulai et al. 2003). Neu is considered to be derived via enzymatic deacetylation (Manzi, Diaz and Varki 1990). No clear idea exists as to why Sia exhibit such a wide and vivid structural variation. However, Angata & Varki (2002) argue that specific recognition of endogenous lectins and evasion of pathogens with Sia recognising lectins, are the driving forces within and outside respectively, and are probably at the heart of this diversification. Yet it is difficult to say to what extent any of these forces acting alone could drive the process of structural variation of the Sia. Put differently, the complex variability of Sia modifications has been suggested as being brought about by the struggle between pathogens and hosts' cells (Gagneux et al. 2001),a classical illustration of evolutionary pressure. The result is the modification of pathogen recognition determinant, Sia by the hosts' cells in order to avoid infection on the one hand. On the other, it leads to pathogens constantly evolving new binding specificities to overcome this hindrance and ensure survival.

By the very nature of their position as terminal sugars on animal glycans, Sia equally play

important roles in masking recognition sites. For instance, pathogenic organism like viruses, e.g. *Influenza* virus A, B and C and bacteria make use of Sia containing glycoconjugates to gain attachment to the surfaces of hosts' cells (Kelm and Schauer 1997). In eukaryotes, Sia have evolved to mediate a wide and diverse range of cell-cell and cell-molecule interactions. This include glycoconjugates and cell membrane stabilisation, mediation of cell-cell regulation and acting as chemical messengers, regulating transmembrane receptor function, affects membrane transport, control of the half-lives of circulating glycoproteins and cells and contributes to perm-selectivity of the glomerular endothelium and slit diaphragm (Schauer 2000). Taken together, the relative importance of these complex molecules in the life function of higher organisms could be appreciated. This was amply demonstrated by early death of transgenic mouse mutants with homozygous defect in Sia biosynthesis (Schwarzkopf et al. 2002).

1.3.1.1. Sia synthesis and/or acquisition by organisms

According to Vimr and Lichtensteiger (2002), pathogenic organisms that modify their surfaces with Sia do this to avoid the host's immune system by masquerading as 'self'. They also do this to subvert the host's innate immune system. It is envisaged that the biological advantages of cell surface sialylation in the host-pathogen or host-commensal interactions to the pathogen or commensal are enormous and due to this, these organisms have evolved, at least four known strategies to sialylate cell surfaces. These strategies include (i) *de novo* synthesis (ii) donor scavenging (iii) *trans*-sialidase and (iv) precursor scavenging (note: precursor scavenging is not discussed here).

The *de novo* Sia biosynthetic pathway was first discovered in *Escherichia coli* K1, where Sia are the repeating subunits of the capsular polysaccharide. The first step in the *de novo* Sia synthesis involves the conversion of the common cell precursor, UDP-GlcNAc to ManNAc by NeuC. NeuC catalyses the formation of a 2-acetamidoglucal intermediate by an elimination reaction. This is followed by irreversible epimerisation of this intermediate to ManNAc, as occurs in the mammalian system (Chou et al. 2003). Although slight variations exist within microorganisms (for e.g. between *E. coli* and the group B meningococci) in terms of NeuC orthologue, in either case, free ManNAc is produced as the obligate substrate for the following condensation reaction between ManNAc and phosphoenolpyruvate catalysed by NeuB (Vann et al. 1997). A detailed review of this process is provided by Vimr et al. (2004). In mammals however, the UDP-GlcNAc epimerase is a homologue of NeuC and that the mammalian orthologue of NeuB uses ManNAc-6-P instead of free ManNAc in the condensation reaction with phosphoenolpyruvate (Ringenberg, Lichtensteiger and Vimr 2001). The free Sia synthesised is activated by CMP-Sia synthetase, NeuA to produce the obligate donor of Sia for prokaryotic and eukaryotic sialyltransferases.

Donor scavenging is the second strategy of cell surface sialylation, only found in the sexually

transmitted bacteria, *Neisseria gonorrhoea*. The bacterium that causes gonorrhoea in man lacks the neuABC genes, yet it is capable of sialylating its cell surface (Vimr et al. 2004). This is accomplishes by an extracellular, membrane bound sialyltransferase which by virtue of its location has access to CMP-Sia which are present in small amounts as normal secretions of the host.

1.3.2. Trans-sialidases

Trypansomes express a unique form of glycosyltransferase called trans-sialidase (TS) which it uses to acquire Sia. It was first described in the blood stream form of the American trypanosome, *T. cruzi* (Schenkman et al. 1991) and has been implicated in pathogenicity of the parasites. In the African trypanosomes, TS was shown to enhance survival and establishment of infections in *T. bruce*i in the tsetse vector (Nagamune et al. 2004). TS was initially thought to be expressed only in the polycyclic forms in African trypanosomes (Pontes de Carvalho et al. 1993, Engstler, Schauer and Brun 1995). Recent evidences have shown that it plays a role in anaemia in animals suffering *Trypanosoma congolense* infections (Nok and Balogun 2003, Coustou et al. 2012) and *T. vivax* infections (Guegan et al. 2013). TS has therefore been established as virulent factors in trypanosomiasis. The enzyme accomplishes this by desialylation of erythrocytes leading to their clearance from the circulating system (Coustou et al. 2012, Guegan et al. 2013). A homology model of TconTS is presented as Figure 4b in Chapter 2.

TS is a modified version of the typical sialidases and catalyses the transfer of the Sia moiety from a donor conjugate to an acceptor substrate. The acceptor substrate must present a β-galactoside or N-acetylgalactosamine as the terminal monosaccharide forming a new α2,3-glycosidic linkage (Schenkman et al. 1991). Unlike the typical sialylytransferases, it does not require monosaccharide donors in form of cytidine-5′-monophosphate activated Sia and is highly specific for α2,3-linked sialoglycoconjugate, though α2-6 linked Sia can also be transferred (Tiralongo et al. 2003b). This trans-sialylation is highly efficient and differs significantly from the irreversible transfer catalysed by known CMP-Sia dependent sialyltransferases. They also act as typical sialidases in absence of an appropriate acceptor by hydrolysing glycosidically linked Sia (Schenkman et al. 1991). In this respect, TS is similar to viral, bacterial and even mammalian sialidases.

1.3.2.1. Expression and diversity in the *Trypanosoma* spp

Trans sialidase occur in *Trypanosoma spp.,* as a multigene family in the genomes. *T. cruzi,* has the highest number of 1430 genes (Cazzulo and Frasch 1992, De Pablos and Osuna 2012), with members encoding for proteins of different sizes ranging from 60 to more than 200 kDa (Cazzulo and Frasch 1992, Colli 1993, Frasch 2000). Out of these large number, only 12 encodes enzymatically active *T. cruzi* trans-sialidase (TcTS) proteins while 725 encodes for inactive gene products and 693 are pseudogenes (Kim et al. 2005). The key element that differentiates an active TcTS from an inactive one is a single amino acid exchange, Tyr342-His (Frasch 2000). The

situation in terms of gene numbers seems more simplified in African trypanosomes, where the TS and TS-like gene families are much smaller. This is illustrated by the presence of 9 TS gene members (TbTS) identified in *T. brucei* (Montagna et al. 2002, Montagna, Donelson and Frasch 2006, Nakatani et al. 2011). In *T. congolense* (TconTS), at least 17 TS-like genes have been identified (Tiralongo et al. 2003a, Tiralongo et al. 2003b, Koliwer-Brandl et al. 2011, Coustou et al. 2012, Gbem et al. 2013). The TconTS genes have been classified into at least four different active groups as TconTS1, TconTS2, TconTS3, TconTS4 and the inactive TconTS-like genes (Gbem et al. 2013). The largest group, TconTS1, has 11 closely related members that share over 95% sequence identity and all are active (Koliwer-Brandl et al. 2011).

The key element mediating the functions of given TS has been ascribed to the N-terminal catalytic domain (CD) harbouring the active site with characteristic conserved amino acids (Cremona et al. 1995, Buschiazzo, Campetella and Frasch 1997, Buschiazzo et al. 2000, Buschiazzo et al. 2002, Montagna et al. 2002, (Amaya et al. 2004, (Montagna et al. 2006). Members of TconTS-like genes lack these critical amino acids and are considered inactive. A recent study (Gbem et al. 2013) pointed to the possibility of the lectin domain (LD), at the C-terminus of these enzymes playing hitherto an unknown contribution in activity. More is however required to make conclusive statements on the role the lectin domain plays in TS activity.

T. vivax was thought to harbour genes expressing only sialidase activity without the corresponding transfer activity (Engstler et al. 1995). Phylogenetic analysis on the WTSI shot-gun sequences using trans-sialidase genes revealed the presence of 6 trans-sialidase gene like sequences (Jackson, et al. 2013) with most conserving the critical amino acids required for trans-sialidase activities. Guegan et al. (2013) found at least 3 TS/SA genes, TvivTS1, TvivTS3 and TvivTS5 to be involved in the animal pathogenicity of *T. vivax* infections. It is possible that other trypanosomes express TS genes as well, but which are yet to be studied. Phylogenetic analysis of the *Trypanosoma* genus using TS genes (Jackson et al. 2013, Guegan et al. 2013, Gbem et al. 2013) showed shared orthologues between *T. congolese* and *T. brucei*. In addition, while these two African trypanosomes were more closely related, *T. vivax* clustered separately away, an indication of a more distant evolution exemplified by a different biology in the tsetse vector.

1.3.2.2. Trans-sialidase structure

Much of what is known about TS structure and mode of action are from studies using TcTS. Structurally, TcTS is an oligomer formed by heterogeneous subunits varying from 120-240kDa. These subunits are composed of an amino terminal domain containing a putative catalytic site and a carboxy-terminal domain made up of variable numbers of tandem repeats. The primary structure of TcTS contains a catalytic domain of 380 amino acids on the N-terminal region. This portion shares 30% identity with some known bacterial sialidases with most of the amino acid residues in the active site conserved (Cremona et al. 1995, Chuenkova and Pereira 1995; Buschiazzo et al.

2000). The catalytic domain is linked by by a long α-helical segment of 23 amino acid residues to the lectin-like domain, made up of 260 amino acid residues (Buschiazzo et al. 2000). The lectin domain in natural TcTS is immediately followed by a repetitive, highly antigenic 12 amino acid residue motif known as shed acute phase antigen, SAPA (Buscaglia et al. 1999). SAPA is known to increase the half-life of TS in the blood (Buscaglia et al. 1999) and has also been speculated to serve as a decoy by TS to evade the host immune system (Frasch 1994). (Campetella et al. 1994) showed a recombinant TcTS without SAPA showing enzymatic activity, demonstrating that it is not required for the transfer reaction. Structural motifs have been found in trans-sialidase, e.g, Asp boxes, FRIP region but these have no direct effect on activity except protein structure (Monti et al. 2010).

T. rangeli a non-human pathogenic close relative of *T. cruzi* expresses a sialidase, TrSA that shares 70% amino acids identity with TcTS without SAPA and it is strictly a hydrolase (Buschiazzo et al. 2000). Crystallographic along with mutagenesis studies helped illustrate a protein structure with a globular core that folds into two distinct structural domains with amino acids involved in transfer reactions identified (Buschiazzo et al. 2000, Buschiazzo et al. Amaya et al. 2004, Paris et al. 2005). The N-terminal domain (residues 1-372) displays a canonical 'β-propeller' topology that harbours the active centre core, similar to viral and bacterial sialidases. The C-terminal domain typically shows the characteristic β-barrel topology of plant lectins (Buschiazzo et al. 2000) and is referred to as the lectin-like domain. Using modelling studies, Tiralongo et al. (2003a) and (Koliwer-Brandl et al. 2011) provided evidence that the TconTS folds in a similar way like the TcTS (see the modelled structure of TconTS in Chapter 2).

It was determined that in TcTS, Tyr120 was essential in binding Sia from donor substrate. Other critical amino acids are the arginine triad of Arg35, 245 and 314. This triad is essential in binding the COOH-group of Sia. Glu230 is required for stabilising the tree arginine residues. Asp59 and Asp96 are involved in proton transfer during catalysis, resulting in the formation of the transient oxocarbenium ion. Tyr342 was found to maintain contact with the oxocarbenium ion at C2 of Sia (Buschiazzo et al. 2000, Watts et al. 2003). A proline residue, P283 has also been found to be necessary as it influences the position of Trp312 in the active cleft. A combination of energy analysis and molecular dynamics (Demir and Roitberg, 2009, Mictchell et al. 2013), computational modelling, surface plasmon resonance (SPR) and nuclear magnetic resonance (NMR) spectroscopy (Buschiazzo et a. 2002, Haselhorst et al. 2004, Todeschini et al. 2004, Blume et al. 2007,) have been used to gain up to date insight in the catalytic mechanism of TS enzymes. The sequence of events that occur in trans-sialylation are presented in mechanistic basis of TS reaction (see **Section 1.3.2.5**).

1.3.2.3. Conserved amino acids in TS catalytic pocket of African Trypanosomes

Molecular approaches have led to defining primary amino acid sequences in both *T. rangeli* and

several pathogenic trypanosomes. Primary amino acid sequences have been used to identify orthologues between different trypanosomes (Coustou et al. 2012, Gbem et al. 2013, Guegan et al. 2013, Jackson et al. 2013). However, the absence of data on both hydrolysis and transfer activities that could be used to make meaningful comparison among trypanosomes have made it difficult to derive detailed inferences. Gbem et al. (2013) reported on the biochemical activities of TconTS1, TconTS2 and TconTS3 enzymes. In combination with specific activities reported for TconTS1 variants (Koliwer-Brandl et al. 2011), comparisons could be made for at least TconTS gene products. Comparison on the basis of amino acid conservations in the catalytic pocket did not give a clear indication of specific activities in between TconTS genes, as critical amino acids were mostly conserved, yet specific activities varied.

1.3.2.4. Mechanistic basis of trans-sialylation and hydrolysis

Knowledge of enzyme catalysis by TS has been gained mainly from studies on the TcTS. The double displacement reaction mechanism for retaining glycosidases has been proven to hold for trans-sialidases (Watts et al. 2003; Amaya et al. 2004), but with a fundamental difference. In most of the known retaining glycoside hydrolases, a negatively charged nucleophile directly attacks the anomeric carbon to generate the covalent intermediate, while a neighbouring residue; for example, tyrosine stabilises the negative charge in the free enzyme. However, as Sia acids bear carboxylate groups adjacent to the anomeric centre, it is most likely that unfavourable electrostatic attractions will arise if the invariant Glu230 located in the active cleft of TSs were to directly function as a nucleophile (Watts et al. 2003). Computational analysis revealed that a long-lived covalent intermediate is formed in in trans-sialidase reactions with the Tyr342/Glu230 couple been invoked to relay charge from the more distant or remote glutamate by tyrosine. This makes the phenolic oxygen of tyrosine transiently achieve appreciable negative ion character at the transition state and the repulsive effects are therefore avoided. It means there must be substrate binding to the enzyme holo form before any transfer reaction is possible.

Comparing crystallographic structures of TcTS and TrSA show the active cleft of the former to be narrower and and more hydrophobic as opposed to the latter. The exclusion of water from the trans-sialidase was suggested to possibly favour trans-sialylation reaction over hydrolysis (Buschiazzo et al. 2002). (Demir and Roitberg 2009) used molecular dynamics calculations and confirmed the presence of Trp312 in the catalytic pocket of TcTS, making the above assertion possible as flexibility in the apo form is ensured due to the loop motion of Trp312. Combining several techniques including X-ray crystallographic studies, atom substitution at certain positions of the Sia moiety, mass spectroscopy, kinetic isotope effects and enzyme kinetics, detailed sequences of events that occur in the trans-sialidase substrate catalysis have been elucidated. These events include the formation of enzyme-substrate complex (Michaelis complex), formation of a covalent glycosyl enzyme intermediate, sugar distortion, nucleophilic charge relay and

acid/base catalysis.

Buschiazzo et al. (2002), Haselhorst et al. (2004) and Blume et al. (2007) showed via surface plasmon resonance (SPR) and NMR spectroscopy that the Sia binding modulates the asialoglycon binding. In other words, the acceptor aglycon would not bind until Sia binds. By retarding bond cleavage events in the enzyme-substrate complex through the introduction of a fluorine atom at C3 of the substrate, Amaya et al. (2004) showed the complete nature of enzyme-substrate formation in the trans-sialidase catalytic activity. Two different substrates, sialyl-lactose (TcTS-SL complex) and methylumbelliferyl neuraminic acid, MUNANA (TcTS-MUNANA complex) showed the aglycon moiety occupies the same binding pocket in the enzyme-substrate complexes and makes strong stacking interactions with Trp312 and Try119. This has been further confirmed by Mitchell et al. (2010) using molecular dynamics simulations, showing that Trp 312 plays a dual role in making the flexibility of the active cleft possible. The stacking interactions of Trp312 with Tyr119 helps orients the substrates for sialylation and once the Sia is delivered, Trp312 via its lever-like action akin to molecular shovel, releases the donor from the active site and loads up the acceptor. Therefore, the donor moiety occupies the same site as its acceptor lactose in the reaction ternary complex. This supports the double displacement reaction mechanism.

Conformational changes are necessary for trans-sialylation reaction (Smith and Eichinger 1997). These changes are caused by the Sia binding in the active pocket leading to distortion in the packing of TS. The carboxyl group of Sia makes a number of hydrogen bonding interactions with the Arg triad. The interaction of the N atom in the *N*-acetyl group with Asp96 causes its movement about 0.8Å from its original position in the native TS binding pocket, with slight movement physically locking the acetamide group in position. This plasticity is necessary for trans-sialalylation reaction and is distinctly absent in hydrolysis. However, this flexibility is much reduced once sialoside ligand is bound.

There is also the interaction with Arg53 and Asp96 by the O4 of Sia, which leads to a further stabilisation of the locked positions of Asp96 and acetamide group. There is a single hydrogen bond interaction with the glycerol side chain of Sia with the terminal hydroxyl of Tyr120, priming the catalytic nucleophilic attack on the anomeric carbon of Sia. Kinetic isotope effects studies (Yang, Schenkman and Horenstein., 2000), Pierdominici-Sottile, Horenstein and Roitberg., (2011) and later confirmed by (Watts et al. 2003 and Amaya et al. 2004) suggested the formation of the next stable species along the reaction coordinate being the glycosyl enzyme intermediate. A covalent bond of about 1.42Å is formed between the oxygen atom of Tyr342 side chain and the anomeric carbon of Sia.The intermediate stabilised by stronger interaction of the glycerol group in the sialyl-trans sialidase reactants as well as the relaxation of the sugar ring conformation offering greater stabilisation of the intermediate relative to ground states (Amaya et al. 2004). This stabilisation increases the life of the glycosyl-enzyme intermediate long enough to allow *trans*-glycosylation

which requires more time than the usual hydrolysis reaction.

It has been observed that the Sia ring in complex with TS adopts a distorted B2,5 conformation. This distortion arises from the slight displacement of O4 that forms a hydrogen bonding interaction with Asp96. This distortion however places the anomeric carbon in a suitable position for in-line attack by the nucleophile (Tyr342) with minimal hindrance from the 1.3 diaxial repulsion and satisfies the stereo-electronic requirements for an incipient oxocarbenium ion (Amaya et al. 2004). Formation of the covalent intermediate is accompanied by a change in the conformation of the sugar ring. The sugar ring now relaxes into an undistorted $^{2}C_{5}$ chair conformation with a β linkage to the enzymatic nucleophile.

Saturation transfer difference (STD)- NMR (Blume et al. 2007) using pNP-Neu5Ac has allowed comparisons of the rate of hydrolysis with that of transfer by TcTS. TcTS is capable of transferring Sia from non-natural donors e.g. *p*-nitrophenyl and 4-methylumbelliferyl α-sialosides. The rate of transfer is however slower for the artificial substrates. Paris et al. (2001) and Haselhorst et al. (2004) showed that the rate of Sia transfer from MUNANA to lactose is only about 0.6 compared to that of hydrolysis while that of hydrolysis is about twice more than that of sialyl-lactose. TconTS enzymes however show poor or no hydrolysis on MMNANA (manuscript from S. Kelm's laboratory). While the molecular events surrounding the differences in the reactions of TcTS with MMNANA and sialyl-lactose on one hand and the difference between TconTS on the other are not clear, it buttresses the subtle difference that exist between TSs from the two *Trypanosoma* spp and warrants further studies.

Equally, if the elements and events in the catalytic pocket were the only determinants of trans-sialylation and hydrolysis reactions, TconTS enzymes would have exhibited close to same magnitude of activity, if similar substrates were to be used. Studies with the four active TconTS enzymes (Tiralongo et al. 2003a, Koliwer-Brandl et al. 2011, Gbem et al. 2013) showed different specific activities for these enzymes. This points to other elements, possibly from the lectin domain being involved in catalysis (Gbem et al. 2013). It will be interesting to see how much is contributed towards activity by the other factors inherent in TS genes. Knowledge gained in this area will be significant in designing inhibitors for the various TS enzymes.

1.3.2.5. Substrates for Trans-sialidase

Substrate specificities have been extensively studied for the American *T. cruzi* TS (reviewed in Schauer and Kamerling 2011). This is because TS was first discovered in this organism and research on the ethiologic agent of Chagas' diseases is by far more advanced than what obtains for the *Trypanosoma* species causing nagana. Substrates specificities are discussed here only in terms of the African trypanosomes.

(Engstler, Reuter and Schauer 1993) showed a variety of sialo-glycoconjugates as excellent

donors for *T. brucei* procyclic stage-derived TS. Serum glycoconjugates, human and bovine erythrocytes all served as substrates from which Sia was transferred. Acceptors of the transferred Sia were terminal β-galactose residues from oligosaccharides and glycoconjugates, all resulting in the formation of α2-3 linkages in the new products. Neu5Ac(α2-3)Gal(β1-4)Glc, Neu5Ac(α2-3)Gal(β1-4)Glc-ol as well as fetuin served as ‘‘good’’ donors when lactose was used as an acceptor. MU-Neu5Ac was equally reported as a good donor. MU-Neu5Ac has also been reported as a donor of Sia when *T. congolense* procyclic stage TS was used (Tiralongo et al. 2003a, Schrader et al. 2003). Equally for *T. congolense*, it was reported that both Neu5Ac(α2-3)Gal(β1-4)Glc and Neu5Gc(α2-3)Gal(β1-4)Glc served as excellent donors with similar relative Sia transfer rates but bovine brain ganglioside, collocalia mucin and bovine submandibular gland were not utilised (Engstler et al. 1995). These substrates were followed by fetuin and MU-Neu5Ac and that *T. congolense* preferentially linked Sia to (α2-3)Galβ1- structures. Like *T. brucei*, *T. congolense* shows minimal utilisation of α2-6 and α2-8 structures (Engstler et al. 2003, Schrader et al. 2003, Tiralongo et al. 2003a) and in addition, Tiralongo et al. (2003a) found out that even though TconTS uses α2-6 structures as donors, only products with α2-3 linkages are formed. Sialylated milk products acted as donors for the *T. congolense* procyclic TS which generally was found to favour terminal Gal(β1-4)GalNAc over Gal(β1-3)GalNAc sequences (Tiralongo et al. 2003a).

Koliwer-Brandl et al. (2011) found very low transfer activities for two variants of TconTS1; TconTS1b (EMBL:HE583284) and TconTS1e-1 (EMBL:HE583287) when using MU-Neu5Ac and Neu5Ac-pNP, another synthetic donor. This is in direct contrast to the earlier reports (Engstler et al. 1993, Tiralongo et al. 2003a, Schrader et al. 2003). This discrepancy possibly arises from the fact that previous assays all made use of proteins from polycyclic *T. congolese* forms and those purifications most likely contained additional TS forms as well as GARP. At least the purification by Tiralongo et al. (2003a) has been shown to contain GARP in addition to other TconTS forms (Koliwer-Brandl et al. 2011, Gbem et al. 2013).

1.3.2.6. Inhibitors of Trans-sialidase

Due to the scourge of the American and African trypanosomiasis, several targets within the parasites have been sought for in order to design inhibitors in a bid to cure infections and disrupt their transmission cycle (Neres, Bryce and Douglas 2008) set out a list of criteria that should be considered before any rational drug design strategy could be embarked upon. These include aspects of parasite biochemistry that makes a strong basis for scientific intervention, if the parasite posses within its arsenal other alternatives that it could possibly employ to thwart such a scientific attack, if such a selected target is specific to the parasite and not common to both parasite and host, positive effects, if parasite is knocked out using means such as antibodies, gene knockout, siRNAs, previously known drugs analogous with similar effects and if mode of inhibition would affect the parasite significantly. On a holistic basis, TS presents an attractive target and inhibitors of

these enzymes have generally been considered and pursued over other approaches in the fight against trypanosomes. This is more important especially as approaches like gene knockouts appear problematic due to multiple gene members.

It has been observed that classical sialidase inhibitors like 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu2en5Ac), also called DANA, 4-amino-2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (4-amino-Neu2en5Ac), 4-guanidino-Neu2en5Ac and *N*-(4-nitrophenyl) oxamic acid are not effective on TcTS (Paris et al. 2005, Neres, 2006). 2,3-Difluorosialic acid (Watts et al. 2003) inhibited wild-type TcTS in a time dependent manner as confirmed from kinetics studies. However, the wild type TcTS rapidly recovered activity by transglycosylation upon removal of excess inhibitor and incubation with lactosides. A derivative of Neu5Ac, 2,3-difluoro-Neu5Ac incorporating an aromatic ring at C9 was used in a bid to confer specificity. 20 mM concentration was found to inhibit TcTS by forming covalent intermediates with the enzymes (Buchini, Buschiazzo and Withers 2008). Hydrophobic interactions between the phenyl ring of Tyr119 was found to prevents lactoside binding in the catalytic pocket by 3-fluorosialyl fluoride. Other compounds designed to mimic Sia but contained phopsphonate instead of carboxylate group while inhibiting classical sialidases in a competitive manner did not inhibit trans-sialidases from *T. cruzi* and *T. brucei* (Engstler et al. 1994). (Streicher and Busse 2006) experimented with cyclohexenephosphonate monoakyl esters and found them to be weakly inhibitive.

Sia donor and acceptor analogues have also been considered and efficacies tested. These have been extensively reviewed (Neres et al. 2008, Schauer and Kimerling, 2011). The principle underlining this approach is for such substrates to occupy the Sia binding site of the donor substrate or target the acceptor site. For example, modification of the ganglioside GM3, though a substrate for trans-sialidase at either C4 or C8 results in reasonable inhibition of TcTS with inhibition concentrations ranging between 10–100 μM (Vandekerckhove et al. 1992). Several of such substrates have been reported, for e.g. by (Agusti et al. 2007).

Lactitol, a lactose derivative was found to out compete lactose as a Sia acceptor, preventing lactose sialylation as well as the sialylation of *N*-acetyl-lactosamine and 4-metylumbelliferyl-β-*D*-galactopyranoside (Agusti et al. 2004). It was found that lactitol prevented sialylation of *T. cruzi* mucins in mM concentrations and reduced infections of mammalian cells up to 27% but without inhibiting TcTS enzymatic activity of. Instead, it acted as a preferential Sia acceptor (Agusti et al. 2004).

From the foregoing, it is obvious that a potent inhibitor of TS is yet to be found. Recently, (Buschiazzo et al. 2012) reported on an effective mouse monoclonal antibody (mAb13G9) with high affinity and specificity for TcTS. The mAb was shown to prevent both immune system and haematological abnormalities caused by *T. cruzi* infections. In *T. congolense*, Coustou et al. (2012) vaccinated mice with the TconTS-Like 2 gene and reported protection when challenged with *T.*

congolense parasites, illustrating the promise of vaccination using TS.

1.3.2.7. Drug transport and resistance in *Trypanosoma* spp

Drug and cross-resistance have become a huge problem in both AAT and HAT treatment. In AAT, three major drugs have been in use for the past 50 yrs. These are diminazene aceturate (DA), homidium chloride, both having prophylaxis and therapeutic properties and isometamidium chloride (ISM) which shows only therapeutic properties. It was reported over 10 years ago that about 35 million doses of these drugs are used within the 37 African countries where trypanosomiasis is endemic (Diarra et al. 1998). It is therefore not surprising that across Africa, drug resistance have been reported for both the AAT and HAT (Geerts et al. 2001). Veterinary resistances to these drugs have been reported in 17 African countries (Delespaux et al. 2008). Drug resistance is equally a problem in HAT (see reviews by (Alsford et al. 2012), (Baker et al. 2013), (Kennedy 2013). Cross-resistance has arisen due to long-term usage due to non-availability of new drugs among other factors. In order to come out with answers to this problem, research focused on mode of actions and mechanisms involved in this phenomenon.

Mitochondrial membrane potential (Werbovetz 2006) and binding to nuclear and kinetoplastid DNA (Carter and Fairlamb 1993) both aid the accumulation of diamidines in trypanosomes, but it is the presence of specific transporters in the plasma membrane that leads to the accumulation of trypanocides in the parasites (de Koning and Jarvis 2001). The purine transporter 2, P2, responsible for transport of adenine and adenosine has been implicated in reduced melarsoprol (a drug for human treatment but regularly used for animal treatment) uptake and cross-resistance to both melamine based arsenicals and diamidines. Both are imported into the cell via the same transporter, P2. In *T. brucei*, P2 is encoded by *TbTA1* (Maser et al. 1999) as *TbTA1* gene deletion and loss of mutation have been described in drug resistant strains (Mäser et al. 1999, Stewart et al. 2010). These same mutations were found in field isolates of *T. brucei* species in which treatment with melarsoprol failed (Matovu et al. 2001, Nerima et al. 2007). Another proof of the involvement of the P2 transporter in the cross-resistance came from the finding that *tbat1*-null trypanosomes lost the adenine sensitive component of adenine and melarsoprol import (Geiser et al. 2005), (Matovu et al. 2003). There is overwhelming evidence to support that DA is exclusively taken up by P2/TbAT1 transporter. Deletion of this gene or loss in the activity of its gene product results in high levels of resistance (de Koning 2001).

It was found that 50% pentamidine flux (pentamine is meant for human treatment but farmers equally employ it for animal treatment) is mediated by the same P2 transporter (de Koning and Jarvis 2001). The realisation of this low sensitivity and net uptake made it clear that other transporters must be involved in the cross-resistance phenotype. This led to the identification of the high affinity pentamidine transporter, HAPT1 (Bridges et al. 2007). Melarsoprol was shown to be a substrate for HAPT1 and loss of HAPT1 activity in *Tbat1* null cells led to increased resistance to pentamidine and melarsoprol (Bridges et al. 2007), (Teka et al. 2011)

The ATP binding cassette (ABC) class of transporters have been implicated in resistance mechanisms (Baker et al. 2013). Changes that occur in the net accumulation of drugs, as as well as the energy-dependent expulsion or extrusion of drugs, prodrugs or active metabolites have been associated with the ABC transporters. Two of these transporters, the P-gylcoprotein and the multi-drug resistance associated proteins, MRPs have been shown to be involved in cases of drug resistance in *Plasmodium* and *Leishmania* (Klokouzas et al. 2003), protozoan relatives of trypanosomes. In *T. brucei*, the efflux of the trypanothione, Mel T was observed when MRP A was over expressed in that organism (Shahi et al. 2002).

Of recent, RNAi experiments led to the identification of a new class of transporters involved in drug resistance (Baker et al. 2012). The *T. brucei* genome encodes three classes of aquaglyceroporins (AQPs), AQP 1-3. The aquaglyceroporin 2, AQP2 restricted to the flagellar pocket has been found to mediate melarsoprol-pentamidine cross-resistivity in trypanosomes. Mutations affecting AQP2 have been linked to clinal resistance (Baker et al. 2013). However, the specific details underlining its involvement are yet to be understood.

1.4. Aims

The work presented here aimed to biochemically characterise trans-sialidase genes and their products from the African trypanosome, *T. congolense*. This is the most prevalent causative agent of nagana. A detailed understanding of gene variations, number of gene copies, substrates specificities and enzyme kinetics would fill the knowledge gaps and engender new approaches in the fight against the veterinary trypanosomosis. HAT and a-HT may equally benefit from these finding. Therefore, we aimed to clone, express, purify and employ different substrates to biochemically characterise TS gene products from this organism. In order to have a basis for comparison, we looked at enzyme activities on a single but relevant glycoprotein, fetuin, We then looked at enzymatic activities on blood glycoconjugates. We made use of data obtained from databases in order to compare TS genes from other trypanosomes to ascertain the occurrence of or lack or orthologues among these genes.

Trans-sialidase represents an attractive target in the new direction as it is critical in the life cycle of the organism, both in the insect vector as well as in the mammalian host. Moreover, TS enzymes are not expressed by the mammalian host. It was thought that TS in African trypanosomes was only expressed in the procyclic insect forms (Engstler et al. 1993, 1995) where it plays a major role in the establishment of infection (Nagamune et al. 2004). The possible biotechnological application of the enzyme was a major attraction for investigating the enzymes from African trypanosomes (Tiralongo et al. 2003a). New evidences have pointed to the presence of these enzymes in the blood stream from of the parasites (Gbem et al. 2013) and as virulent factors in trypanosomosis (Nok and Balogun, 2003, Coustou et al. 2012, Guegan et al. 2013). With this findings, the attractiveness of TS as a therapeutic target in African trypanosomes has been greatly enhanced.

1.5. References

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Results

Publication 1

Biochemical characterization of trans-sialidase TS1 variants from *Trypanosoma congolense*

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BMC Biochemistry, 2011, 12:39
http://www.biomedcentral.com/1471-2091/12/39

Contributions of Thaddeus Gbem

- TconTS1 gene cloning and sequencing
- Expression of TconTS1 and *T. cruzi* TS proteins
- Protein purification
- Gene sequences analysis and alignments
- Drafted above parts in manuscript

Koliwer-Brandl et al. BMC Biochemistry 2011, 12:39
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RESEARCH ARTICLE Open Access

Biochemical characterization of trans-sialidase TS1 variants from *Trypanosoma congolense*

Hendrik Koliwer-Brandl, Thaddeus T Gbem, Mario Waespy, Olga Reichert, Philipp Mandel, Eric Drebitz, Frank Dietz and Sørge Kelm*

Abstract

Background: Animal African trypanosomiasis, sleeping sickness in humans and Nagana in cattle, is a resurgent disease in Africa caused by *Trypanosoma* parasites. Trans-sialidases expressed by trypanosomes play an important role in the infection cycle of insects and mammals. Whereas trans-sialidases of other trypanosomes like the American *T. cruzi* are well investigated, relatively little research has been done on these enzymes of *T. congolense*.

Results: Based on a partial sequence and an open reading frame in the WTSI database, DNA sequences encoding for eleven *T. congolense* trans-sialidase 1 variants with 96.3% overall amino acid identity were amplified. Trans-sialidase 1 variants were expressed as recombinant proteins, isolated and assayed for trans-sialylation activity. The purified proteins produced α2,3-sialyllactose from lactose by desialylating fetuin, clearly demonstrating their trans-sialidase activity. Using an HPLC-based assay, substrate specificities and kinetic parameters of two variants were characterized in detail indicating differences in substrate specificities for lactose, fetuin and synthetic substrates. Both enzymes were able to sialylate asialofetuin to an extent, which was sufficient to reconstitute binding sites for Siglec-4. A mass spectrometric analysis of the sialylation pattern of glycopeptides from fetuin revealed clear but generally similar changes in the sialylation pattern of the *N*-glycans on fetuin catalyzed by the trans-sialidases investigated.

Conclusions: The identification and characterization of a trans-sialidase gene family of the African parasite *T. congolense* has opened new perspectives for investigating the biological role of these enzymes in Nagana and sleeping sickness. Based on this study it will be interesting to address the expression pattern of these genes and their activities in the different stages of the parasite in its infection cycle. Furthermore, these trans-sialidases have the biotechnological potential to be used for enzymatic modification of sialylated glycoconjugates.

Background

Animal African trypanosomiasis, called sleeping sickness in humans and Nagana in cattle, is a resurgent disease in Africa. Nagana is caused by *Trypanosoma congolense* (*T. congolense*), *Trypanosoma vivax* (*T. vivax*) and *Trypanosoma brucei* (*T. brucei*) subspecies. Most research on African trypanosomes has focused on *T. brucei*, whereas only few studies have been done with other African trypanosomes including *T. congolense*. In wild animals, these parasites cause relatively mild infections while in domestic animals they cause a severe, often fatal disease. Because of Nagana, stock farming is very difficult within the tsetse belt of Africa [1].

Although of crucial importance for their survival, cyclical transmission and hence pathogenicity of trypanosomes, trypanosomes lack the biochemical metabolic machinery synthesizing sialic acids (Sia), but use a unique enzyme, trans-sialidase (TS) to transfer Sia onto the parasites surface molecules from the environment. Structurally TS belong to the family of sialidases (SA). In contrast to the usual sialyltransferases, TS does not utilize CMP-activated Sia as monosaccharide donors, but catalyzes the transfer of carbohydrate-linked Sia to another glycan forming a new α2,3-glycosidic linkage to galactose or *N*-acetylgalactosamine.

Whereas more detailed studies exist on the role of TS in the pathogenicity of *T. cruzi*, the etiologic agent of

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Chagas diseases in South America, where TS was first discovered [2], the current knowledge about the corresponding enzymes in the African trypanosomes is very limited. Of all the African trypanosomes, only *T. brucei* full length TS genes have been cloned and studied [3]. Sialylation of parasite surfaces is believed to protect the parasites from the action of glycolytic enzymes as well as from immunocompetent substances present in the tsetse gut and blood meal respectively, as well as influencing the interaction of parasites with the gut epithelial cells [4-6]. In the African trypanosomes where TS is thought to be expressed only in the procyclic insect stages [5,7], TS has been shown to increase the survival, maturation and hence establishment of the parasites in the vector midgut [8].

Two TS forms, named TS-form 1 and TS-form 2, have been purified from procyclic *T. congolense* cultures [6]. Interestingly, glutamic acid and alanine-rich protein (GARP) was co-purified with TS-form 1, suggesting that GARP may be a natural substrate for TS-form 1. Interestingly, TS-form 1 had significantly less SA activity and higher TS activity, whereas SA activity was predominately found in preparations of TS-form 2. An anti-*T. congolense* TS antibody (mAb 7/23) was developed using TS form 1 as antigen. This antibody is specific for *T. congolense* TS recognizing TS-form 1 and TS-form 2, but does not bind to *T. brucei* TS. Peptides micro sequencing revealed the amino acid sequence VVDPTVVAK in TS-form 1. Subsequently, fragments of two TS genes (TS1 and TS2) were sequenced, sharing about 50% sequence identity [9]. TS1 encoded this peptide sequence, whereas in TS2 this sequence ended in VVK. These data strongly suggested that the gene product of TS1 has been present in TS-form 1. Nevertheless, it has remained unclear whether only TS1 and/or TS2 gene products were present in TS-form 1 and TS-form 2 preparations. Due to the fact that the monoclonal antibody mAb 7/23 bound both TS preparations, it is quite possible that TS-form 2 contained at least some amounts of TS1 gene product, which might have been responsible for the TS activity of this preparation.

Here, we report the cloning of eleven trans-sialidase TS1 variants from *T. congolense* and their recombinant expression in CHO_{Lec1} cells. Furthermore, the enzymatic properties of two of these recombinant TS1 variants were compared with TS from *T. cruzi*.

Results

Diversity of TS1 genes and structural model

Based on the partial sequence of TS1 [GenBank: AJ535487.1] [9], an open reading frame in the Welcome Trust Sanger Institute (WTSI) database was identified. The full-length translation product consists of 750 amino acids extending the partial sequence of TS1 by 153 amino acids at the N-terminus and by 84 amino acids at the C-terminus. It contains a 16 amino acids N-terminal signal peptide and a catalytic domain (residues 17-467), which is connected through a long α helix (residues 468-491) to a lectin domain (residues 492-732) followed by a potential C-terminal GPI-anchor attachment site (residues 733-750, identified by big-PI predictor [10]). Furthermore, nine potential *N*-glycosylation sites were identified (Figure 1).

T. congolense TS1 shares about 57% sequence identity with *T. brucei* TS [EMBL: AAG32055.1] and 48% with *T. cruzi* TS [EMBL: BAA09334.1] (Figure 2). The *T. brucei* TS has a prolonged N-terminus of approx. 90 amino acids, which is conserved in *T. congolense* TS1 sharing 50% amino acids, but is absent in *T. cruzi* TS. The catalytic domain of both African proteins has 60% and the lectin domain 43% sequence similarities. *T. congolense* TS1, like *T. brucei* TS and *T. rangeli* SA, has no C-terminal SAPA domain typical for *T. cruzi* TS [11]. Almost all amino acid residues reported to be required for TS activity are identical in TS1 with the exception of A325 (corresponding to P231 in *T. cruzi* TS) [11], R127, G344-Q346 and Y408 (corresponding to Y248 and W312, respectively in *T. cruzi* TS) [12] (Figure 2).

To produce recombinant protein for enzyme characterization, the DNA encoding amino acids 17-732 was amplified using genomic *T. congolense* DNA as a template and inserted into a mammalian expression vector as described in Methods. 13 clones were picked from two independent cloning experiments and sequenced. Interestingly, not all the 13 clones had identical sequences and eleven different sequences were obtained (TS1a through TS1j), exhibiting an overall amino acid identity of 96.3%. A more detailed search of the WTSI database using these sequences as queries confirmed the presence of these TS1 genes in the *T. congolense* genome database. As shown in Figure 3, changes are not evenly distributed over the protein sequences. Eight were found in the lectin domain and 17 in the catalytic domain, some close to the predicted active site as shown in Figure 4A.

For a better understanding of how these differences may affect TS function, we calculated a model structure (Figure 4) for TS1 e-1 by homology modeling based on the crystal structure of *T. cruzi* TS [12], which was complexed with the Sia derivative 3-fluoro-5-*N*-acetyl-9-benzamido-2,9-dideoxy-neuraminic acid. The superimposed structures of *T. cruzi* TS and the *T. congolense* TS1 e-1 model had a root mean square deviation (RMSD) of 0.685 Å over 594 aligned residues.

In Figure 4A, amino acids of the active site are highlighted. Most of the amino acids reported to be relevant for TS activity are identical in all *T. congolense* TS1 variants (white labels). However, differences to *T. cruzi* TS

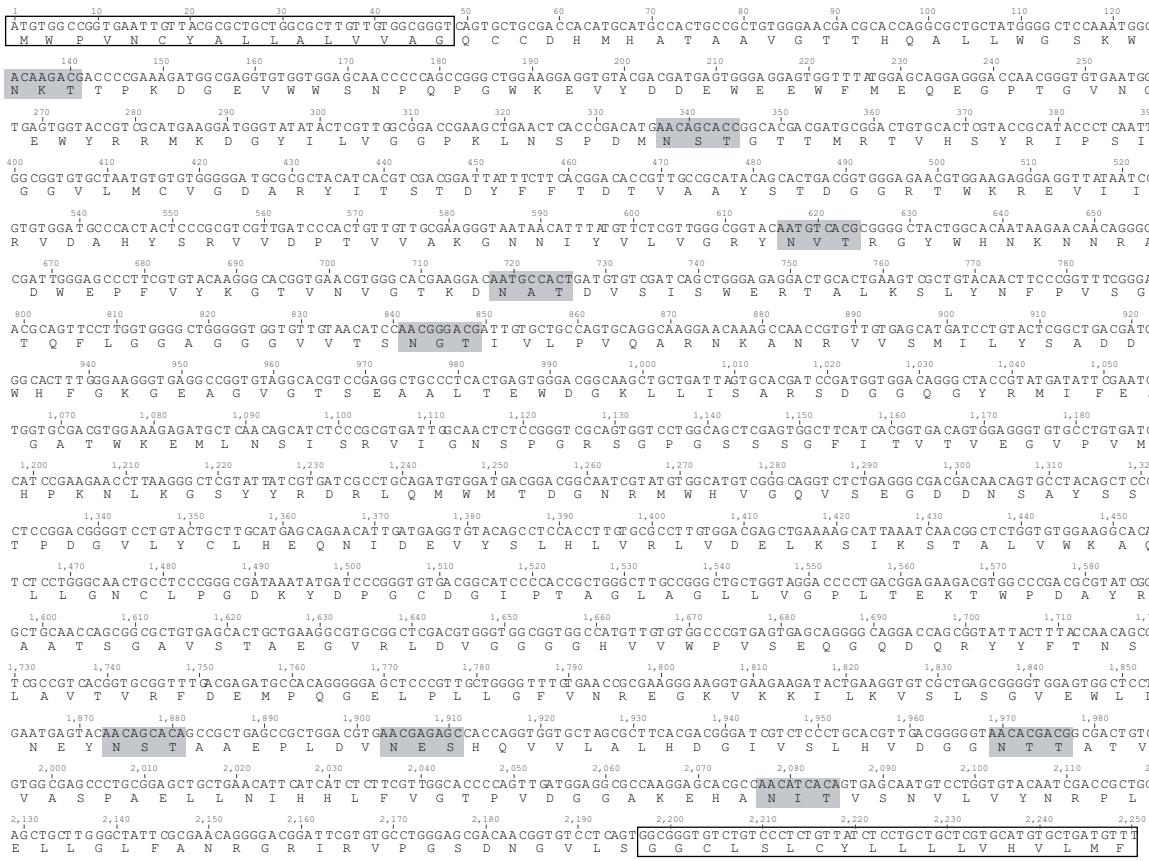

Figure 1 Primary sequence of TS1a. The full length coding domain sequence [EMBL: HE583283] with corresponding amino acid translations is shown. The recombinant protein was generated without the N-terminal signal peptide and without the C-terminal region predicted to be replaced by a GPI anchor in the native protein (framed boxes). Predicted *N*-glycosylation sites are highlighted by light grey boxes.

were identified at three positions (yellow labels in Figure 4A). (I) At position 325 all *T. congolense* TS1 variants have an alanine, like in *T. brucei* TS, replacing a proline occurring in *T. cruzi* TS (P231); (II) Y408 of all *T. congolense* TS1 variants corresponds to a tryptophan in *T. cruzi* TS (W321) and *T. brucei* TS; (III) the group of G342, G343 and Q344 replaces a tyrosine (Y248) in *T. cruzi* TS. In addition, near the catalytic site at position 407 (red label) in *T. congolense* TS1 variants, a serine or valine occurs instead of arginine (R311) in *T. cruzi* TS. Interestingly, similar differences occur also in *T. brucei* TS (Figure 2). Since these amino acids are close to the active site, they could influence the acceptor binding specificity. The arginine at position 144 (blue label) is conserved in all TS, with the exception of *T. congolense* TS1g, where it is a cysteine.

In Figure 4B the amino acid positions are highlighted, which have different side chains in TS1a-TS1j (Figure 3). It should be noted that these are all on the same side of the protein as the catalytic site. Striking is a cluster of amino acid variations in the lectin domain (position 599 to 602 and 643) suggesting that these changes may influence substrate binding of larger substrate molecules, such as glycoproteins.

Characterization of *T. congolense* TS1 enzyme activity

All eleven TS1 gene products (TS1a-TS1j) were expressed as recombinant proteins and were recognized by the anti- *T. congolense* TS antibody (mAb 7/23) [6] (data not shown). For all TS1 variants similar robust TS activity could be determined, except for TS1g. This variant, which carries cysteine instead of arginine at position 144, had only very low TS activity. However, in contrast to the other variants, TS1g released free Sia from fetuin at about 50% of the transfer to lactose. Two of the *T. congolense* TS1 variants, TS1b and TS1 e-1, were further characterized. They differ in eleven of the total 25 positions with amino acid variations listed in Figure 3, three in the catalytic domain and eight in the lectin domain.

The donor substrates fetuin 3'SL or pNP-Neu5Ac and the acceptor substrates lactose, galactose or Gal-MU were employed to determine sialidase and trans-sialidase activities. For this purpose, a new assay was established

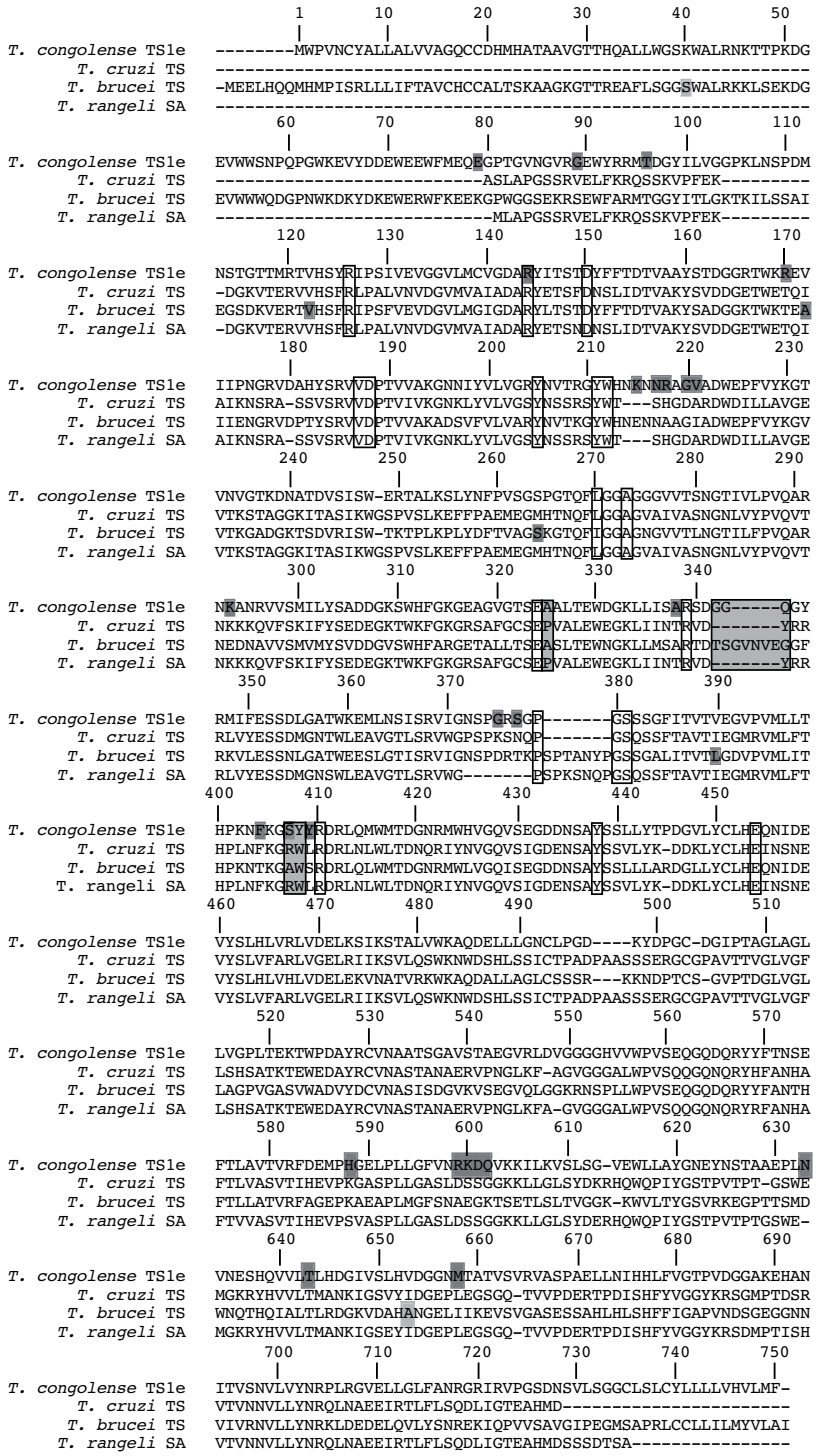

Figure 2 Primary sequence alignment of trypanosomal trans-sialidases. The amino acid sequence of *T. congolense* [EMBL: HE583287] TS1 e-1 was aligned with *T. cruzi* TS [EMBL: BAA09334.1, PDB: 3B69], *T. brucei* TS [EMBL: AAG32055.1] and *T. rangeli* SA [EMBL: AAC95493.1] based on a structural alignment of *T. congolense* TS1 e-1 with *T. cruzi* TS generated with Yasara during homology modeling. Amino acids, which have been proposed to be relevant for enzymatic activity are marked with black frames if conserved or with black frames and light grey background if not conserved. Positions with variations occurring in *T. congolense* TS1 or *T. brucei* TS [3] are highlighted by dark grey boxes.

position	TS1a	TS1b	TS1c	TS1d	TS1e	TS1e'	TS1f	TS1g	TS1h	TS1i	TS1j
Catalytic domain											
79	E	K	E	E	E	E	E	E	E	E	E
89	G	T	G	G	G	G	G	G	A	G	G
96	K	K	T	T	T	T	K	K	K	K	T
144	R	R	R	R	R	R	R	C	R	R	R
170	R	R	R	S	R	R	S	R	R	R	R
215	K	K	R	Q	K	K	Q	K	K	Q	Q
217	N	N	D	D	N	N	D	N	N	D	D
218	R	R	K	E	R	R	E	R	R	E	E
220	G	G	A	G	G	G	A	G	G	G	G
221	V	V	I	V	V	V	I	V	V	V	V
293	K	K	K	K	K	K	K	K	K	K	N
338	A	A	A	A	A	A	T	A	A	A	A
375	G	G	K	G	G	G	P	K	K	K	S
377	S	S	N	S	S	S	S	N	N	N	S
404	L	L	L	L	F	L	L	L	L	L	L
407	S	S	S	V	S	S	S	S	S	S	S
409	Y	Y	Y	S	Y	Y	Y	Y	Y	Y	Y
Lectin domain											
588	Q	Q	Q	Q	H	H	H	Q	R	R	R
599	R	R	R	R	R	R	R	H	H	R	R
600	E	E	E	E	K	K	K	K	K	K	K
601	G	G	G	G	D	D	D	G	G	G	G
602	K	K	K	K	Q	Q	Q	K	K	K	K
633	D	D	D	D	N	N	N	N	L	D	D
643	A	A	A	A	T	T	T	T	A	A	A
658	T	T	T	T	M	M	M	T	T	M	T

Figure 3 Amino acid variations found in *T. congolense* TS1a-TS1j. TS1a [EMBL: HE583283], TS1b [EMBL: HE583284], TS1c [EMBL: HE583285], TS1d [EMBL: HE583286], TS1 e-1 [EMBL: HE583287] , TS1 e-2 [EMBL: HE583288], TS1f [EMBL: HE583289], TS1g [EMBL: HE583290], TS1h [EMBL: HE583291], TS1i [EMBL: HE583292], TS1j [EMBL: HE583293]. Differences in amino acids are highlighted (light grey: conservative; dark grey: modest; black: drastic change).

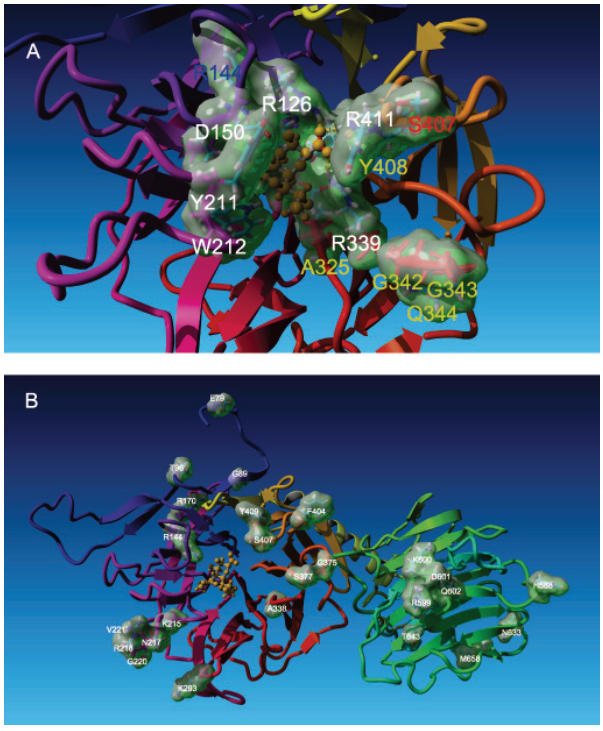

Figure 4 Homology model of *T. congolense* TS1. The crystal structure of *T. cruzi* TS [12] in complex with 3-fluoro-5-*N*-acetyl-9-benzamido-2,9-dideoxy-neuraminic acid was used as template to calculate a model structure for *T. congolense* TS1 e-1. Only the Neu5Ac part (orange) of the ligand in the binding site of the complex is illustrated. (A) Conserved amino acids of the active site are labeled in white. Amino acids at positions reported to be important for ligand binding in *T. cruzi* TS [12], which are not conserved in *T. congolense* are labeled in yellow. The red labeled position 407 is a serine or valine in *T. congolense* TS1 forms. R144, which is exchanged to a cysteine in TS1g, is labeled in blue. (B) Green clouds mark positions at which different amino acids occur in other *T. congolense* TS1 forms.

as described under Methods, using HPAEC-PAD to quantify sialylated oligosaccharide products with the detection limit of 20 pmol 3'SL corresponding to 0.5 μM in the reaction mixture. In standard assays, 50 μL TS reactions were set up with 50 ng TS1b or TS1 e-1, 100 μg fetuin (approx. 600 μM bound Sia) as donor substrate and 100 nmol acceptor substrate (2 mM e. g. lactose or galactose). Under these conditions, linear product formation was obtained for up to 2500 pmol corresponding to 50 μM 3'SL (Figure 5).

If lactose is used as a donor substrate under standard conditions, 3'SL concentration increases linearly for about 30 minutes before the reaction velocity started to decrease (Figure 5A). It should be noted that lactose was sialylated almost twice as fast by TS1b than by TS1 e-1. In contrast to lactose, galactose was sialylated at the same rate by both enzymes, but at about 20-fold lower velocity than lactose.

Different specific activities were obtained for *T. congolense* TS1b, TS1 e-1 and *T. cruzi* TS (Figure 6). The reaction velocity was linearly dependent on the amount of TS as long as the concentration of the product 3'SL was below 50 μM. Under standard conditions 50 μM 3'SL was produced in 30 minutes with 50 ng TS. If 200 ng TS or more were used, product formation was independent of the amount of TS, probably due to the increased use of 3'SL as a donor substrate in the reverse reaction, finally leading to an equilibrium between lactose, 3'SL, sialylated and desialylated glycans on fetuin. This equilibrium apparently was reached in 30 minutes with 500 ng TS (266 ± 4 μM 3'SL for *T. cruzi* TS, 194 ± 6 μM 3'SL for TS1b and 165 ± 7 μM 3'SL for TS1 e-1). After 20 h incubation, 50 ng TS was sufficient to reach the equilibrium. Independent of the amount of enzyme used, for all three TS applied similar final concentrations of 3'SL were obtained after 20 h incubation (Table 1).

The HPAEC-PAD method used allowed not only determining the TS, but also SA activity, since free Sia and 3' SL could be quantified from the same chromatogram. In standard reactions (50 ng TS, 30 min incubation time) no SA activity could be detected, both in the presence or absence of lactose as an acceptor substrate. This suggests that these TS1 variants usually need an acceptor substrate like lactose to cleave Sia from a donor substrate. However, after 20 h incubation, free Sia was detected. The quantity of Sia released was dependent on the amount of TS used (Table 1). Besides standard TS reactions with fetuin as donor and lactose as acceptor substrate, TS reactions with 2 mM 3'SL as donor and 2 mg/mL ASF as acceptor substrate were performed. In these reactions, free Sia was detected after short reaction times and after incubation for 24 h, 0.5-1 mM free Sia were produced (data not shown).

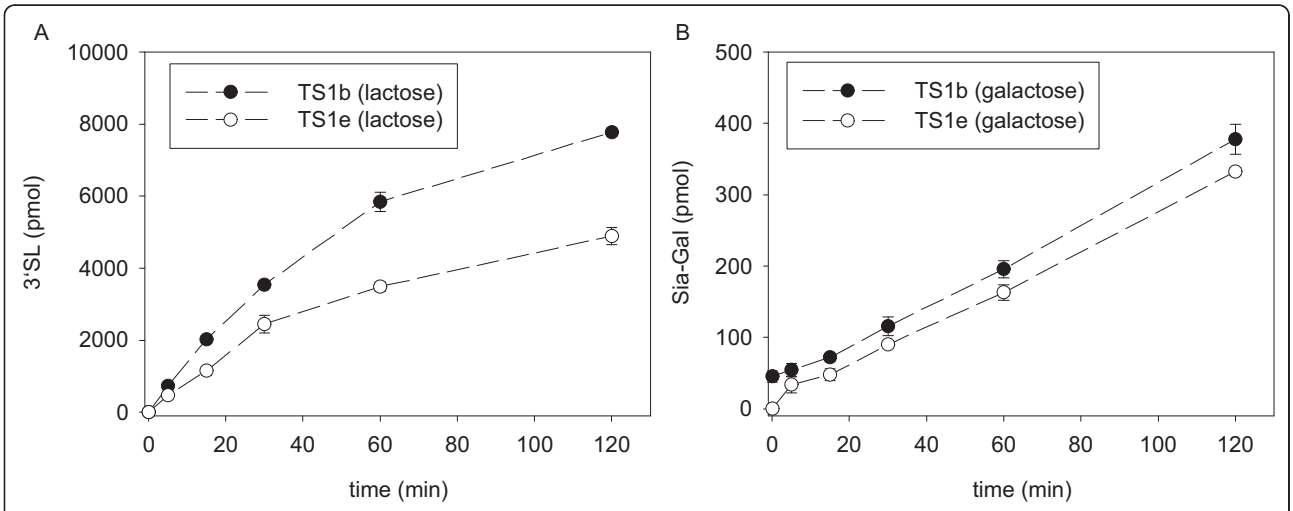

Figure 5 Time dependence of TS reactions. Reactions of 100 μg fetuin with 100 nmol lactose (A) or 100 nmol galactose (B) were started with 50 ng TS in 50 μL and incubated at 37°C. The amount of 3'SL produced was determined by HPAEC-PAD as described under Methods. Data points are means ± standard deviations of three replicates.

For kinetic experiments, assays were incubated for 30 minutes using 50 ng TS, since under these conditions 3'SL production was linear for all three TS. To determine the kinetic parameters for the acceptor substrates lactose (Figure 7A) or Gal-MU (Figure 7C), 100 μg fetuin (600 μM bound Sia) was used as donor substrate. The lowest K_M for lactose was found for *T. cruzi* TS with 327 μM compared to 1683 μM for TS1b and 727 μM for TS1 e-1 (Table 2). Furthermore, *T. cruzi* TS was able to produce twice more 3'SL than TS1b and fourfold more than TS1 e-1 under these conditions.

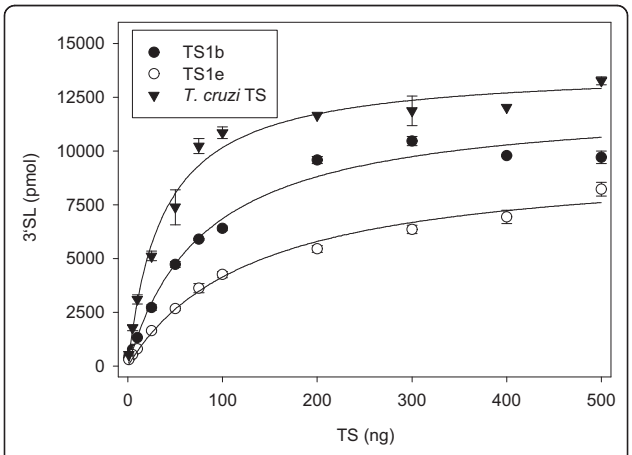

Figure 6 Concentration dependence of TS reactions. Reactions of 100 μg fetuin with 100 nmol lactose were started with varying amounts of TS in overall 50 μL 10 mM phosphate buffer, pH 7.4 and incubated at 37°C for 30 min. The amount of 3'SL produced was determined by HPAEC-PAD as described under Methods. Data points are means ± standard deviations of three determinations.

To determine the kinetic parameters for the donor substrate fetuin (Figure 7B), 2 mM lactose was used as acceptor substrate. Both *T. congolense* TS1 had similar v_{max}-values, whereas the v_{max} for *T. cruzi* TS was about fivefold higher. Different to the K_M of lactose, the lowest K_M for fetuin was exhibited by TS1b with 359 μM, which is about fivefold lower compared to TS1 e-1 with 1617 μM and about 12-fold lower compared to *T. cruzi* TS with 4124 μM.

Kinetic studies with TS were also performed for the acceptor substrate Gal-MU (Table 2) and the donor substrate pNP-Neu5Ac. Almost similar K_M and v_{max}-values were found for both *T. congolense* TS1. The substrate pNP-Neu5Ac was only weakly used as a donor substrate by all three TS species. Therefore, no reliable kinetic parameters could be determined.

Table 1 Free Sia and 3'SL production at equilibrium of the reaction

	Neu5Ac [pmol]	3'SL [pmol]
0 ng TS	262 ± 46	0
50 ng *T. cruzi* TS	580 ± 8	12597 ± 115
250 ng *T. cruzi* TS	1820 ± 13	11199 ± 288
50 ng TS1b	415 ± 7	15378 ± 117
250 ng TS1b	1095 ± 129	14435 ± 2226
50 ng TS1 e-1	348 ± 83	12719 ± 3057
250 ng TS1 e-1	1186 ± 9	13655 ± 491
only fetuin	116 ± 8	0

Neu5Ac and 3'SL produced by TS after 20 h incubation under standard conditions were determined as described under Material and Methods. Data points are means ± standard deviations of three replicates.

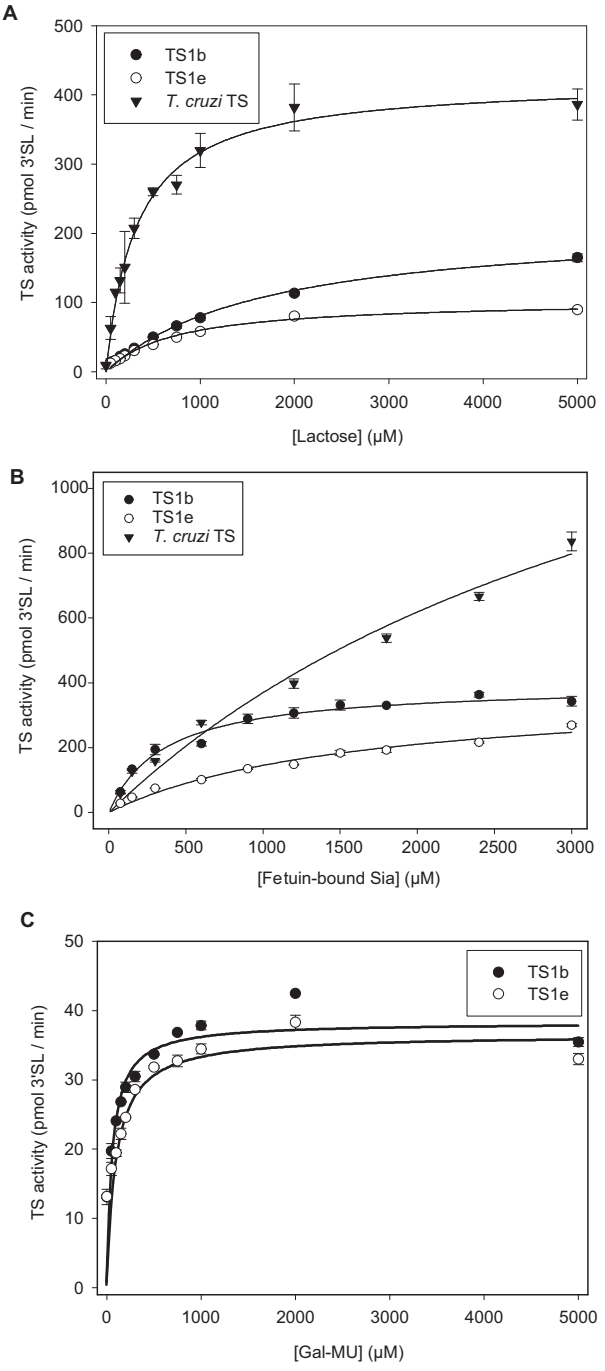

Figure 7 Michaelis-Menten kinetics of TS reactions. All reactions were started with 50 ng TS in a final volume of 50 μL and incubated for 30 min at 37°C. **(A)** 600 μM fetuin-bound Sia was used as donor substrate with varying concentrations of lactose as acceptor substrate. **(B)** Varying concentrations of fetuin-bound Sia were used as donor substrate with 2 mM lactose as acceptor substrate. **(C)** Varying amounts Gal-MU were used as acceptor substrate and 50 nmol 3'SL as donor substrate. The amount of 3'SL (in the case of **(A)** and **(B)**) or 3'Sia-Gal-MU (in the case of **(C)**) produced was determined by HPAEC-PAD as described under Methods. Data points are means ± standard deviations of three replicates.

Sialylation pattern of glycopeptides

Structural differences between TS1 variants may influence the enzymes preference for glycans on glycoproteins, such as fetuin. This could possibly result in different sialylation patterns on glycoproteins after incubation with TS. Fetuin contains 3 *N*-glycosylation sites and 3 *O*-glycans, which all can serve as Sia donors in TS reactions [13]. To investigate the specificity of TS towards different *N*-glycans on fetuin, we used MALDI-TOF-MS to determine the sialylation pattern of glycopeptides (GPs) from trypsin-digested fetuin after incubation with TS and lactose (Figure 8).

The sialylation patterns of three glycopeptides, GP 127-141 (dibranched or tribranched), and GP 54-85 (tribranched) were determined. All 14 potential sialylation variants of these glycopeptides could be identified unambiguously and quantified from the MALDI-TOF-MS spectra. In untreated fetuin most branches on the three *N*-glycans investigated were sialylated, whereas upon treatment with TS and lactose after 30 minutes a clear shift towards incompletely sialylated glycans was observed. After 24 h TS incubation the relative amounts of unsialylated glycans was further increased and monosialylated glycans represented the most prominent species on both, di- and tribranched glycans (Figure 9).

Sialylation of glycoproteins

As described above, *T. congolense* TS1b and TS1 e-1 readily used fetuin as donor substrate for the production of 3'SL. However, long-term TS reactions or experiments with higher amounts of TS had suggested that the reverse reaction also takes place. Therefore, we investigated whether *T. congolense* TS1b and TS1 e-1 can restore sialylated glycans on *Vibrio cholerae* sialidase-treated fetuin (ASF) as model glycoprotein. Resialylation experiments were performed with 100 μg ASF as acceptor and 100 nmol 3'SL as donor substrate as well as 50 ng TS1 in 50 μL to start the TS reaction and were incubated up to 24 h. This resialylation partially reversed the shift in electrophoretic mobility in SDS-PAGE observed for sialidase-treated fetuin (Figure 10). Also by MALDI-MS of glycopeptides, the sialylation of unsialylated glycans was confirmed (data not shown).

Furthermore, we addressed the question of whether through this reaction recognition sites for siglecs can be restored. For this purpose, TS-treated ASF was immobilized to a microtitre plate and used as target for Siglec-4, which preferentially binds α2,3-linked Sia. Under these conditions robust Siglec-4 binding was observed of ASF that had been treated with TS for 4 h. A prolonged (up to 24 h) TS reaction only led to little further increase reaching 40% of binding levels observed with native fetuin (Figure 11).

Table 2 Kinetic parameters of *T. cruzi* TS, *T. congolense* TS1b and TS1 e-1

	Acceptor substrates				Donor substrates	
	Lactose		Gal-MU		Fetuin-bound Sia[x]	
	v_{max} (μmol/(min × mg TS))	K_M (μM)	v_{max} (μmol/(min × mg TS))	K_M (μM)	v_{max} (μmol/(min × mg TS))	K_M (μM)
TS1b	4.3 ± 0.1	1683 ± 101	0.77 ± 0.03	57 ± 14	7.9 ± 0.3	359 ± 45
TS1 e-1	2.1 ± 0.1	727 ± 48	0.72 ± 0.03	74 ± 17	7.6 ± 0.5	1617 ± 223
***T. cruzi* TS**	8.4 ± 0.3	327 ± 31	n.d.	n.d.	37.9 ± 6.0	4124 ± 985

K_M and v_{max} were calculated from Michaelis-Menten kinetics (Figure 7) by SigmaPlot. Data points are means ± standard deviations of three replicates. × Approximately 30 nmol Sia per 100 μg fetuin. Abbr.: n.d.: not determined.

Discussion

In 2003 Tiralongo et al. published a partial sequence for *T. congolense* TS1 [9]. Our approach to obtain the full length sequence of TS1 led to the discovery of 11 variants of this gene with an overall sequence identity of 96.3% in the genome of this parasite. The previously published partial TS1 sequence, which had been assembled from 47 independent PCR reactions, turned out to represent a mixture of fragments from the 11 TS1 forms identified in this study. Therefore, it is likely that that sequence doesn't exist in nature.

Similar TS-like gene families occur also in other trypanosomes. In *T. brucei* the situation appears to be less complex, since only 8 TS closely related genes have been identified [3] and these differences lead to 6 amino acid mutations. The largest TS gene family has been found in *T. cruzi*. Its 140 members fall into three different groups [14]. Blood stream trypomastigotes express two of these groups, one with TS activity and the other only with lectin activity. A third group has TS activity and is expressed by epimastigotes. At present it is unknown whether the expression of the different TS genes in African trypanosomes is also stage-dependent.

T. congolense TS1 shares about 57% identical amino acids with *T. brucei* TS and 48% sequence identity with *T. cruzi* TS. By comparison, the American *T. rangeli* SA and *T. cruzi* TS are more closely related with approximately 70% sequence identity [15].

The homology model for *T. congolense* TS1 based on crystal structures from *T.cruzi* TS and *T. rangeli* SA provided (I) insight in differences in the active site and its surrounding between TS from these parasites and (II) revealed the spatial distribution of the amino acid differences between the TS1 variants.

Compared to *T. cruzi* TS only three amino acids are changed in TS1 (A325, S407 and Y408). At position 325 a proline at the corresponding position of *T. cruzi* TS appears to be relevant for full TS activity [11]. However, in both, *T. brucei* TS and in *T. congolense* TS1, position 325 is an alanine. Tiralongo et al., (2003) postulated A325 might be common to African trypanosomes and does not seem to have an effect on enzymatic activity [9]. Our data have demonstrated that an alanine at this position is well compatible with TS activity, since all active TS1 variants have A325. The exchanges at 407 and 408 may be relevant for the different substrate

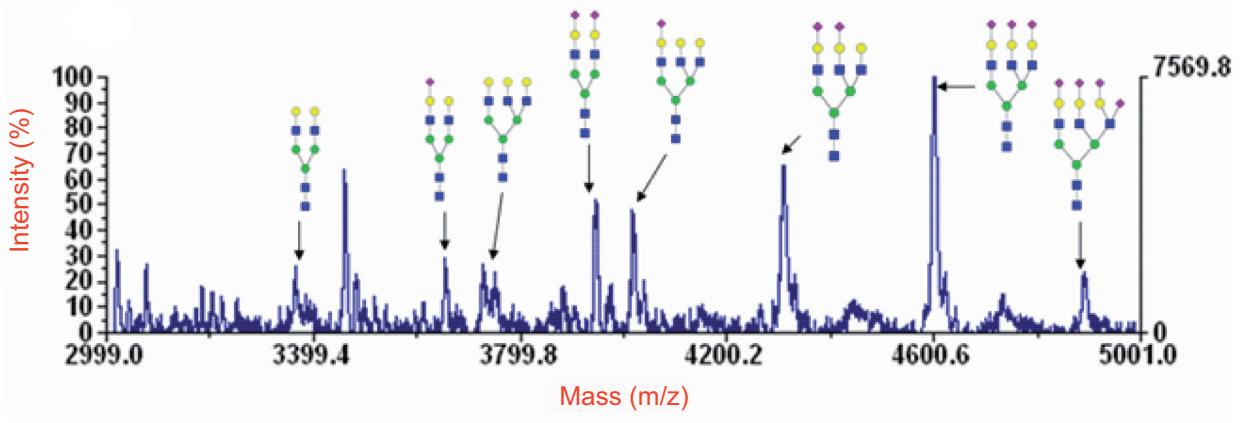

Figure 8 MALDI-TOF-MS of glycopeptide 127-141 sialylation variants. 100 μg fetuin were treated with trypsin and analyzed by MALDI-TOF-MS as described under Methods. The symbols code for the monosaccharide units is according to the nomenclature of the Consortium for Functional Glycomics (http://www.functionalglycomics.org): GlcNAc (blue squares), Man (green circles), Gal (yellow circles), Neu5Ac (purple diamonds).

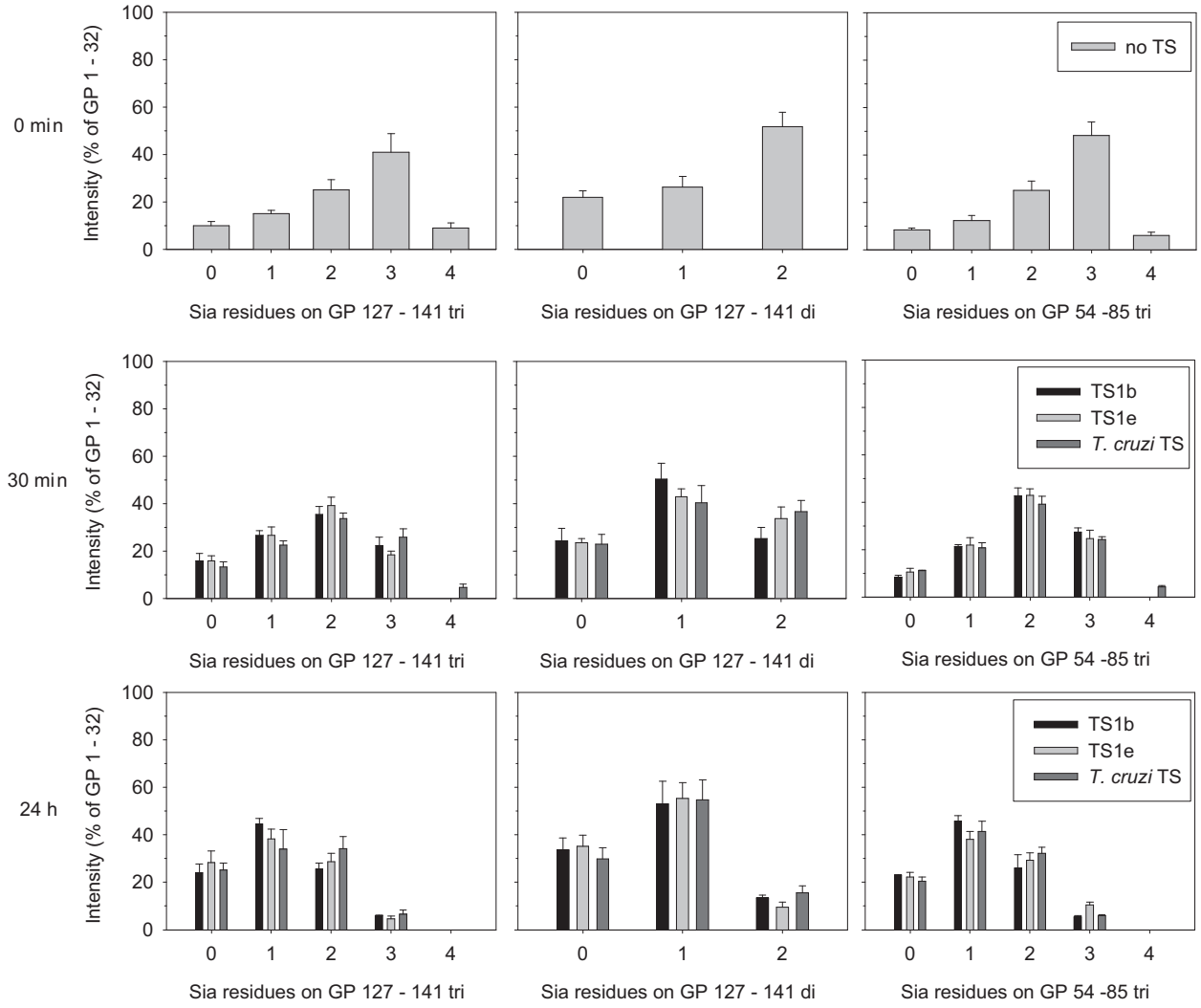

Figure 9 Relative changes of the sialylation pattern of fetuin *N*-glycans. Shown are the sialylation patterns of the glycopeptides GP 127-141 with dibranched (GP 127-141 di) or tribranched (GP 127-141 tri) glycans and of the glycopeptide GP 54-85 with tribranched glycans (GP 54-85 tri) relative to the non-sialylated GP 1 -32. 100 μg fetuin was incubated with 50 ng TS1b, TS1 e-1 or *T. cruzi* TS and 100 nmol lactose for the times indicated, trypsinized and glycopeptides analyzed by MALDI-MS as described under Methods. Data points are averages ± standard deviations of three to ten replicates.

specificities of TS species, since this area possibly participates in the interaction with the underlying galactose.

With the full length coding sequence of TS1 available, the enzymes were expressed and characterized as recombinant proteins to address the question of whether TS1 accounts for the TS activity in the two preparations (TS-form 1 and TS-form 2) from procyclic parasite cultures [6]. The amino acid sequences of the eleven TS1 variants described here contain the peptide VVDPTVVAK present in TS-form 1. Furthermore, all recombinant TS1 variants reacted with the anti-*T. congolense* TS antibody (mAb 7/23) [6]. Based on this information, it is now clear that TS1 was present in both, TS-form1 and TS-form 2. However, two observations made with recombinant TS1 suggest that the native enzyme preparations of TS-form 1 and even more for TS-form 2 contained additional TS1-like proteins. (I) The reaction velocities with the synthetic substrates pNP-Neu5Ac (as donor) and Gal-MU (as acceptor) were much lower than with fetuin and lactose for all three TS. Tiralongo et al., 2003 [6] determined a K_M of 500 μM for Gal-MU, which is approximately 10-fold higher than for TS1b (57 μM) and TS1 e-1 (74 μM). These differences could be due to the presence of other TS1-like enzymes in the preparations of TS-form 1 and TS-form 2. (II) Using the substrate Neu5Ac-MU no SA or TS activity could be detected for the *T. congolense* TS1 variants investigated here, whereas cleavage of

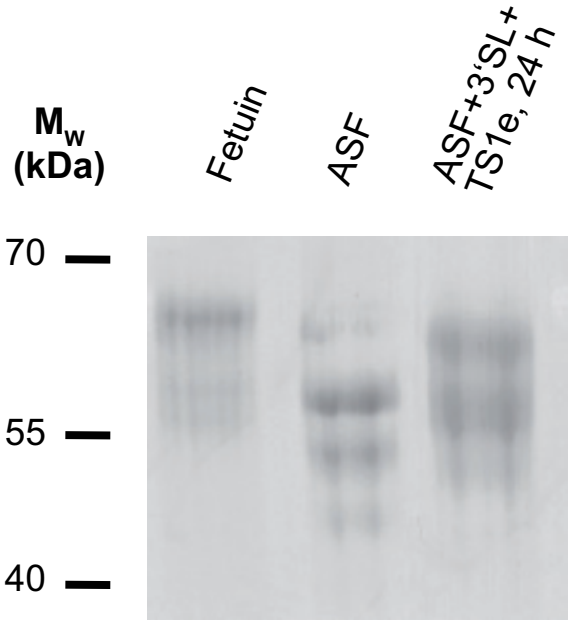

Figure 10 SDS-PAGE of resialylated fetuin. ASF (45 to 58 kDa): *Vibrio cholera* sialidase-treated fetuin (58 to 65 kDa). For resialylation ASF was treated with TS1 e-1 and 3'SL for 24 h. 2 μg protein per lane were run on a 12% polyacrylamide gel. Abbr.: M_W: molecular weight

this substrate indicated SA activity in preparations of TS-form 1 and TS-form 2 [6], as well as TS activity in procyclic parasites [16]. Also this apparent discrepancy can easily be explained by the presence of other TS1-like enzymes accepting Neu5Ac-MU as TS or SA substrate.

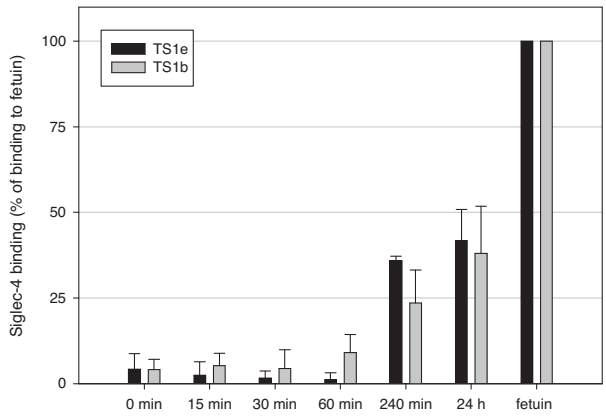

Figure 11 Binding of Siglec-4 to resialylated fetuin. *Vibrio cholera* sialidase-treated fetuin (ASF) was treated with TS1 e-1 and 3'-SL for the times indicated as described under Material and Methods. Siglec-4 binding relative to untreated fetuin is shown. Data points are means ± standard deviations of three independent determinations.

Ten of the eleven recombinant TS1 variants revealed TS activity, which is in agreement with the TS-form 1 purified by Tiralongo et al. [6]. Only TS1g, which carries a cysteine at position 144 instead of an arginine, showed very low TS but clear SA activity. This suggests that R144 is important but not essential for the transfer reaction and hinders SA activity in *T. congolense* TS1. In a previous study on *T. rangeli* SA [17] R144 has been postulated to form a hydrogen bond to the O4 of Sia. However, it remains unclear how this could relate to TS activity. Furthermore, the homology model of TS1 does not provide evidence for such a hydrogen bond for the binding site of *T. congolense* TS1.

The TS1 homology model (Figure 4B) displays also the position of amino acid exchanges in the eleven TS1 forms identified. It could be speculated that these positions are relevant determinants of antigenic sites and that these variations help to escape recognition by the immune system. However, if TS1 is expressed in the procyclic form in the insect vector, this is unlikely to be relevant. Interestingly, these exchanges are located on the same side of the protein where substrate binding occurs, which opens the possibility that these changes influence the binding specificities. The cluster of changes from position 599 to 602 and 643 could be involved in recognition of larger substrate molecules, such as glycoproteins. Positions, 407 and 408 (Figure 4B), as well as positions 375, 377 and 404 (Figure 4A) are closer to the active site in the catalytic domain, possibly contributing to interactions with substrates. It should be noted that the six amino acid differences occurring in *T. brucei* TS are not found in clusters like in *T. congolense* TS1, and furthermore, they are not even on the same side of the protein.

For a more detailed characterization of their differences in activity, we choose two TS1 variants, TS1b and TS1 e-1. The two amino acid sequences of TS1b and TS1 e-1 differ mainly in the lectin domain (Figure 4), whereby the exchange from E600 in TS1b to K600 in TS1 e-1 represents the most drastic change.

The observation that lactose is a much better acceptor substrate than Gal is in agreement with previously reported relative transfer activity data for the preparations TS-form 1 and TS-form 2 [9]. Interestingly, with fetuin as donor substrate *T. cruzi* TS, *T. congolense* TS1b and TS1 e-1 produced different amounts of 3'SL in 30 minutes under identical conditions. It is likely that these differences are due to distinct substrate specificities for the sialoglycans of fetuin. However, after 20 h, equilibriums for the reactions were attained and the three TS applied produced almost the same amounts of 3'SL. Most likely this reflects a similar equilibrium for these three TS.

Clear differences were found in the kinetic parameters of TS1b and TS1 e-1 for lactose and fetuin. Reliable kinetic parameter for 3'SL as donor substrate could not be determined, because of the inaccurate quantification of the high concentrations of 3'SL as donor substrate. Whereas TS1b has a slightly higher K_M (1683 μM) for lactose compared to TS1 e-1 (727 μM), the opposite and more pronounced difference was observed for fetuin-bound Sia, where TS1 e-1 has approximately five-fold higher K_M (81 μM) compared to TS1b (17 μM). In combination, this implies that the ratio of K_M for lactose/K_M for fetuin-bound Sia is approximately 100 for TS1b, whereas it is only 10 for TS1 e-1. By comparison, for *T. cruzi* TS the K_M for both substrates is quite similar (326 over 206 μM) and its v_{max} was fourfold higher than for the TS1 isoforms. The differences in the kinetic parameters for fetuin observed for TS1b and TS1 e-1 are possibly related to altered affinities resulting from amino acid divergences in the lectin domain of TS1b and TS1 e-1. This would suggest a mechanism linking the lectin domain to the enzymatic properties of TS.

No release of free Sia could be detected after 30 minutes of TS reactions with fetuin as donor and lactose as acceptor substrates, demonstrating the absence of SA activity. However, after 20 h incubation, free Sia was detected clearly indicating SA activity. This activity correlated with the TS amount present, implying SA activity to be a side reaction observable only in extended reactions. Interestingly, in the reverse TS reactions with 3'SL as donor substrate for sialylation of galactose residues of ASF, free Sia is detected very early in the reaction (data not shown). This suggests that the free Sia detected in extended TS reactions times with fetuin as donor substrate is mainly the product of a SA side activity of the reaction using 3'SL as substrate. Therefore, the amount of free Sia could provide indirect information on the velocity of the reverse reaction. This assumption is further supported by the fact that lower amounts of TS can led to the same amount of final 3'SL but produce less free Sia as side product (Table 1). In this context it should be noted, that TS from *T. cruzi* clearly produced more free Sia than *T. congolense* TS1b or TS1 e-1. The structural basis for this phenomenon is unclear but may be related to the kinetics of the reaction. TS have been reported to follow ping-pong bi bi kinetics [12,18,19]. It will be interesting to investigate the structure-function relationship of this phenomenon and whether this is related to the SA activity of TS1g.

The TS substrate specificities for the glycans of the donor substrate fetuin were investigated by a MALDI-TOF analysis of TS treated glycopeptides from trypsin digested fetuin, since the glycosylation of fetuin is well established [13,20-23]. Three glycopeptides coming from two of the three *N*-glycosylation sites (di- and tribranched GP 127-141, and tribranched GP 54-85) could be analyzed reliably. The predicted masses of GP 142-169 di could not be identified in any spectra. GP 54-85 di and GP 142-169 tri differed only in one Da, which were not resolved by the equipment available and were excluded from the analysis. The peptide containing the three *O*-glycosylation sites could not be detected due to its high mass. But it is important to note, that TS1 clearly utilizes sialylated *O*-glycans as donor substrates as indicated by a rapid unmasking of peanut agglutinin recognition sites (data not shown).

Only minor differences in the sialylation pattern of fetuin GPs were observed using the different TS species. In summary, these were too small to draw a conclusion that these TS differ in their substrate specificities for fetuin glycans. In general, the TS applied cleaved Sia from *N*-glycans of glycopeptides investigated, but also transferred Sia back to these *N*-glycans. *N*-glycans that carried three and four Sia molecules in the case of the tribranched *N*-glycans as well as *N*-glycans that carried two Sia molecules in the case of dibranched *N*-glycans before TS incubation, were reduced to predominantly 0-2 Sia molecules in the case of tribranched and 0-1 Sia molecule in the case of dibranched *N*-glycans after 24 h TS incubation.

Whereas in the reactions discussed above Sia was transferred from fetuin to synthesize 3'SL, we could also show that *T. congolense* TS1 as well as *T. cruzi* TS transfer Sia in the reverse direction from 3'SL to glycoproteins. Furthermore, the TS reaction restores binding of Sia-binding proteins, such as Siglec-4. Due to the reversibility of the reaction, a complete resialylation of an acceptor substrate applying TS cannot be expected under these conditions. Nevertheless, differences in the kinetic parameters as shown for two of the eleven *T. congolense* TS1 variants could be used in kinetically controlled reactions to optimize the TS reaction to one or the other product, making the TS1 variants interesting tools for biotechnological applications. Thus, TS1 can be utilized to transfer Sia in α2,3-linkage on biologically relevant glycoproteins containing terminal galactose as Sia acceptor.

Conclusions

For the first time, full length TS from the African parasite *T. congolense* has been cloned and sequenced, opening new perspectives for investigations on the biological role of these enzymes in the pursuit of a cure for Nagana and sleeping sickness. Eleven *T. congolense* TS1 variants were identified and expressed as recombinant proteins. The eleven TS1 differ in 25 of 702 amino acid positions and a structural model revealed that these variations occur in three clusters on the side of the protein that is open to substrate binding. Ten of these TS1

variants share predominantly TS and little SA activity. Only one, TS1g, has much lower TS but increased SA activity, probably due to an exchange of an arginine to a cysteine at position 144. Interestingly, the kinetic parameters of two characterized TS1 variants reveal subtle differences in substrate specificities. However, these did not lead to major differences in the sialylation pattern of *N*-glycans on fetuin after treatment with different TS variants. Finally a proof of principle has been provided that these TS can be used to sialylate glycoconjugates to create binding sites for Sia-binding proteins like Siglec-4.

It will be interesting to investigate the expression patterns of TS1 variants in the parasite's life cycle in future investigations addressing their importance for the manifestation of midgut colonization and maturation in the tsetse vector with possible implications for the transmission to the mammalian host.

Methods

Materials

Complete Mini, EDTA free protease-inhibitor tablets and *Vibrio cholerae* sialidase were purchased from Roche Diagnostics, Mannheim, Germany. *Pfu* DNA polymerase and restriction enzymes *Bam*HI and *Spe*I, isopropyl β-D-1-thiogalacto-pyranoside (IPTG), PageBlue and molecular weight marker (PageRuler) were from Fermentas, St. Leon-Rot, Germany. Trypsin was from Promega, Mannheim, Germany, 2,5-Dihydroxybenzoic acid from Bruker Daltonics, Billerica, USA. Ultrafiltration units Vivaspin6 and VivaCell250 were from Sartorius, Göttingen, Germany. BCA Protein Assay Kit was purchased from Thermo Scientific Pierce, Rockford, USA. Anti-SNAP-tag rabbit polyclonal antibody was from GenScript, Piscataway, USA. Anti-*Strep*-tag rabbit polyclonal antibody, StrepTactin beads and buffers were purchased from IBA, Göttingen, Germany and hygromycin from PAA, Pasching, Austria. 2'-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt hydrate (MU-Neu5Ac), 4-methylumbelliferyl ß-D-galactoside (MU-Gal), 4-methylumbelliferone (MU), glucoronic acid, Ex-cell® CD CHO media, fetuin and PEI transfection reagent were purchased from Sigma-Aldrich, Munich, Germany. X-ray film, enhanced chemiluminescence system, Ni-NTA and Q-Sepharose FF were purchased from GE Healthcare, Munich, polyvinylidene difluoride membranes and ZipTips from Millipore, Schwalbach, Germany.

Cloning and expression of recombinant TS1

The published partial sequences of *Trypanosoma congolense* TS1 [Genebank: AJ535487.1] [6,9] was used as starting query for searching the *T. congolense* genomic database for pathogen genomics at the WTSI (http://www.sanger.ac.uk/). Based on the obtained sequence fragments, an open reading frame encoding TS1 was assembled. Based on this, primers were designed to amplify TS1 from *T. congolense* (strain STIB 249) genomic DNA [9] using *Pfu* DNA polymerase in a nested PCR reaction leaving out the N-terminal signal peptide sequence and the C-terminal GPI anchor attachment site. Both outside primers (forward ATG CGG CCG GTG AAT TGT TAN and reverse CAT CAG CAC ATG CAC GAG CAN) were degenerate at the 3' end, whereas the internal primers (forward CGA CTA GTC AGT GCT GCG ACC ACA TGC AN and reverse CGG GAT CCG TCG CTC CCA GGC ACA CGA AN) were designed to introduce *Spe*1 and *Bam*HI restriction sites, respectively. These restriction sites were used to ligate the PCR-products in frame into a modified pDEF [24] vector (pDEF-T3C/SNAPstrep) providing in frame a Transin cleavable signal peptide [25], a 3C-protease cleavage site [26] followed by SNAP (Covalys, Witterswil, Switzerland) and *Strep* (IBA, Göttingen, Germany) tags. The pDEF-T3C/SNAPstrep was obtained as follows: The coding sequence for the Transin signal peptide has been introduced into pcDNA 3 Amp *Strep-tag* [27] using the *Hind*III/*Bam*HI-digested linker obtained by hybridization of the following oligonucleotides: sense 5'-CGAAGCTTATGAAAGGGCTCCCAGTCCTGC TGTGGCTGTGTACGGCTGTGTGCTC

ATCCTACCCATTGCATGGCAGTGAAGAAGAT GCTGGCATGGAGACTAGTGGATCCCG

and antisense primer 5'-

CGGGATCCACTAGTCTCCATGCCAGCATCTT CTTCACTGCCATGCAATGGGTAGG

ATGAGCACACACAGCCGTACACAGCCACAGCA GGACTGGGAGCCCTTTCATAAGCTTCG.

This Transin linker introduced a unique *Spe*I restriction site. The coding sequence of the hAGT protein (SNAP) was amplified using the pSNAP-tag® (T7) vector (NEB, Ipswich, MA, USA) as a template and subcloned in frame into *Bam*HI/*Eco*RI digested pcDNA3 Amp Transin *Strep*-tag providing pcDNA3-T3C/SNAP-strep. The primers used for amplification were: sense 5'-CGGGATCCCTGGAGGTGCTGTTCCAGGGCCC-CATGGACAAAGACTGCGAAATGAAGCG-3' including the coding sequence for the 3C protease recognition site of the human rhinovirus HRV 3C (LEVLFQGP, underlined) and antisense 5'- CGGAATTCACCCAGC CCAGGCTTGCCCAGA.

CHO_{Lec1} cells were used for TS1 expression due to their ability to express only high-mannose glycans, since these cells are lacking *N*-acetyl glucosaminyltransferase I [28]. Transfection of CHO_{Lec1} cells grown in αMEM supplemented with 10% fetal calf serum at 37°C, 5% CO_2 was accomplished with polyethylenimine (PEI) transfection reagent following the manufacturer's

instructions. 24 h after transfection, cells were passaged into 96-well plates in a selection media of αMEM containing varying amounts of hygromycin, ranging from 400 μg/mL to 1000 μg/mL. Expression of recombinant TS1 (120 kDa including SNAP and *Strep* tags) was tested by analyzing cell culture supernatant using Western blots with anti TS1 monoclonal antibody (mAb 7/23) as primary antibody [9]. The presence of SNAP and *Strep* tags was confirmed using anti-SNAP-tag rabbit polyclonal antibody and anti-*Strep*-tag rabbit polyclonal antibody respectively in Western blots analysis. Selected cells were then adapted to Ex-cell® CD CHO media supplemented with 8 mM L-glutamine.

Purification of *Trypanosoma congolense* trans-sialidase

The harvested tissue culture supernatant was supplemented with 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM DTT and 0.02% sodium azide (all final concentrations) and centrifuged at 125,000 × g for 1 h. The cleared supernatant was then concentrated 100-fold by ultrafiltration (100 kDa cut off). Buffer was exchanged twice using 250 mL 100 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA (buffer A) in the same ultrafiltration unit and concentrated to a total volume of 10 mL for 1 L tissue culture supernatant. This was further clarified by centrifugation at 21,000 × g for 30 minutes before applying on a column of 1 mL *Strep*Tactin® beads equilibrated with buffer A. After loading the column was washed with 5 column volumes wash buffer (100 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA) and TS1 was eluted with 3 column volumes of elution buffer (100 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin) in fractions of 0.5 mL. The affinity purified TS1 was dialyzed (10 kDa cut off) against 10 mM phosphate, pH 7.4. Purification products were analyzed by SDS-PAGE and quantified by BCA assay with bovine serum albumin as standard.

Expression and purification of *Trypanosoma cruzi* trans-sialidase

Recombinant *T. cruzi* TS was produced in *E. coli* M15 (pREP4) according to Agusti et al., 2004 and Neubacher et al., 2005 [29,30]. In brief, cells were grown in 1 L "terrific broth" medium overnight at 18°C. Protein expression was initiated with 0.5 mM IPTG. The cells were dissolved in 40 mL lysis buffer (50 mM phosphate, 300 mM NaCl, pH 8.0 and 0.05% Lubrol), 1 tablet protease-inhibitor (Complete Mini, EDTA free) and 1 spatula tip of lysozyme were before incubation of 30 minutes at 4°C. The cells were disrupted by 5 cycles of sonification on ice. Cells debris was removed by centrifugation at 40,000 × g for 60 minutes at 4°C and the supernatant was filtered using a 0.2 μm pore size filter. 20 mM imidazol was added before application on 0.5 mL Ni-NTA beads. Target proteins were eluted in the same buffer and 250 mM imidazole. The eluted protein was dialyzed against 20 mM Tris, 30 mM NaCl, pH 8.0 and further purified using a Q-Sepharose FF column in the same buffer with a linear gradient up to 1 M NaCl. The activity of the purified protein was tested by a sialidase activity assay using MU-Neu5Ac as donor substrate and lactose as acceptor substrate as described below.

Vibrio cholerae sialidase treatment of fetuin

Asialofetuin (ASF) was prepared from fetuin by *Vibrio cholerae* sialidase (VCS) treatment as described [31]. In brief, fetuin was digested with VCS in 50 mM sodium acetate, 9 mM $CaCl_2$, pH 5.5 overnight at 37°C in a dialysis bag against the same buffer and afterwards against distilled water. Sialylated fetuin and sialidase was separated from ASF by anion exchange chromatography using Q-Sepharose. The proteins were eluted by a linear gradient from 0 to 1 M NaCl in 10 mM Tris, pH 7.4. Collected fractions were assayed for protein at 280 nm and for SA activity with MU-Neu5Ac acid as substrate as described below. The fractions containing ASF but no SA activity were pooled and the buffer was exchanged against 10 mM phosphate, pH 7.4 using VivaSpin6 ultrafiltration units (10 kDa cut off).

Sialidase activity assay

Sialidase activity (+/- lactose) was determined by applying a microtitre plate assay detecting free 4-methylumbelliferone (MU) released from Neu5Ac-MU [6]. In brief, 50 μL sample were incubated with 1 mM Neu5Ac-MU (final concentration) in a black 96-well microtitre plate. To determine *T. cruzi* TS 1 mM lactose was added as acceptor substrate. The plate was centrifuged for 1 minute at 1,000 × g and incubated for 30 minutes at room temperature in the dark. The reactions were stopped with 200 μL 100 mM glycine, pH 10 and the fluorescence intensities were measured at 355 nm excitation and 460 nm emission using a fluorimeter (Ascent Fluoroscan).

Trans-sialidase reactions

The principle of this assay is based on the quantification of sialylated oligosaccharides by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described below. In these reactions either fetuin is used as donor substrate, e.g. lactose as acceptor or 3'-sialyllactose (3'SL) is used as donor substrate with ASF as acceptor. A final volume of 50 μL 10 mM phosphate buffer were used for all TS reactions. Stock solutions of donor and acceptor substrate were mixed in 40 μL buffer (10 mM Tris/HCl, pH 7.5) and the reactions were started with 10 μL TS (50 ng in standard reactions) and incubated at 37°C. The

reactions were stopped with 200 μL ice-cold acetone containing 28 μM glucuronic acid and incubated overnight at -20°C. After centrifugation for 15 minutes at 20,000 × g and 4°C, 225 μL supernatant were removed and both, protein pellets and supernatants were lyophilized.

The dried supernatant of the acetone precipitation was dissolved in 125 μL H_2O for HPAEC-PAD, which was carried out by using a DX600 system (Dionex, Sunnyvale, CA, USA) with an electrochemical detector (ED50), a gradient pump (GP50) and an autosampler (AS50). Carbohydrates were separated by HPAEC on a CarboPAC PA1 (4 × 250 mm) analytical column (Dionex) together with a guard column (4 × 50 mm) using a constant flow rate of 1 mL/min. Sample volumes of 25 μL were injected and the chromatography was performed as follows: 100 mM NaOH for 2 min, followed by 100 mM NaOH/100 mM NaOAc for 22 min. The column was regenerated by washing for 5 min with 100 mM NaOH/500 mM NaOAc, followed by 5 min with 100 mM NaOH. For PAD the typical quadruple waveform was used as described previously [32]. The Dionex software Chromeleon 6.40 SP8 was used for data acquisition and data evaluation.

Calculation of kinetic parameters

V_{max} and K_M were calculated using the curve fit module of SigmaPlot 11 employing the Michaelis-Menten equation $v = v_{max} \times c_s/(c_s + K_M)$.

Siglec-4 binding assay

Murine Siglec-4_{d1-3}-Fc was purified by protein-A affinity chromatography from tissue culture supernatants of stably transfected CHO Lec3.2.8.1 as described before [33]. The protein solution was dialyzed against 10 mM phosphate buffer pH 7.4, sterile filtered and stored at 4°C. Binding assays with Siglec-4 were performed as described previously [33]. In brief, 4 μg/mL fetuin, ASF or TS-treated fetuin were immobilized in microtitre plates and binding of serially diluted Siglec-4_{d1-3}-Fc (8 dilutions starting with 16 μg/mL) was determined using alkaline phosphatase-labeled anti-Fc antibodies. The concentrations sufficient for 50% binding (relative to Siglec-4_{d1-3}-Fc binding to fetuin) were determined from corresponding binding curves. At least three independent titrations were performed.

SDS-PAGE and Western Blot analysis

Samples were separated by SDS-PAGE (MiniProtean III; Bio-Rad, München, Germany) according to Laemmli [34] and stained with PageBlue.

For Western blot analysis, samples were transferred onto polyvinylidene difluoride membranes after SDS-PAGE. The membranes were blocked with 5% BSA in Tris-buffered saline (TBS) buffer containing 0.15% Tween20 (TBS-T) for 1 h. Washing of the membrane was done five times for 5 minutes each using TBS-T. Immunodetection was performed by incubating membranes with a primary antibody diluted in blocking buffer overnight at 4°C. The following antibodies were used: anti-*T. congolense* TS mAb 7/23 (1:1000) and rabbit anti-*Strep*-tag (1:1000). Following four washes with TBS-T of 10 minutes each, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase for 2 h at room temperature. After washing four times with TBS-T, blots were developed with the enhanced chemiluminescence system using X-ray film.

Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF-MS)

TS reactions were carried out with 50 ng TS, 100 nmol lactose and 100 μg fetuin as described above. The dried protein pellets after TS reaction were dissolved in 200 μL 50 mM ammonium hydrogen carbonate, pH 7.8 and 1.6 μL 45 mM DTT were added. After 30 minutes incubation at 50°C, 1.6 μL 100 mM IAA were added and further incubated for 30 minutes at 37°C. The tryptic digestion was started with 2 μg trypsin dissolved in 1 μL 50 mM acetic acid and incubated overnight at 37°C and stored at -20°C.

5 μL of the tryptic digest were mixed with 5 μL H_2O and 1 μL 1% trifluoroacetic acid and were directly mixed with matrix and 1 μL was applied to the MALDI-TOF-MS target plate. The remaining 9 μL were desalted using C18-reversed phase pipette tips (ZipTip). Peptides were eluted with 3 × 100 μL 12% and 30% acetonitrile in H_2O and lyophilized. 2,5-Dihydroxybenzoic acid in 0.1% trifluoroacetic acid was used as MALDI matrix. The dried peptides were directly dissolved in 10 μL DHB solution and 1 μL of the mixture were spotted to the target plate for crystallization.

The mass spectrometry was performed using a Voyager DE Pro MALDI-TOF N_2-Laser with a wavelength of 337 nm (Applied Biosystems, Foster City, USA). All spectra were measured in the linear detector mode. Laser intensities and the number of records per spectrum were varied manually.

Voyager software was used for data acquisition and peak detection. For quantification the peak intensity of each peptide was determined relative to the non-sialylated glycopeptide 1-32 (3459.66 Da) as internal standard. This peptide is detected in all spectra of the sialylation variants of the glycopeptides and is not changed by TS reaction (Figure 8). All measurements were repeated for at least three times.

Homology Modeling

A homology model of *T. congolense* TS1 e-1 was calculated using the software Yasara 10.11.8 [35-40]. The

crystal structure of *T. cruzi* TS (UniProt: Q26964; PDB entry: 3B69), previously reported by Buschiazzo et al., 2002 [12] was used as the template structure. A benzoylated *N*-acetylneuraminic acid derivative used as a ligand for *T. cruzi* TS in the template structure was kept in the binding site during the calculation of the homology model.

The following parameters of the Yasara homology modeling module were modified manually from the default settings of the program: Modeling speed: slow, PsiBLASTs: 6, EValue Max: 0.5, Templates total: 1, Templates SameSeq: 1, OligoState: 4, alignments: 10, LoopSamples: 50, TermExtension:10.

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Authors' contributions

HKB carried out all the HPAEC-PAD based TS assays and its data acquisition, the Siglec-4 binding assay and SDS-PAGE to determine resialylation of asialofetuin, homology modeling, alignment of amino acid sequences, supported MALDI data acquisition and drafted the manuscript. TTG carried out *T. congolense* TS1 cloning, sequencing and expression as well as *T. cruzi* TS expression. MW performed the TS1g enzyme assays. OR participated in development of the HPAEC-PAD based TS assay. PM carried out MALDI-MS analysis and its data acquisition. ED participated in MALDI-MS analysis and its data acquisition. FD established the pDEF-Transin-TS-3C-SNAP-Strep vector construct and carried out *T. congolense* TS1 cloning. SK designed and coordinated the study and supported drafting of the manuscript. All authors read and approved the final manuscript.

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3.0.

Results

Publication 2

Biochemical diversity in the *Trypanosoma congolense* trans-sialidase family

Thaddeus T. Gbem, Mario Waespy, Bettina Hesse, Frank Dietz, Joel Smith, Gloria D. Chechet, Jonathan A. Nok, Sørge Kelm

PLoS Negl Trop Dis 7 (12): e2549.doi: 10.1371/journal.pntd.0002549

Contributions of Thaddeus Gbem

- TconTS2 through TconTS4 gene cloning and sequencing
- Expression of TconTS proteins
- Trans-sialylation/sialidase reactions
- HPAEC-PAD data acquisition and analysis
- Data mining for otherTS/sialidase gene sequences
- Drafted above parts in manuscript

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PLOS | NEGLECTED TROPICAL DISEASES

Biochemical Diversity in the *Trypanosoma congolense* Trans-sialidase Family

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Abstract

Trans-sialidases are key enzymes in the life cycle of African trypanosomes in both, mammalian host and insect vector and have been associated with the disease trypanosomiasis, namely sleeping sickness and nagana. Besides the previously reported TconTS1, we have identified three additional active trans-sialidases, TconTS2, TconTS3 and TconTS4, and three trans-sialidase like genes in *Trypanosoma congolense*. At least TconTS1, TconTS2 and TconTS4 are found in the bloodstream of infected animals. We have characterised the enzymatic properties of recombinant proteins expressed in eukaryotic fibroblasts using fetuin as model blood glycoprotein donor substrate. One of the recombinant trans-sialidases, TconTS2, had the highest specific activity reported thus far with very low sialidase activity. The active trans-sialidases share all the amino acids critical for the catalytic reaction with few variations in the predicted binding site for the leaving or acceptor glycan. However, these differences cannot explain the orders of magnitudes between their transfer activities, which must be due to other unidentified structural features of the proteins or substrates selectivity. Interestingly, the phylogenetic relationships between the lectin domains correlate with their specific trans-sialylation activities. This raises the question whether and how the lectin domains regulate the trans-sialidase reaction. The identification and enzymatic characterisation of the trans-sialidase family in *T. congolense* will contribute significantly towards the understanding of the roles of these enzymes in the pathogenesis of Animal African Trypanosomiasis.

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Introduction

Trypanosoma congolense (subgenus: *Nannomonas*) is a major causative agent of the Animal African Trypanosomiasis (AAT) otherwise known as nagana. Other parasites implicated in nagana include *T. vivax* (subgenus: *Duttonella*) and *T. brucei brucei* (subgenus: *Trypanozoon*). These protozoan parasites are transmitted by several species of hematophagous biting flies of the genus *Glossina*. Nagana exhibits a severe negative impact on stock farming, milk and meat production [1]. The impact of the disease is thought to be underestimated as most affected areas are remote, limiting access and hence accurate data acquisition. The need for an alternative arsenal against AAT is heightened as existing drugs are either toxic or rapidly becoming ineffective due to drug resistance [2].

The role of TS in Chaga's disease caused by the South American *T. cruzi* has been extensively studied [3]. On the other hand, studies on the *trans*-sialidases from African trypanosomes responsible for the Human African Trypanosomiasis (HAT) as well as AAT are scanty. In *T. brucei*, TS has been implicated in the cyclical survival of the parasite as evidence supports enhanced survival of parasite in midgut of the insect host [4]. No data exist on *T. congolense* in this regard. Though scanty, the role of blood stream TS and sialidase in anaemia in animals suffering trypanosomiasis caused by *T. congolense* [5] [6] and *T. vivax* [7] has been established.

Multiple copies of TS-like genes exist in *Trypanosoma* genomes. The highest number occurs in *T. cruzi*, but most of the over 1000 genes encod enzymatically inactive proteins [8]. In African trypanosomes, the TS-like gene families are much smaller. For example, 9 members have been identified in *T. brucei* [9] [10] [11]. In *T. congolense*, at least 17 TS-like genes have been identified [12] [13] [14] [6]. Eleven of these, forming the TconTS1 family, are closely related and share over 95% sequence identity [14]. The key element mediating the functions of TS has been ascribed to the N-terminal catalytic domain (CD) harbouring the active site with characteristic conserved amino acids [15] [16] [17] [9] [18], whereas hardly anything is known about possible functions of the lectin-like domain (LD) at the C-terminus of these enzymes.

Here we report that besides TconTS1, three additional members of the *T. congolense* TS gene family transfer sialic acids between glycoconjugates, but have much lower sialidase activities. The identification and biochemical characterisation of *T. congolense* TS genes will enable new studies investigating the role of these genes in nagana disease.

Methods

Unless where stated, all chemicals and reagents used were cell culture and analytical grade. *Vibrio cholerae* sialidase was purchased

Author Summary

Trypanosomiasis is a disease also known as sleeping sickness in humans (Human African Trypanosomiasis) and nagana in animals (Animal African Trypanosomiasis). This disease is caused by protozoan parasites of the genus *Trypanosoma*. Tsetse flies are responsible for the transmission of these parasites. *Trypanosoma congolense* is the main causative agent of nagana in cattle. The clinical signs of the disease have been linked to the presence of an enzyme called trans-sialidase. Interestingly, the enzyme alternates in different forms in the mammalian and the insect vector. Previous knowledge had shown that the parasite requires the enzyme for survival in the fly vector. Our current work has revealed other forms of the enzyme that could be essential for the persistence of the disease in mammalian and vector hosts. These enzymes, though similar in structural architecture, show differences in their activities that could be key in delineating their individual roles in the pathophysiology of the disease.

from Roche Diagnostics (Mannheim, Germany). *Pfu* DNA polymerase, *Hind*III, *Xba*I, *Spe*I, *Eco*RI and *Dpn*I, PageBlue, molecular weight marker (PageRuler), BCA assay protein kit were all purchased from Thermo Scientific (St. Leon-Rot, Germany). VivaSpin 6 and VivaCell250 ultracentrifugation units were from Sartorius (Göttingen, Germany). Anti-SNAP-tag rabbit polyclonal antibody was from GeneScript (Piscataway, USA) while anti-*Strep*-tag rabbit polyclonal antibody, *Strep*-Tactin resin beads and buffers were from IBA (Göttingen, Germany). Hygromycin and Gentamycin were purchased from PAA, (Pasching, Austria). Polyethylenimin transfection reagent, glucuronic acid, *N*-acetyl-neuraminic acid (Neu5Ac), 3'sialyllactose (3'SL) and lactose were purchased from Sigma-Aldrich (Steinheim, Germany). Ex-cell CD CHO media from SAFC, USA, X-ray film, Enhanced Chemiluminescence system, and recProtein-A Sepharose Fast Flow were purchased from GE Healthcare (Uppsala, Sweden). Polyvinylidene difluoride membrane was from Millipore (Schwabach, Germany).

Cloning, sequencing, expression and purification of trans-sialidase genes

The Basic Local Alignment Search Tool (BLAST) was used to search the shot-gun sequences of *T. congolense* at the WSTI (http://www.sanger.ac.uk). Using the BLASTN algorithm, the "*T. congolense* reads" were queried with the partial nucleotide sequences (Genbank Accession numbers TS1: AJ535487 and TS2: AJ535488) previously described [13]. Perfect BLAST hits (smallest sum probability P(N)<10-10) were arranged into contiguous sequences using Contig Express (Invitrogen, Carlsbad, USA). By searching the database with ends of the contiguous sequences, the assembled contigs were expanded until open reading frames (ORF) were obtained. On the basis of the obtained ORFs, primers (Supporting Information, Table S1) were designed to amplify by nested PCR the ORF including flanking regions encoding for TconTS2, TconTS3 and TconTS4 using genomic DNA of *T. congolense* strain STIB249 [13]. The resulting products were cloned into the pBlueScript KS- vector (Stratagene, Santa Clara, Ca, USA) via *Spe*I and *Bam*HI (TconTS2) or via *Eco*RI and *Sma*I (TconTS4) or into the mammalian expression vector pcDNAIII Amp (Invitrogen, Carlsbad, USA) via *Hind*III and *Xba*I (TconTS3) and sequenced (Supporting Information, Table S2).

Cloning and sequencing of *T. brucei* TS genes followed a similar strategy as described for *T. congolense* above except that genes were cloned in pJET1.2/blunt vector (Thermo Scientific) following instructions of the manufacturer (for primers see Supporting Information, Table S1).

For the expression of secreted TconTS proteins in mammalian fibroblasts, corresponding DNA sequences without those encoding the signal peptides and GPI anchors were subcloned into a modified pDEF vector providing a 3C protease recognition site, SNAP and *Strep* tags using *Spe*I and *Bam*HI restriction sites [14]. For this purpose, the *Bam*HI site in TconTS3 as well as the *Spe*I and *Bam*HI sites in TconTS4 were removed by site directed mutagenesis without changing the amino acid sequence encoded (for primers see Supporting Information, Table S1). All sequences and mutations were confirmed by Sanger dideoxy DNA sequencing at the Max Planck Institute for Marine Microbiology, Bremen, Germany.

Recombinant TconTS proteins were purified as described [14]. Briefly, CHO_{Lec1} cells (ATCC CRL-1735) were transfected with polyethylenimine, transfection reagent (Sigma, Steinheim, Germany) and stably expressing cell lines selected with hygromycin. Expression of recombinant protein was tested from cell culture supernatant by SDS-PAGE and Western blots methods using rabbit anti-*Strep* and anti-SNAP antibodies. CHO_{Lec1} cells producing TconTS proteins were subsequently adapted to chemically defined Excel CD CHO media.

Purification of anti-TS1 monoclonal antibody

The 7/23 hybridoma cells [12] were grown for 3 days in RPMI media supplemented with IgG depleted 10% FCS. The tissue culture supernatant was cleared by ultracentrifugation at 105×g for 60 min and anti-TconTS antibody was purified using recProtein-A Sepharose Fast Flow and eluted with 0.1 M glycine/HCl pH 3.0. Antibody containing fractions were neutralised with 1M Tris pH 8.0 and dialysed against 10 mM phosphate buffer. Purified antibodies were used in the detection of TconTS proteins in SDS-PAGE and Western Blot analysis as described [14].

Trans-sialidase and sialidase reactions

Purified recombinant proteins were assayed for sialidase and TS activities using Neu5Ac-MU and fetuin as sialic acid donor substrates and lactose as acceptor substrate as described before [14]. In brief, reactions of 50 µL containing substrates and enzymes were incubated at 37°C for the times indicated. Sialidase activity was determined as free sialic acids released from Neu5Ac-MU, 3'SL or fetuin in the absence and/or presence of an acceptor substrate. TS activity on the other hand was determined as 3'SL produced in the presence of lactose. Both, free Neu5Ac and 3'SL were quantified using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using the Dionex system, DX600 (Dionex Sunnyvale, CA, USA) [14]. The curve fit module of SigmapPlot 11 was used to calculate v_{max} and K_M employing the Michaelis-Menten equation $v = v_{max} \times C_s/(C_s+K_M)$.

Phylogenetic analysis

For the phylogenetic analysis TconTS1b, TconTS2, TconTS3 and TconTS4 were aligned with TS and sialidase sequences from *T. brucei*, *T. vivax*, *T. cruzi* and *T. rangeli*. As outgroup the sialidase from *Vibrio cholerae* was used (genes listed in Supporting Information Table S2). Full length protein sequences were first aligned using ClustalW in Geneious and then truncated at the N-terminus. CDs started from the FRIP region to the N-terminus of the conserved α-helix (HL) linking the CD to the LD. The LDs were taken immediately after the α-helix linkage to the C-terminus

without the stop codon. DNA sequences encoding either full length proteins, the CDs or the LDs were aligned based on the alignment obtained for the amino acid sequences by T-Coffee algorithm in RevTrans, version 2.0 (http://www.cbs.dtu.dk/services/RevTrans-2.0/web/). DNA sequences used in the phylogenetic calculations are shown in Supporting Information Files S1, S2 and S3. Best parameters (HKY substitution model with 6 gamma rate categories) for phylogenetic constructions were determined using MEGA5 and applied in the phylogenetic calculations using the "MrBayes" plug-in of Geneious.

Results

T. congolense sialidase/*trans*-sialidase genes

Partial coding sequences of TconTS1 and TconTS2 genes had been described [13]. From "reads" of the WTSI *T. congolense* genome sequencing project (http://www.sanger.ac.uk), we assembled the full length sequences coding TconTS1 and TconTS2. Further BLAST hits with smallest sum probabilities (P(N)<1–10) were identified and arranged into contiguous sequences leading to further five genes with sequence similarities. Two of the putative gene products shared over 40% sequence identity with TconTS1 and TconTS2 and contained all the conserved amino acids required for transfer reactions [17] [18] [10]. Consistent with the naming of TconTS1 and TconTS2 [13], we refer to them as TconTS3 and TconTS4. The other three genes were distantly related with 20–30% amino acid identity (Table 1) and lack several of the conserved amino acid residues. We assume that these set of genes are likely without sialidase or TS activity and were named TconTS-Like1, TconTS-Like2, and TconTS-Like3.

In order to compare sequence similarities between TconTS genes, we cloned and sequenced full lengths TconTS2, TconTS3 and TconTS4. In an earlier study, we amplified eleven highly similar (about 96% identical amino acids) but clearly different sequences of TconTS1 from *T. congolense* genomic DNA [14]. Sequencing several clones of TconTS2, TconTS3 and TconTS4 provided no evidence for such heterogeneity of these genes. The alignment of these genes is given in Figure 1.

Amaya *et al.* [18] identified amino acids in *T. cruzi* TS involved in the catalytic and substrate binding. Whereas these amino acids are not conserved in the three TconTS-like gene products, they are almost completely conserved in TconTS1, TconTS2, TconTS3 and TconTS4 (Table 2). Most of these are conservative changes, with the exception of position 293 (numbering in the consensus sequence), where a Tyr is replaced by Pro in TconTS2. Furthermore, based on mutagenesis experiments [19] [17] with *T. cruzi* TS, two proline residues corresponding to positions 411 and 465 appear to be required for full TS activity. Whereas at position 465 Pro is conserved across the TconTS, it is not found in the TconTS-like gene products (Table 2). However, at position 411 the Pro is not conserved in TconTS, but replaced by Ala or Ser.

TS orthologues occur in *T. congolense* and *T. brucei* but not in *T. vivax*

To decipher the phylogenetic relationship between TS and TS-like genes of African trypanosomes, we compared the four TconTS and three TconTS-Like sequences together with seven sequences from *T. brucei* and five from *T. vivax* (Supporting Information Table S2) using the alignment of DNA sequences reverse transcribed from the protein alignment (see Supporting Information Files S1, S2 and S3 for DNA sequences used). As shown in Figure 2A, for each TconTS and TconTS-Like gene a corresponding orthologue was identified in *T. brucei*, whereas *T. vivax* gene products cluster separately from TS of the other African trypanosomes. TconTS-Like2 and TconTS-Like3 form a branch together with their *T. brucei* orthologues separate from all South American TS genes. In contrast, TconTS-Like1 and its *T. brucei* orthologue appear to be more closely related with the more distant South American branch than the African genes.

Trypanosomal TS contain an N-terminal CD followed by a C-terminal LD. Besides the phylogenetic analysis with the entire ORFs, analysis were performed using sequences coding for each domain separately. The phylogenetic tree obtained for the CDs resembles that for complete ORFs (not shown). Surprisingly, a different situation was observed for the LDs of TconTS1, TconTS2, TconTS3 and TconTS4 and the *T. brucei* TS genes (Figure 2B). First, within the TconTS genes, the LD of TconTS2 is most closely related to that of TconTS1, whereas the CD of TconTS3 is more closely related to TconTS2. Second, it should be noted that not the same *T. congolense* and *T. brucei* genes group as orthologous pairs, if LDs are compared. Amplification and sequencing ORF of *T. brucei* TS genes confirmed that the combination of the CDs and LDs were as predicted from the contigs in the databases.

Monclonal anti-TS1 antibody cross-reacts with TconTS2, recognising an epitope on the lectin domain

To biochemically characterise TconTS genes, recombinant proteins were made for TconTS2, TconTS3 and TconTS4 as previously described for TconTS1 [14]. Recombinant TconTS proteins were expressed in CHO_{Lec1} [20] and purified by affinity chromatography to obtain pure protein from eukaryotic cells with high mannose-type *N*-glycans. The apparent molecular masses of the recombinant TconTS proteins including the SNAP and *Strep* tags are between 110 and 125 kDA as resolved on SDS-PAGE. The generic *Strep* tag fused to the proteins is recognised by anti-*Strep* polyclonal Ab in all the recombinant TconTS proteins as shown in Figure 3 (upper panel). Surprisingly, the monoclonal anti-TS antibody [12] reacted with both TconTS1 and TconTS2,

Table 1. *Trypanosoma congolense trans*-sialidase sequence similarities expressed as percentage of identical amino acids in pair-wise alignments.

Trans-sialidase	TconTS2	TconTS3	TconTS4	TconTS-Like1	TconTS-Like2	TconTS-Like3
TconTS1	42.2%	43.6%	46.2%	21.1%	26.3%	29.8%
TconTS2	-	48.3%	42.8%	20.8%	26.2%	29.3%
TconTS3		-	48.9%	21.1%	25.1%	29.9%
TconTS4			-	21.3%	27.8%	29.8%

Tcon = *Trypanosoma congolense*

doi:10.1371/journal.pntd.0002549.t001

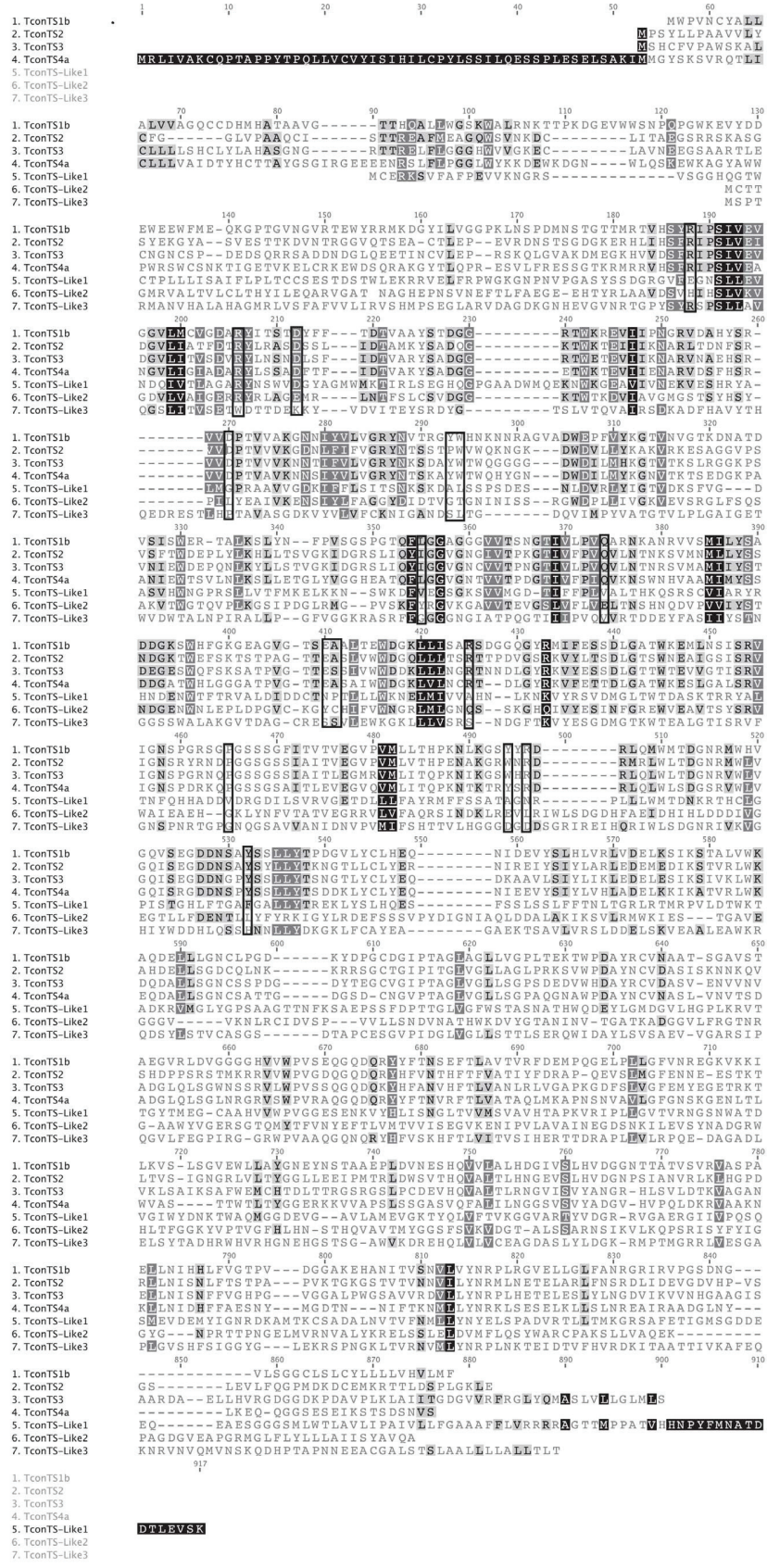
1. TconTS1b
2. TconTS2
3. TconTS3
4. TconTS4a
5. TconTS-Like1
6. TconTS-Like2
7. TconTS-Like3

Figure 1. Primary amino acid sequence alignment of TconTS and TconTS-Like genes. Amino acid sequences of TconTS1b (EMBL:HE583284), TconTS2, TconTS3 and TconTS4 were obtained by sequencing of cloned genes. Those of TconTS-Like 1, TconTS-Like 2 and TconTS-Like 3 were obtained from the WSTI database. CluwstalW alignment plugin of the Geneious software was used employing BLOSUM12 with gap openings and extension penalties of 10 and 0.1, respectively. Increasing darkness of background indicates increasing numbers of identical amino acids at each position. The numbers on top of the sequences indicate the positions in the consensus sequence. Amino acid residues postulated to be critical for catalysis, substrate binding and structure as given in Table 2 are boxed.
doi:10.1371/journal.pntd.0002549.g001

but not TconTS3 and TconTS4 (Figure 3; lower panel). This result points to a similar epitope being present in both TconTS1 and TconTS2. Further experiments provided evidence that the epitope is located in the LDs.

TconTS2, TconTS3 and TconTS4 are trans-sialidases

In order to investigate the enzymatic activities we used the glycoprotein fetuin as donor and lactose as acceptor substrates. Free sialic acid (the product of sialidase activity) and 3'SL (the product of TS activity) could be quantified simultaneously from HPAEC-PAD chromatograms of the reactions. Under standard conditions 25 ng TconTS2 produced about 330 pmol/min 3'SL leading to 200 μM 3'SL in the reaction mix within 30 minutes (Figure 4A). Product formation by TconTS2 was linear for up to 50 ng enzyme under these conditions. The reaction catalysed by TconTS3 was slower than that of TconTS2, since 500 ng of enzyme generated only 4.2 pmol/min 3'SL corresponding to 10 μM 3'SL after 4 h (Figure 4B). 3'SL formation by TconTS3 was almost linear for 4 h. TS activity was also detected for TconTS4. However, the activity was even lower than that of TconTS3 and 500 ng of TconTS4 produced less than 0.1 pmol/min 3'SL (Figure 4C). Therefore, 24 h incubations were routinely used to determine TconTS4 activity.

To determine kinetic parameters (Table 3) reactions were conducted under standard conditions for 30, 120 and 1440 minutes for TconTS2, TconTS3 and TconTS4, respectively. TconTS2 exhibited the lowest K_M for fetuin (299 μM), similar to that reported for TconTS1b (395 μM) [14], whereas those determined for Tcon TS3 (6090 μM) and TconTS4 (949 μM) were higher. The lowest K_M for lactose was found for TconTS2 (602 μM), followed by TconTS3 (1104 μM) and TS4 (1806 μM). Comparing the results with those obtained for two variants of TconTS1 [14], TconTS2 has a K_M value for lactose similar to TconTS1e-1 but about 3-fold less than TconTS1b. For both substrates, TconTS2 showed about 2-, 200- and 2000-fold higher v_{max} values than TconTS1, TconTS3 and TconTS4, respectively.

Sialidase activities

Sialidase activity has been shown for TconTS purified from *T.congolense* axenic culture [12] and for *T. congolense* infected animals [5] [7]. Therefore, we investigated the sialidase activities of TconTS using fetuin as a model glycoprotein. Whereas no release of free Neu5Ac was observed for TconTS1, TconTS2 and TconTS3 under standard conditions of TS assays, TconTS4 clearly showed sialidase activity producing 0.76 pmol/min Neu5Ac up to 48 h (Figure 5A). Indeed, the sialidase activity of TconTS4 is relatively stable and retained a residual sialidase activity of 40% even after incubation at 37oC for 120 days.

We also investigated the effect of lactose on TconTS4 sialidase activity. At lactose concentrations above 3 mM, release of Neu5Ac dropped to undetectable levels (Figure 5B). This result indicates the existence of a competition between lactose and water for the cleaved Neu5Ac from the donor fetuin. This is confirmed by the increased amount of 3'SL produced with increasing lactose concentration.

When we incubated TconTS2 or TconTS3 with fetuin and lactose for extended periods, it was observed that increasing amounts of Neu5Ac were released, similar to TconTS1 [14]. Interestingly, free Neu5Ac appeared only after 3'SL has accumulated as a product of transialylation. For example, in TconTS2 reactions, Neu5Ac was detectable (0.68 μM, 27 pmol) when the 3'SL concentration had reached almost 600 μM, the maximum 3'SL concentration reached. Whereas further incubation did not result in higher 3'SL concentrations, the amount of free Neu5Ac continuously increased. This observation suggests that TconTS2releases free Neu5Ac from 3'SL but not from fetuin. Similar observations were made for TconTS1, but the highest 3'SL concentration reached was about 300 μM. These data indicated that for TconTS1 and TconTS2 at 300 μM and 600 μM 3'SL, respectively, the transfer of Neu5Ac between fetuin and 3'SL has reached an equilibrium. For TconTS3, we could not reach such equilibrium; probably since the maximum 3'SL concentration obtained was 50 μM due to the low specific activity of this enzyme. Nevertheless, small amounts of free Neu5Ac were detected in prolonged TconTS3 reactions leading to 50 μM 3'SL. Also in this case, Sia appears to be released from 3'SL only, since in the absence of lactose no release of free Neu5Ac could be observed.

These observations suggest that TconTS1, TconTS2 and TconTS3 can release free Neu5Ac from glycoconjugates, but the level of product released is too low to be detected under standard TS assay conditions. To address this, higher amounts (500 ng) of TconTS1, TconTS2, TconTS3 and TconTS4 were incubated with 600 μM fetuin-bound Neu5Ac without lactose for 2 h. Under these conditions sialidase activities could also be detected for TconTS1 and TconTS2, but not for TconTS3 (Table 4). However, compared to the corresponding TS activities, the sialidase activities were very low.

Discussion

The enzymatic properties of four TconTS were compared using fetuin as a model for a blood glycoprotein donor substrate. Two of these enzymes, TconTS1 and TconTS2, exhibit about 100- or 1000-fold higher specific TS activities than TconTS3 and TconTS4, respectively. The K_M values for lactose were around 1 mM (0.6 to 1.8 mM) for all four TconTS and did not correlate with their specific activities. The K_M values for the donor fetuin were more different ranging from 0.4 to 6 mM glycoprotein bound sialic acids. The K_M for fetuin also did not correlate with the specific activity, since the highest K_M was determined for TconTS3, one of the enzymes with low activity, and TconTS4 has a similar K_M as TconTS1, but is 1000-fold less active. Since the K_M values are lower than the substrate concentrations used, especially for TconTS4, the specific activities given in Table 3 are lower than those to be expected, if the acceptor substrate lactose would be at saturating concentrations.

Table 2. Amino acids in the catalytic domains of TS and TS-Like genes from *T. congolense* involved in enzymatic activities*.

Consensus	Tcon TS1	Tcon TS2	Tcon TS3	Tcon TS4	Tcon TS-Like1	Tcon TS-Like2	Tcon TS-Like3
catalysis							
212	D150	D135	D142	D207	D110	E85	K86
410	E324	E309	E316	E381	N291	Y257	S262
532	Y438	Y423	Y430	Y493	F404	L375	H382
substrate binding							
188	R126	R111	R118	R183	E86	H61	R62
425	R339	R324	R331	R396	A306	Q272	S277
496	R410	R395	R402	R465	N375	L339	D346
206	R144	R129	R136	R201	R104	R79	W80
270	D188	D173	D180	D245	G160	L122	P132
293	Y211	P196	Y203	Y268	A183	G145	S155
294	W212	W197	W204	W269	L184	T146	L156
374	Q289	Q274	Q281	Q364	V255	E222	V227
494	Y408	W393	W400	Y463	A373	E337	D344
structure							
411	A325	A310	S317	A382	P292	C258	S263
465	P379	P364	P371	P434	V344	G308	G315

*The indicated amino acids have been selected based on structural [18] and mutation [19] [23] studies with *T. cruzi* TS and on the sequence alignment of TconTS1b with *T. cruzi* [14]. Amino acid positions have been numbered based on the consensus of alignment (Figure 1) or starting methionine of each ORF.
doi:10.1371/journal.pntd.0002549.t002

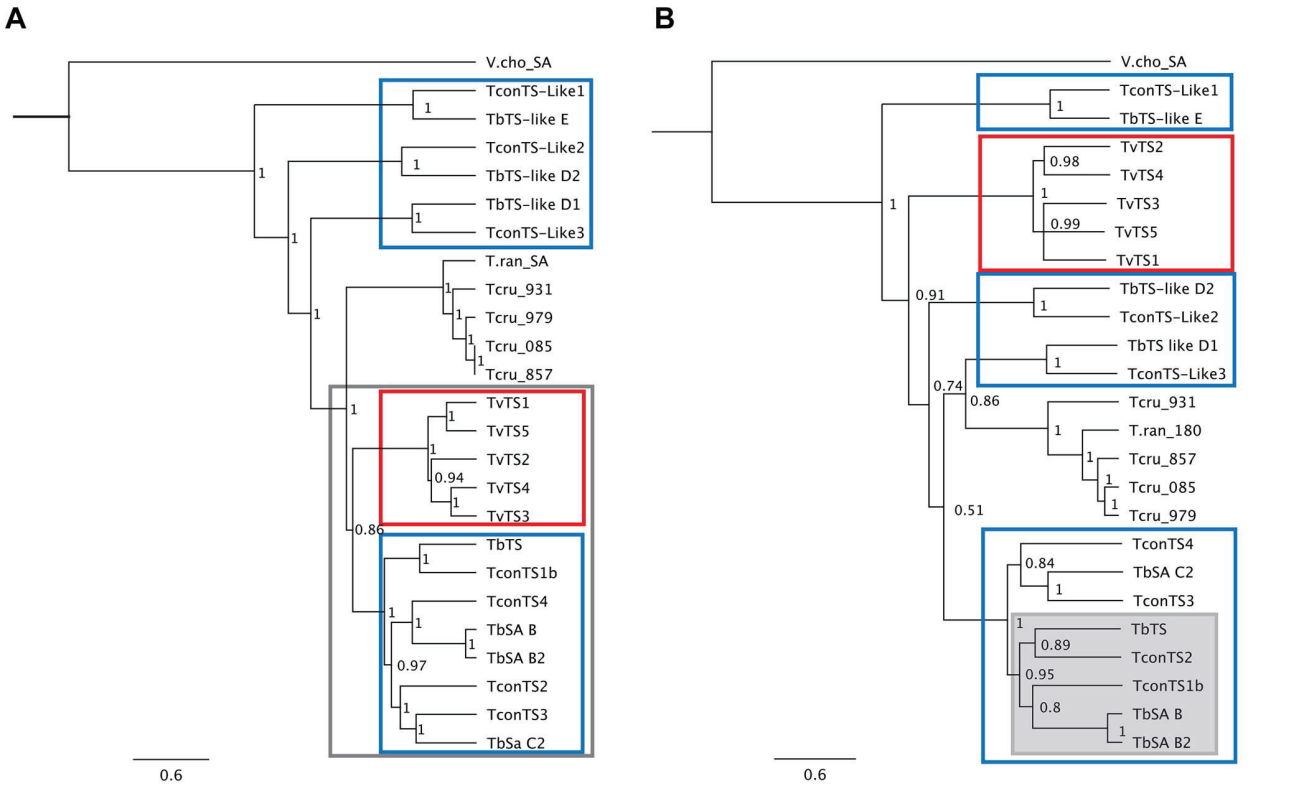

Figure 2. Phylogenetic analysis TS genes. Phylogenetic analyses of DNA sequences were performed as described under Methods using ''MrBayes'' plug-in of Geneious. Trees are midpoint rooted and nodes supported by posterior probability values and non-parametric bootstraps generated by maximum likelihood analysis in ''MrBayes'' as described under Methods. TS genes from *T. congolense* and *T. brucei* are marked by blue frames, TS genes from *T. vivax* by red frames. **A**: Phylogenetic tree for full ORFs, the ''African TS-branch'' is marked by a grey frame; **B**: Phylogenetic tree for LDs, the most active TconTS and their orthologues from *T. brucei* are highlighted by a grey box.
doi:10.1371/journal.pntd.0002549.g002

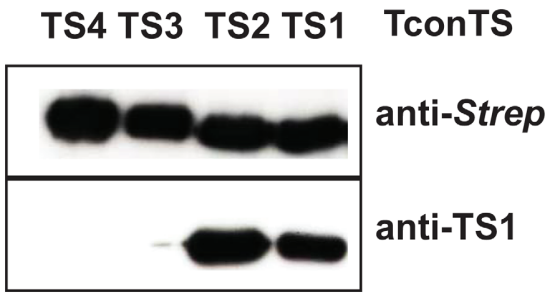

Figure 3. Recognition of TconTS proteins by antibodies. 100 ng of affinity purified TconTS proteins were analysed by Western blot as described under Methods. Blots were probed either with polyclonal rabbit anti-Strep (upper pane) or with monoclonal mouse anti-TS1 antibody, which shows cross reactivity with TconTS2 (lower pane).
doi:10.1371/journal.pntd.0002549.g003

Tiralongo *et al.* [12] purified two TconTS forms with different TS/sialidase activity ratios from procyclic *T. congolense* cultures. Peptide sequences from these preparations have demonstrated that they contained at least TconTS1 [12] [14]. Recombinant TconTS1 variants expressed in eukaryotic cells had lower specific activities for synthetic substrates than described for the purified enzyme [12] suggesting that these TconTS preparations also contained other enzymes and/or factors influencing the TS/sialidase activities [14]. For example, in those preparations, Glutamic Acid-Rich Protein, GARP, a natural substrate for TconTS was co-purified with one of the TS forms [12]. Although it is unclear what role GARP might have played, its presence, as well as other TS enzymes, might have been responsible for the reported higher specific activities of these TS preparations for synthetic substrates. In summary, it appears that TconTS1 and TconTS2 are responsible for most of the TS activity of *T. congolense*. However, it may well be that TconTS3 and TconTS4 are more active on other donor substrates, such as glycoproteins and/or glycolipids from blood components, the natural substrates for trypanosomal TS.

It has been established that procyclic forms of African trypanosomes express TS [21] and emerging evidences point to expression also in the blood stream forms [6] [7]. So far, no information is available on which TS genes are expressed at what stage of the parasite's life cycle. Recently, we have identified mRNAs for TconTS1, TconTS2 and TconTS4 in the blood of infected goats (data not shown). Also the stability and persistence of shed enzymes in the blood stream has to be taken into account. *In vitro* TconTS1 and TconTS3 are the most stable of the four enzymes investigated, retaining full TS activities even after four months at 37°C. Under these conditions TconTS2 lost its activity completely, while TconTS4 retained 40% residual sialidase but no transfer activity (data not shown). It would be interesting to investigate whether this long-term stability correlates with a sustained persistence of enzyme activity in the blood stream.

Lactose was found to suppress the sialidase activity of TconTS4. *In vitro*, lactose appears to be a better acceptor than water (Figure 5B). Therefore, in presence of lactose, the transfer activity of TconTS4 is more efficient than its hydrolytic activity. Anaemia in animals suffering African *Trypanosoma* infections has been attributed to the effects of sialidases [22] [5] [7]. Desialylation of erythrocytes by sialidases exposes underlying galactose residues and their subsequent degradation. The presence of lactose in mammalian blood would lead to lowered efficiency of parasites sialylation and eventual clearance by the immune system. However, desialylation of parasites is equally possible in presence of lactose due to the action of TS. Along this line it is interesting to note that infusion of lactose in the blood of sheep suffering experimental anaemia from *T. congolense* suppressed anaemia (unpublished observation).

All amino acid residues shown to be involved in the catalytic reaction or interaction with the substrate for *T. cruzi* TS are conserved in the TconTS enzymes (Table 2). Only the two residues interacting with the methylumbelliferyl aglycon or the lactose part of 3'SL in the *T. cruzi* TS [18], positions 293 and 494 in consensus sequence (Figure 1), are different in the two most active TconTS1 and TconTS2. This could explain why these enzymes do not use Neu5Ac-MU as substrate (data not shown). Furthermore, these changes could lead to a weaker interaction with the leaving groups and thus facilitate their release during catalysis. In this context it is interesting to note that the most drastic change, Tyr to Pro at position 293, occurs as P196 in TconTS2, the most active enzyme with the highest TS/sialidase ratio (Table 4). Certainly, this modification will reduce the interaction with hydrophobic aglycons or the leaving galactose residue of the donor substrate.

Amaya *et al.* [18] also provided evidence that in *T. cruzi* TS Y119 (position 293 in the consensus sequence) also contributes to hydrogen bonding with O9 of the covalently bound Sia following a conformational change induced by the reaction. Such an interaction would not be possible in TconTS2, but could be compensated by hydrogen bonding with the conserved side chains W197 and Q274 of TconTS2 corresponding to W120 and Q195 in *T. cruzi* TS, two amino acids contributing to the hydrogen bonding network of O9 in the covalently bound Sia [18].

Similarly, although to a lesser degree, the replacement of a Trp at position 494 (corresponding to W312 in *T. cruzi* TS) with a Tyr, as found in TconTS1, is expected to reduce the hydrophobicity of this site leading to a reduced affinity for the leaving group. In *T. cruzi* TS substitution of this Trp (W312) by Ala basically abolished Neu5Ac transfer but only slightly decreased hydrolytic activity for 3'SL [23]. Interestingly, in contrast to the wild type *T. cruzi* TS, this mutant was not able to hydrolyse Neu5Ac-MU, similar to TconTS1, which also does not accept Neu5Ac-MU as a substrate [14].

It appears that TS activity depends on well controlled conformational changes [17] influenced by specific proline residues. This is supported by the potential of the *T. rangeli* sialidase to acquire transfer ability due to a change of Gln to Pro at position 284 [24] and the loss of enzymatic activity in *T. cruzi* TS by the reverse mutation [16]. At the corresponding position 465 Pro is found in all active TconTS (Table 2). The relevance of conformational changes in the enzyme rather than a direct specific interaction of the amino acid were also indicated by mutation of Pro231 to Ala in *T. cruzi* TS [19], corresponding to position 411 in the consensus sequence. Although this mutation led to a significant decrease in *T. cruzi* TS activity, all active TconTS have an Ala or Ser at this position. It would be interesting to see, if higher TS activities can be obtained by introducing a Pro at this position, particularly in TconTS3 or TconTS4, the two enzymes with low TS activities.

Besides these critical amino acids listed in Table 2, other structural features obviously control the ratio of TS versus sialidase activities, since TconTS2 and TconTS4 share identical amino acids at all these positions. Yet, TconTS4 has the highest sialidase to TS ratio amongst the TconTS enzymes, whereas TconTS2 has the lowest ratio (Table 4). Koliwer-Brandl *et al.* [14] observed for TconTS1 that a natural mutation that replaced R144 (206 in

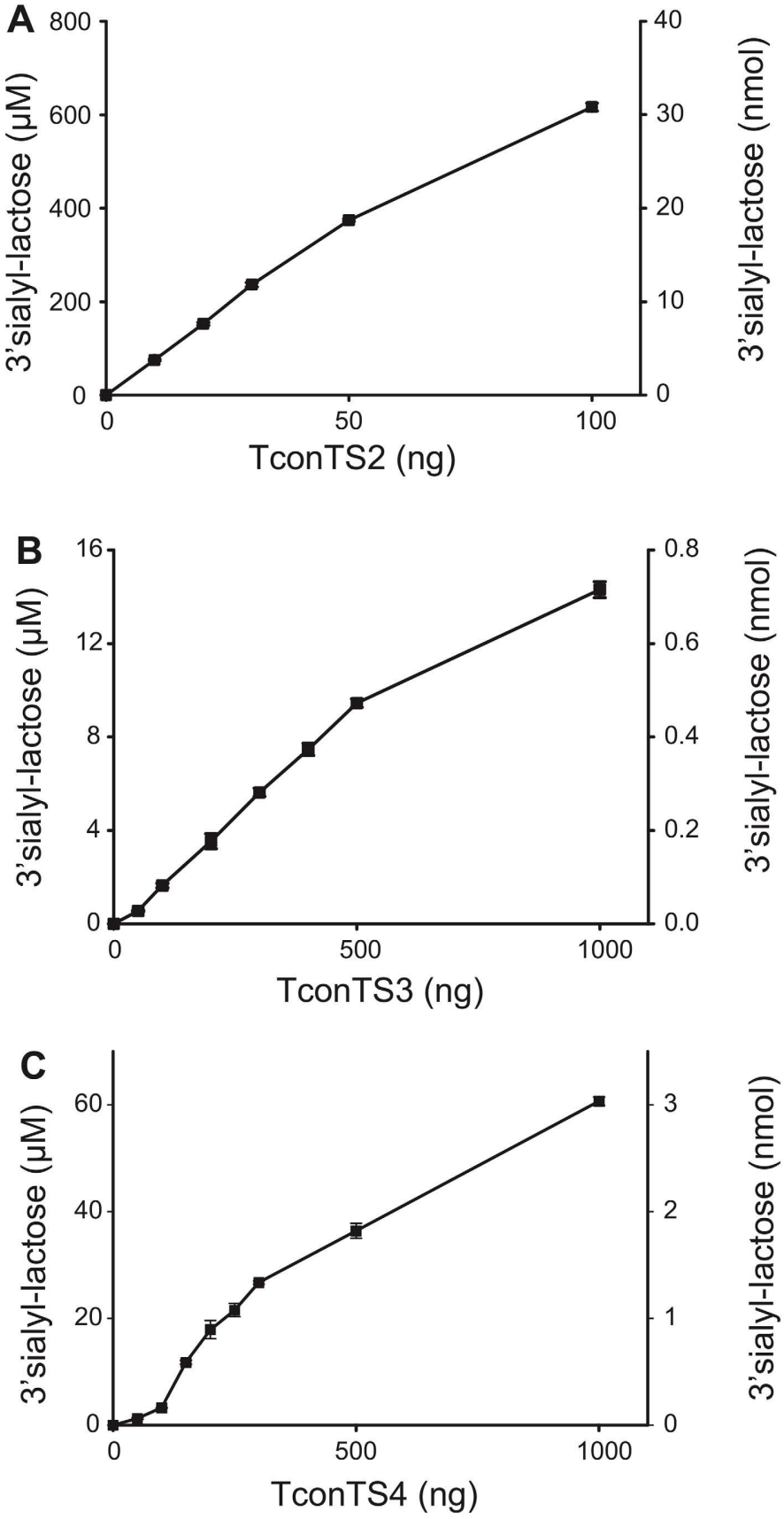
A
800
600
400
200
0
40
30
20
10
0
0
50
100
3'sialyl-lactose (μM)
3'sialyl-lactose (nmol)
TconTS2 (ng)
B
16
12
8
4
0
0.8
0.6
0.4
0.2
0.0
0
500
1000
3'sialyl-lactose (μM)
3'sialyl-lactose (nmol)
TconTS3 (ng)
C
60
40
20
0
3
2
1
0
0
500
1000
3'sialyl-lactose (μM)
3'sialyl-lactose (nmol)
TconTS4 (ng)

Figure 4. Concentration dependency of TS activity. The indicated amounts of recombinant TconTS proteins were incubated with 100 μg fetuin (600 μM bound Sia) and 2 mM lactose for 30 (TconTS2), 240 (TconTS3), or 1440 (TconTS4) minutes. 3'SL produced was determined by HPAEC-PAD as described under Methods. Data points are means ± standard deviations of three independent experiments, each as triplicates. **A**: TconTS2, **B**: TconTS3, **C**: TconTS4.
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consensus) sequence with Cys in the variants TconTS1g (EMBL: HE582290) did not terminate but only reduced TS activity, while increasing relative hydrolytic activity. It can be assumed that a weaker interaction with the hydroxyl group at C4 of sialic acid is responsible for the catalytic properties of TS1g, since the Arg (R53 in *T. cruzi* TS) is in close contact with the bound sialic acid and probably supports the stabilisation of the enzyme-substrate complex [18].

Conservation and/or substitution of amino acids in the active centre of the catalytic domain did not give clear indications of activity differences between the TconTS enzymes. In this context, an interesting aspect is how the different specific activities of TconTS enzymes correlate with those of related gene products from other African trypanosomes. Phylogenetic analyses have allowed the clear assignment of orthologues for *T. brucei*, but not for *T. vivax*, where TS genes clustered exclusively together and away from the TS genes of *T. congolense* and *T. brucei* (Figure 2) [7] [25]. A direct comparison of the TS activities between *T. congolense* and *T. brucei* is difficult, since limited comparable data for enzymatic activities is available. In *T. brucei* TbTS and TbSA C2 have been identified as active TS [9] [10] [11]. This is consistent with the observation that their orthologues (TconTS1 and TconTS2, respectively) are the most active TS in *T. congolense*. RNA*i* based experiments provided evidence that in *T. brucei* TS and sialidase activities are encoded by different genes, TbTS and TbSA C [10], whereas assays with purified recombinant proteins demonstrated both enzyme activities for TbTS and TbSA C2 [11]. Noticeably, the diversity of TS-related genes in *T. vivax* is lower than what was obtained for *T. congolense* and *T. brucei*. Equally, *T. vivax* is distinctively different from *T. congolense* and *T. brucei* in terms of development in the insect host. While the later two develop in the insect midgut and proboscis or salivary glands respectively, *T. vivax* develops exclusively in mouthparts of the tsetse. Moloo and Gray [26] showed that *T. vivax* ingested with blood meal to the midgut is disintegrated. TS-like genes from *T. vivax* share all but two (consensus sequence positions 411 and 494) of the conserved amino acids listed in Table 2 with the active TconTS. Recently Guegan *et al.* [7] reported that TvivTS2 has TS activity. Furthermore, they obtained evidence for the presence of at least TvivTS1, TvivTS3 and TvivTS5 in the bloodstream form and none in epimastigotes and the possible involvement of these proteins in anaemia in infected mice. In summary, it may be possible that *T. vivax* is missing a suitable TS to survive and colonise the fly vector midgut. To this end, it would be interesting to see the survival ability of transgenic *T. vivax* expressing a TS, which is expressed by *T. congolense* in the midgut of tsetse flies.

The presence of multiple highly similar TS genes, as described for TconTS1 [14], suggests that these genes undergo active rearrangements, which could lead to strain specific differences. For example, Coustou *et al.* [6] referred to two highly related TconTS3 genes in the IL3000 strain identified in GeneDB and TrytrypDB databases. However, we could not find evidence for their existence in the STIB294 strain used in this study. Similarly, closely related genes with over 80% sequence identity have been identified for TbSA B and TbSA C in *T. brucei* [11].

TS and sialidase genes of African trypanosomes are organised in two major domains; the CD and the LD. The LDs of TconTS are more varied (40% pairwise identity) when compared with the CDs (58% pairwise identity). Surprisingly, the phylogenetic relationships between the TS are clearly different, if only the LDs are included in the analysis (Figure 2). Furthermore, the LDs of the two most active enzymes TconTS1 and TconTS2 are more closely related than the CDs, where TconTS2 is most closely related to TconTS3. Interestingly, the monoclonal anti-TS1 antibody also binds TconTS2, recognising an epitope in the LD. First preliminary experiments obtained with recombinant proteins, in which the LDs have been swapped between TconTS, provided supporting evidence that the LD influences TS and sialidase activities of the enzymes (data not shown). However, the specific activities of these proteins expressed in bacteria is much lower than those of the proteins expressed in fibroblasts described here,

Table 3. Kinetic parameters of TconTS.

	Donor substrate (fetuin-bound Sia)*		Acceptor (lactose)**	
	v_{max}*** (μmol/(min×mg TS))	K_M*** (μM)	v_{max}*** (μmol/(min×mg TS))	K_M*** (μM)
TS1b****	7.9±0.3	359±45	4.3±0.1	1683±101
TS1e-1****	7.6±0.5	1617±223	2.1±0.1	727±48
TconTS2	17.62±0.13	299.00±7.0	17.85±0.13	602±16
TconTS3	0.17±0.02	6090.00±1267	0.0567±0.0014	1104±79
TconTS4	0.0067±0.0002	949±50	0.0075±0.0002	1806±112

*Approximately 30 nmol Sia per 100 μg fetuin; 2 mM lactose was used as acceptor substrate.
**600 μM fetuin-bound Sia was used as donor substrate.
***K_M and v_{max} were calculated from Michaelis-Menten kinetics (see Supplementary Information, Figure S1) by SigmaPlot. Data points are mean ± standard deviations of three independent experiments, each replicated thrice.
****values from Koliwer-Brandl et al. [14].
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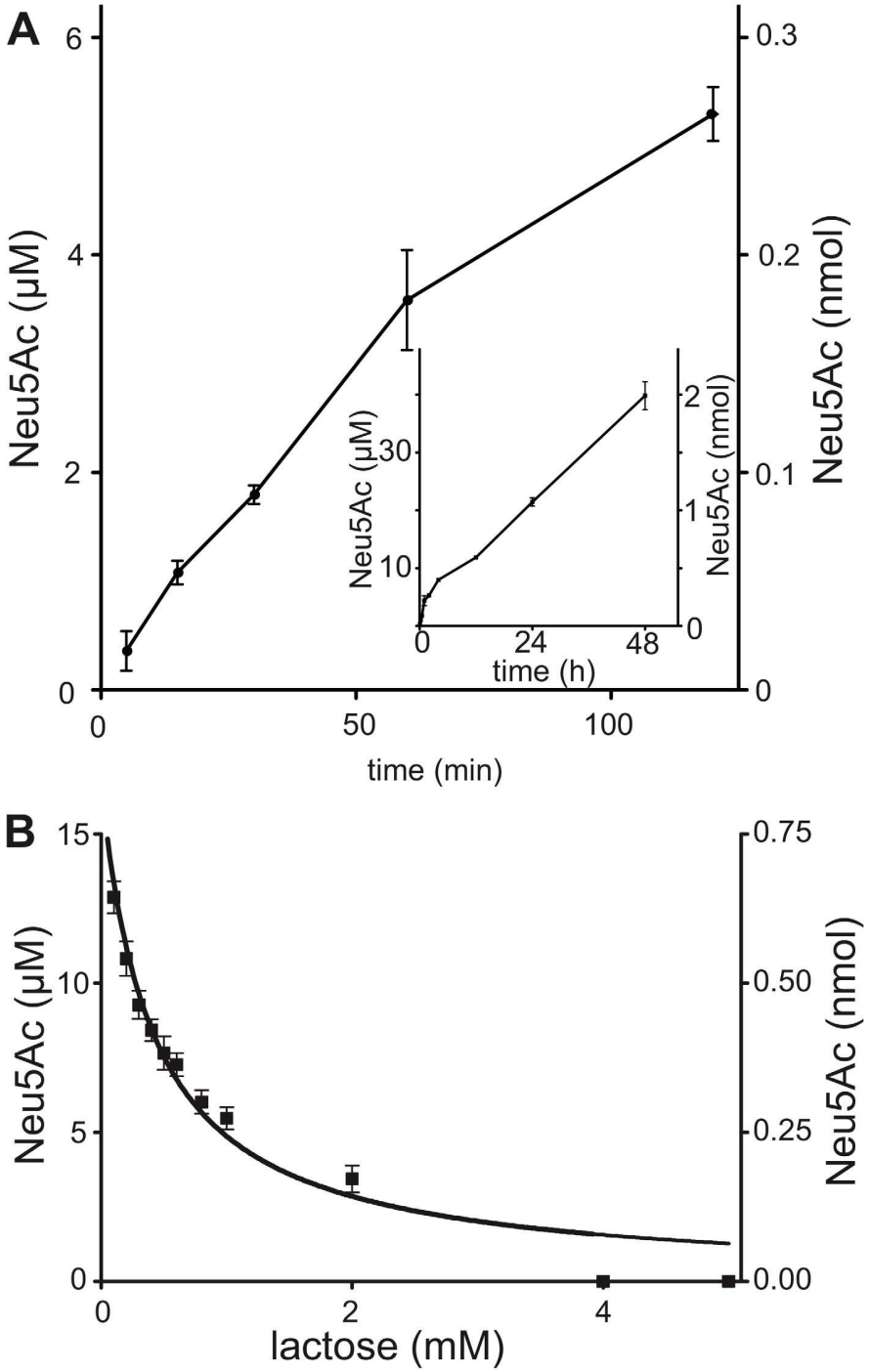

Figure 5. Sialidase activity of TconTS4. A: Recombinant TconTS4 was incubated with 100 μg fetuin (600 μM bound Sia) for the times indicated (see insert for long term reactions) and free Sia determined by HPAEC-PAD as described under Methods. Data points are means ± standard deviations of 3 independent reactions each triplicated. **B**: Recombinant TconTS4 was incubated for 1440 min with 100 μg fetuin (600 μM bound Sia) in the presence of the lactose concentrations indicated and free Sia determined by HPAEC-PAD as described under Methods. Data points are means ± standard deviations of 3 independent reactions each as triplicates.
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suggesting that for conclusive interpretation the domain swapped TconTS have to be expressed in eukaryotic cells and that further studies are necessary to optimise the fusion of the two domains. In summary, these data indicate a more significant role for the LD for the TS activities of TS1 and TS2 and thus possibly in the pathogenesis of African trypanosomiasis.

Table 4. Sialidase activities of TconTS.

	Sialidase* (nmol/(min×mg TS))	Trans-sialidase** (nmol/(min×mg TS))	TS/sialidase
TconTS1	59.7±2.3	4,000±100	67
TconTS2	73.5±4.0	17,850±130	243
TconTS3	n.d.***	34±1	>30
TconTS4	2.7±0.07	9±0.2	3.4

*Sialidase activities were determined by incubating 500 ng of the TconTS indicated for 2 h with 100 µg fetuin (600 µM fetuin-bound Sia). Data points are mean ± standard deviations of three replicates.
**TS activity values see Table 3.
***n.d. not detected.
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Supporting Information

Figure S1 Trans-sialidase reaction velocities depending on substrate concentrations. Product (3′-sialyl-lactose) amounts were determined as described under Methods. v_{max} and K_M for lactose shown in Table 3 were calculated from these data. Data points are mean ± standard deviations of three independent experiments, each replicated thrice. **A) TconTS2 with different donor substrate concentrations.** 50 ng TconTS2 were incubated for 30 minutes with 2 mM lactose and the indicated concentrations of fetuin-bound Sia. **B) TconTS2 with different acceptor substrate concentrations.** 50 ng TconTS2 were incubated for 30 minutes with 600 µM fetuin-bound Sia and the indicated lactose concentrations. **C) TconTS3 with different donor substrate concentrations.** 250 ng TconTS3 were incubated for 120 minutes with 2 mM lactose and the indicated concentrations of fetuin-bound Sia. **D) TconTS3 with different acceptor substrate concentrations.** 500 ng TconTS3 were incubated for 120 minutes with 600 µM fetuin-bound Sia and the indicated lactose concentrations. **E) TconTS4 with different donor substrate concentrations.** 500 ng TconTS4 were incubated for 1440 minutes with 2 mM lactose and the indicated concentrations of fetuin-bound Sia. **F) TconTS4 with different acceptor substrate concentrations.** 500 ng TconTS4 were incubated for 1440 minutes with 600 µM fetuin-bound Sia and the indicated lactose concentrations.
(PDF)

File S1 Nucleotide sequences of the sialidase and trans-sialidase genes used in the phylogenetic comparison of "full length open reading frames" (catalytic plus lectin domains). The gaps inserted for the alignment as described under Methods are indicated by dashes. The file is a text file in FASTA format with the gene names (see Table S2) in the first line for each gene.
(TXT)

File S2 Nucleotide sequences of the sialidase and trans-sialidase genes used in the phylogenetic comparison of "catalytic domains". The gaps inserted for the alignment as described under Methods are indicated by dashes. The file is a text file in FASTA format with the gene names (see Table S2) in the first line for each gene.
(TXT)

File S3 Nucleotide sequences of the sialidase and trans-sialidase genes used in the phylogenetic comparison of "lectin domains". The gaps inserted for the alignment as described under Methods are indicated by dashes. The file is a text file in FASTA format with the gene names (see Table S2) in the first line for each gene.
(TXT)

Table S1 List of primers used for cloning and mutagenesis. Listed are the primers used in this study for cloning, expression plasmids and mutagenesis as described under Methods.
(PDF)

Table S2 Trans-sialidase and sialidase genes used for phylogenetic analysis. Listed are accession numbers and literature references for the genes used in the phylogenetic analysis in this study.
(PDF)

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Author Contributions

Conceived and designed the experiments: SK JAN. Performed the experiments: TTG MW BH JS GDC SK. Analyzed the data: TTG MW JS SK. Contributed reagents/materials/analysis tools: FD. Wrote the paper: TTG MW JAN SK.

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4.0.

Results

Manuscript

Enzymatic activities of trans-sialidases from T*rypanosoma congolense* on blood glycoconjugates

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Contributions of Thaddeus Gbem

- Purified TconTS proteins
- Carried out TS/sialidase reactions
- Carried out HPAEC-PAD analysis
- Drafted the manuscript.

Enzymatic activities of trans-sialidases from T*rypanosoma congolense* on blood glycoconjugates

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Abstract

Nagana, the animal form of trypanosomiasis, caused by *Trypanosoma* species is a serious problem in Africa, the pathology of which trans-sialidases (TS) play a key role. Four active TS genes from *T. congolense*, TconTS1, TconTS2, TconTS3 and TconTS4 are expressed in the bloodstream of infected animals. We studied the enzymatic activities of these diverse gene products on blood glycoconjugates and report that all TconTS enzymes transfer sialic acids (Sia) with TconTS1 and TconTS2 exhibiting highest specific activities for transfer, with little sialidase activity. Sialidase activities of TconTS1, TconTS2 and TconTS4 were higher on serum glycoconjugates than on fetuin. A combination of recombinant proteins leads to cleavage of MU-Neu5Ac, a sialoside not used by single recombinant proteins at detectable levels. The enzymes exhibit different pH optima with TconTS2 showing a wide pH range, being active even at alkaline pH above 9.

Author Summary

Trans-sialidases (TS) have been established as virulent factors in the Animal African Trypanosomiasis (AAT). Clinical signs of nagana include but not limited to accumulation of free Sia in blood and serum, however, it has remained unclear where this free Sia comes from and which enzymes are involved. Our study revealed high sialidase activities of some of the TS on blood glycoconjugates, possibly accounting for the high free Sia observed in blood of infected animals. The discovery that one of these enzymes, TconTS2, has a wide pH range and can be active at alkaline pH is in contrast to other sialidases which show preferences to acidic pH. The implication is that TconTS2 would be active even in the tsetse midgut which has an alkaline pH of about 10. While single recombinant TS enzymes do not utilise MU-Neu5Ac in detectable amounts, when combined, recombinant TconTS accepts MU-Neu5Ac. This suggests that combining different TS would enhance the parasites' ability to handle a wide variety of natural substrates as occurs in the mammalian host.

Introduction

Trypanosoma congolense (subgenus: Nannomonas) is a major causative agent of the Animal African Trypanosomiasis (AAT) otherwise known as nagana. Nagana has profound negative

impact on stock farming, milk and meat production on the African continent (Steverding 2008). Other *Trypanosoma* spp. implicated in nagana include but not limited to *T. vivax* (sungenus: Duttonella) and *T. brucei brucei* (subgenus: Trypanozoon), which are transmitted by bites of *Glossina* spp. These protozoans express a unique enzyme called trans-sialidase (TS). Their role in establishing infection in the tsetse vector has been established for *T. brucei* (Nagamune et al. 2004). While no such information is available for *T. congolense*, TS genes are likely to play a similar role, since *T. congolense* and *T. brucei* share several aspects of their biology, for e.g. they both establish infection first in the tsetse midgut where TS plays a role. Both *T. brucei* and *T. congolense* share TS orthologues, which are distinctively absent in *T. vivax* (Guegan et al. 2013, Jackson et al. 2013, Gbem et al. 2013). Though transmitted by tsetse as well, *T. vivax* exhibits a different mode of development in the fly vector only restricted to the mouth parts (Moloo and Gray, 1989).

In the mammalian host, TS/sialidase are shed in the serum of infected animals and have been identified as virulent factors in trypanosomiasis caused by *T. congolense* (Coustou et al. 2012) and *T. vivax* (Guegan et al. 2013). A significant level of sialidase activities in the blood of *T. congolense* (Nok and Balogun 2003) as well as sialidase and TS in *T. vivax* (Guegan et al. 2013) of infected mice have been reported. Similarly, the mechanism of anaemia induction has been established. It has been shown that removal of Sia from erythrocytes' major surface sialoglycoproteins, the glycophorins leads to erythrophagocytosis in the course of *T. vivax* (Guegan et al. 2013) and *T. congolense* (Coustou et al. 2012) infections.

Serum contains a wide array of glycoproteins and glycolipids that are heavily sialylated. For a detailed list of serum components, refer to the serum metabolomics where a comprehensive list of serum components has been compiled (Psychogios et al. 2011) and can be assessed at SMDB: http://www.serummetablome.ca/http://www.serummetablome.ca. Pathogens can alter the chemical and physiological composition of serum and blood plasma. For example, the intracellular *Trypanosoma* parasite *T. cruzi* is poorly sialylated when released from cells but becomes densely sialylated upon contact with serum (Schenkman et al. 1991). In investigating the role of TS and sialidases in trypanosomosis however, attention has focussed mainly on whole blood (Esievo et al. 1982, Nok and Balogun 2003, Coustou et al. 2012, Guegan et al. 2013), but it has remained unclear where the free Sia come from; the blood cells, soluble glycoproteins, glycolipids or a combination thereof. On these glycoconjugates, Sia occur linked as α2,3, α2,6 or α2,8 to a variety of glycan structures. Further diversity comes from the different naturally occurring Sia modifications (Kelm and Schauer 1997). Bulai et al. (2003) reported that 96% Sia on human erythrocytes are glycoprotein-associated comprising 9 other Sia besides Neu5Ac, the main Sia. These diverse sialylated glycoconjugates on RBC surfaces and on serum components represent potential

substrates for TS and could be removed by these enzymes. While a proportion is transferred to the parasites, as described for *T. cruzi* (Zingales et al. 1987, Schenkman et al. 1991), a certain proportion is lost becoming free Sia in the serum. The desialylated blood glycoconjugates products are rapidly cleared via galactose specific mechanisms (Kelm and Schauer 1997), a mechanism which is most likely an important reason for pathological effects of nagana, such as anaemia and impaired immune response.

T. congolense expresses several TS, which are also expressed in the bloodstream of infected animals (Coustou et al. 2012, Gbem et al. 2013), but the biological relevance for this diversity has not been addressed. Previously, we expressed recombinant TconTS1, TconTS2, TconTS3 and TconTS4 proteins and studied their kinetic activities on single natural substrates. We showed that they are all able to trans-sialylate, but with distinct enzyme kinetics and levels of sialidase activities. Investigating the effects of TconTS activities on biologically relevant substrates is necessary for a better understanding of their possible role in the pathology of nagana. Here, we investigated the activities of TconTS genes on serum, a biologically relevant substrate and conclude that expressing more than one enzyme at a stage may be beneficial to the parasite.

Materials and Methods

Methods

All chemicals and reagents used were cell culture and analytical grade. Anti-SNAP-tag rabbit polyclonal antibody was from GeneScript (Piscataway, USA) while anti-*Strep* tag rabbit polyclonal antibody, *Strep*-Tactin resin beads and buffers were from IBA (Göttingen, Germany). *Vibrio cholerae* sialidase was purchased from Roche Diagnostics (Mannheim, Germany). *Pfu* DNA polymerase, *Hind*III, *Xba*I, *Spe*I, *Eco*RI and *Dpn*I, PageBlue, molecular weight marker (PageRuler), BCA assay protein kit were all purchased from Thermo Scientific (St. Leon-Rot, Germany). X-ray film, Enhanced Chemiluminescence system, and recProtein-A Sepharose Fast Flow were purchased from GE Healthcare (Uppsala, Sweden). Polyvinylidene difluoride membrane was from Millipore (Schwabach, Germany). VivaSpin 6 and VivaCell250 ultracentrifugation units were from Sartorius (Göttingen, Germany). Hygromycin and gentamycin were purchased from PAA, (Pasching, Austria). Polyethylenimin transfection reagent, glucoronic acid, *N*-acetyl-neuraminic acid (Neu5Ac), 3'sialyllactose (3'SL), Neu5Ac-MU, MU-Gal and lactose, HCl and orcinol were purchased from Sigma-Aldrich (Steinheim, Germany). Ex-cell CD CHO media from SAFC, USA, and 96-well plates were from Sarstedt (Hamburg, Germany). Asialofetuin (ASF) was prepared using *Vibrio cholerae* sialidase as previously described (Koliwer-Brandl et al. 2011).

Recombinant trans-sialidases

Cell lines derived from $CHO_{Lec\ 1}$ cells (Kumar and Stanley 1989) expressing recombinant TconTS1

through TconTS4 with a SNAP and a Strep tag and the purification of these proteins has been described (Koliwer-Brandl et al. 2011, Gbem et al. 2013).

Serum preparations

To obtain serum, human blood was collected without anticoagulant and and was allowed to clot at room temperature for at least 30 minutes. The clotted components were separated from serum by centrifugation at 1500 min^{-1} for between 10 minutes at 4^{o}C. To quantify Sia content, serum was serially diluted in 40 µL amounts per well and total sialic acid determined using the resorcinol/Fe^{3+} reagent as described (Reuter and Schauer 1994).

Trans-sialidase and sialidase reactions

Enzymes used in control assays were heat inactivated. All reactions were incubated at 37^{o}C in 10 mM phosphate buffer, pH 7.4. Fetuin, human serum and synthetic substrates including Neu5Ac-MU, 3'SL, pNP-Neu5Ac served as Sia donor substrates while lactose, Gal-MU and ASF served as acceptor substrates. Except for the experiments involving different mixtures of TconTS on Neu5Ac-MU and that involving determination of pH optima, all other tests were carried out as described (Koliwer-Brandl et al. 2011, Gbem et al. 2013). For determination of pH optima for TconTS enzymes, pH was adjusted to the required pH of 6, 7, 8, 9 and 10, while reactions in which pH 7.4 was maintained served as controls. The reactions involving Neu5Ac-MU were done by combining 250 ng individual TS enzymes into different enzyme mixtures in a total reaction volume of 50 µL with 1 mM Neu5Ac-MU as Sia donor and 2 mM lactose as acceptor. Quantification of Neu5Ac, 3'SL and Neu5Ac-GalMU were carried out using the HPAEC-PAD system described (Koliwer-Brandl et al. 2011, Gbem et al. 2013).

In order to establish kinetic parameters, the ligand binding (single site saturation) curve fit module of SigmapPlot 11 was used to calculate v_{max} and K_M employing the Michaelis-Menten equation $v = v_{max} C_s/(C_s + K_M)$.

Results

Sialidase activities on fetuin

Sialidase activities of TconTS genes have been implicated in anaemia in animals suffering *Trypanosoma* infections (Nok and Balogun 2003, Coustou et al. 2012, Guegan et al. 2013). Desialylation of erythrocytes by *Trypanosoma* TS enzymes subsequently leading to their clearance from the host circulatory system has been linked to causes of anaemia. Serum is a complex substrate with different sialylated glycolipids, and glycoproteins. We needed to have a comparative basis by using a single substrate. We firstly characterised sialidase activities of TconTS enzymes on the blood glycoprotein fetuin for the following reasons; (i) it has been extensively employed to

biochemically characterize the transfer activities of these genes (Koliwer-Brandl et al. 2011, Gbem et al. 2013), (ii) it is a single and less complex substrate allowing easy comparison and inferences and (iii) though found in only foetal serum and may be less relevant in this respect, substrates like acute-phase proteins (APPs) e.g. the Alpha-1-acid glycoprotein (AGP) present in serum are highly sialylated and thought to play a role in modulating immune and inflammatory responses (Logdberg and Wester 2000) may be cleared.

Gbem et al. (2013) reported that TconTS enzymes exhibited low sialidase activities with TconTS4 showing the highest activity when fetuin was used as donor in TS reactions with sialidase activities of TconTS1, TconTS2 and TconTS3 detected only when 3'SL accumulated in the reaction. This indicated the desialylation of the 3'SL product but not the donor fetuin. Similarly, we reported that in the absence of lactose, except for TconTS4, sialidase activity were observed for TconTS1 and TconTS2 when 10-fold (500ng) enzyme amount were incubated with fetuin for 2 h. TconTS3 did not show sialidase activity even under these conditions. However, the study did not give v_{max} and K_M values for sialidase activities of these enzymes. Equally, enzyme amounts higher than 50 ng were not incubated with fetuin for longer times and therefore, no definite statement could be made as to wether or not lower enzyme amounts exhibited sialidase activities.

In the present study, sialidase reactions contained approximately 600 µM fetuin-bound Sia without an acceptor substrate. Reaction velocities were measured at indicated time points (Figure 1a, Table 3). 200 ng TconTS1 released 0.82 nmol Sia in 8 h (1.7 pmol/min). Incubations over extended periods up to 24 h released 2 nmol Sia (40 µM), showing reduced reaction velocity. TconTS2 showed a similar pattern but higher Sia release. 200 ng TconTS2 released 1 nmol Sia in 8 h (2.2 pmol/min) with the velocity of free Sia release decreasing with a total of 2.5 nmol free Sia (50 µM) within 24 h. 250 ng TconTS3 released 0.14 nmol Sia in 4 h (0.75 pmol/min). Velocity decreased as prolonged incubations released a total of 0.6 nmol Sia (12 µM) after 24 h. 250 ng TconTS4 released 0.15 nmol Sia in 2 h (1.25 pmol/min). At prolonged incubations up to 24 h, reactions velocities decreased until a total of 1.1 nmol Sia (22 µM). Since fetuin-bound Sia contained 600 µM Sia, the amount of Sia available could not be responsible for the decrease in the velocity of Sia release. However, in typical bacteria sialidases, free Sia has been shown to inhibit sialidase activity (Garcia Sastre et al. 1991). Varied enzyme amounts up to 1000 ng were incubated for 24 h to monitor if Sia release from fetuin depended on the amount of enzyme (Figure 1b). Under these conditions, Sia release from fetuin by TconTS1 and TconTS2 was concentration dependent. 500 ng enzyme released 6 nmol Sia (4.2 pmol /min) and 1000 ng released 12 nmol (8.3 pmol Sia/min) for both TconTS1 and TconTS2. At below 500 ng enzyme amounts, differences exist in the amount of Sia released by the two TconTS enzymes. At 200 ng enzyme amounts, TconTS1 released 2 pmol Sia/min while TconTS2 released 2.4 pmol Sia/min, indicating faster Sia release by TconTS2. The amount of Sia released by TconTS3 followed a similar pattern with those of TconTS1 and TconTS2

but with lower Sia amounts released as 500 ng released 2.1 nmol (40 μM) while 1000 ng released 4.2 nmol Sia (80 μM) after 24 h. Increasing Sia release was observed with increasing TconTS amounts. 500 ng TconTS4 enzyme released 4.5 nmol while 1000 ng released 6.5 nmol in 24 h.

Kinetics parameters were determined for sialidase activities of TconTS enzymes on fetuin (Tables 1, Figure S1a-d). To achieve this, we used 200 ng each for TconTS1 and TconTS2, and 250 ng each for TconTS3 and TconTS4 were incubated with 600 μM fetuin-bound Sia for 2 h for both TconTS1 and TconTS2, and 24h for both TconTS3 and TconTS4. TconTS1 and TconTS2 exhibited similar magnitudes of specific activity on fetuin (about 0.1 μmol/(min x mg TS)). This is 13- and 33-fold higher than that exhibited by TconTS4 and TconTS3 respectively on the same substrate. K_M values were also similar for TconTS1 (20 μM) and TconTS2 (30 μM). The K_M values for TconTS3 (137 μM) and TconTS4 (441 μM) were higher than those of TconTS1 and TconTS2.

Serum glycoconjugates as substrates for TconTS enzymes

Sia transfer was determined from 3'SL production in reactions containing serum as donor and lactose as acceptor substrates, respectively. Sialidase activity was monitored by Neu5Ac release from serum either in the absence or presence of lactose. A summary of these results is presented in Table 4. 200 ng TconTS1 produced 42 pmol 3'SL/min during the first 2 h (Figure 2a). At longer incubation times, the reaction velocity decreased until a maximum of 6 nmol 3'SL was reached. This did not appear to be due to donor substrate exhaustion, since this amount corresponds to 120 μM 3'SL, which is only 20% of the 600 μM serum-bound Sia available in the reaction. Even at prolonged incubations for up to 24 h with up to 1000 ng TconTS1 no more 3'SL was obtained (Figure 2b) suggesting that at 120 μM 3'SL, an equilibrium has been attained or the residual serum-bound Sia is not available for transfer by TconTS1, for example due to sialidase activity. However, free Sia could only be detected after longer incubations or with high amounts of TconTS1 and the maximum Sia released was 1.5 nmol after 24 h or with 1000 ng enzyme, representing less than 3 % of total serum-bound Sia added. In the absence of lactose, higher sialidase activity was detected and 200 ng TconTS1 released 8 pmol Sia/min for up to 4 h, after which the velocity started to decrease, attaining 5 nmol free Sia after 24 h (Figure 2a). Even 1000 ng TconTS1 hydrolysed only slightly more Sia (6.5 nmol). Interestingly, this is similar to the 6 nmol 3'SL formed by the same amount of enzyme.

A similar pattern of 3'SL formation was observed for TconTS2. 200 ng TconTS2 produced 75 pmol 3'SL/min for the first 2 h (Figure 2c). Reaction velocity decreased at longer incubation times until a maximum of 8 nmol 3'SL was reached. Increasing the enzyme to 1000 ng TconTS2 did not lead to higher amounts of 3'SL (Figure 2d) corresponding to 160 μM 3'SL, which represents 27% of the total serum-bound Sia available in the reaction. In the presence of 2 mM lactose, free Sia could only be detected after longer incubation times and reached a maximum of 0.25 nmol free Sia, less

than 1% total serum-bound Sia available in the reaction (Figure 2c and 2d). Higher sialidase activities were observed in the absence of lactose, where 200 ng TconTS2 released 13 pmol free Sia/min in the first 2 h (Figure 2c). Reaction velocity decreased at longer incubation times. The maximum free Sia obtained was 5 nmol and did not increase, even if 1000 ng TconTS2 was incubated for 24 h.

Sia transfer from serum donor substrate by TconTS3 was almost two orders of magnitude slower than with TconTS2, since 250 ng TconTS3 produced 0.83 pmol 3'SL/min. This reaction velocity was linear during the first 8 h (Figure 2e) before it decreased and a total of 0.65 nmol 3'SL was reached after 24 h. Increasing the enzyme to 1000 ng TconTS3, 1 nmol 3'SL could be obtained (Figure 2f). This corresponds to 20 µM representing 3 % of the total 600 µM serum-bound Sia available in the reaction. Under these conditions, free Sia was not detected in TconTS3 reactions containing the acceptor lactose, even at longer incubation times and higher enzyme amounts. In absence of lactose however, sialidase activity was detected and 1000 ng enzyme released 0.3 nmol free Sia in 24 h.

TconTS4 showed faster Sia transfer from serum donor than TconTS3 with 250 ng enzyme producing 4 pmol 3'SL/min in 1 h (Figure 2g). Reaction velocity decreased over extended incubation times leading to 0. 8 nmol 3'SL in 24 h. Incubation with 1000 ng TconTS4 for 24 h produced 1.1 nmol (Figure 2h). In the presence of lactose, 1000 ng TconTS4 released 0.21 nmol free Sia in 24 h and the amount increased to 4.5 nmol in absence of lactose (Figure 2h).

Kinetic parameters of serum-bound Sia as substrate for sialidase and trans-sialidase activities were determined in the presence of 2000 µM lactose as acceptor substrate for trans-sialidase (Table 2, Figure S2a-d). Similarly, K_M and v_{max} values of lactose as acceptor substrate for trans-sialidase was determined in the presence of 600 µM serum-bound Sia (Table 2, Figure S3a-d). In summary, TconTS1 and TconTS2 have higher specific activities than TconTS3 and TconTS4. TconTS1 and TconTS2 have lower K_M values for the acceptor substrate lactose, whereas TconTS3 and TconTS4 have lower K_M for serum-bound Sia.

Kinetic parameters were also established for sialidase activities by incubating reactions at standard conditions without lactose (Table 1, Figure S4a-c). Since 250 ng TconTS3 did not show sialidase activity within a 24 h incubation, no further reaction was set up for this enzyme. TconTS1 had the lowest K_M (0.16 mM) followed by TconTS2 (0.41 mM). TconTS4 had the highest K_M (1.28 mM) but lowest free Sia release from serum-bound Sia. Free Sia release by TconTS4 is about 50- and 80-fold less than that released by TconTS1 and TconTS2, respectively.

TconTS show poor catalytic activities on synthetic donor substrates

(Tiralongo et al. 2003b) reported higher specific activities for synthetic substrates by two TconTS forms purified from procyclic *T. congolense* cultures. One of those preparations was co-purified with Glutamate and Alanine Rich Protein (GARP), the natural substrate for TconTS. Expressed as

recombinant TconTS1 variants in eukaryotic fibroblasts, lower specific activities on synthetic substrates were reported for TconTS1 variants, which were equally unable to release Neu5Ac from Neu5Ac-MU (Koliwer-Brandl et al. 2011). While GARP might have played a role in the higher specific activities observed by Tiralongo et al. (2003a) evidence was provided (Koliwer-Brandl et al. 2011, Gbem et al. 2013) that other TconTS enzymes were probably present in the TconTS forms purified from polycyclic cultures.

We tested individual recombinant TconTS2, TconTS3 and TconTS4 on different synthetic substrates. Like TconTS1 (Koliwer-Brandl et al. 2011), TconTS2, TconTS3 and TconTS4 enzymes were poor at utilising synthetic donor substrates (results not shown). Neu5Ac-MU has been widely used in establishing TconTS enzyme activities (Schrader et al. 2003, Schrader et al. 2006). Apart from the activities of TconTS enzymes of polycyclic origin (Engstler et al. 1993, Engstler et al. 1995, Tiralongo et al. 2003b, Nok and Balogun 2003, Coustou et al. 2012) reported activities of TS/sialidases from blood of *T. congolense* infected animals on Neu5Ac-MU. However, for recombinant TconTS1, TconTS2, TconTS3 and TconTS4 hardly any TS or sialidase activities on Neu5AcMU were detected, even after 24 h incubation (Koliwer-Brandl et al. 2011, Gbem et al. 2013). Bearing in mind that the procyclic TconTS forms most likely contained other TconTS forms, we tested different combinations of TconTS gene products on Neu5Ac-MU. Interestingly, a mixture of TconTS1 and that of TconTS2 with any other TconTS transferred Sia from Neu5Ac-MU, while TconTS3 mixed with TconTS4 did not. No free Sia was detected in the reactions (Figure 3).

Serum presents different donor and acceptor substrates possibilities for TconTS enzymes

We had earlier established that among the TconTS enzymes, TconTS1 and TconTS2 have the highest specific activities on the donor blood glycoprotein fetuin and lactose as acceptor (Gbem et al. 2013). We speculated on the basis of that finding the possibility of substrate selectivity detecting differences in specific activities of the enzymes; for example, TconTS3 and TconTS4 exhibiting higher specific activities on other donor substrates, possibly blood glycoproteins and/or glycolipids. The HPAEC-PAD methods used in quantification of products made it possible to quantify different sialylated as well as desialylated products (shown by reduced peak areas) from chromatograms of single reactions. If TconTS3 was used as enzyme with human serum as substrate, at least one additional product was observed, which was eluted after 3'SL. Quantification of this unknown product (t_R of 8 mins) revealed higher peak areas when compared with the 3'SL peak (Figure 4a). Interestingly, when serum-bound Sia was varied and lactose kept constant, the unidentified product was favoured at low serum-Sia concentrations, whereas 3'SL was formed with higher priority at high serum-Sia concentrations (Figure 4b). Control serum samples usually contained inherent compound peaks that are detected by the HPAEC-PAD method used. We observed that when TconTS2 was used, three of these inherent peaks were desialylated (Figure 5a). No desialylated

peak was observed for TconTS1 (Figure 5b) as well as for TconTS3 and TconTS4 (not shown). This indicates a preference for different donor substrates in serum between TconTS2 and the other TconTS enzymes.

TconTS activities exhibit different pH optima

The pH in the midgut of the tsetse vector is between 9 and 10 (Dyer et al. 2013). In the mammal, the parasite encounters a pH of 7.4. pH optimum may give a likely indication of which TconTS gene product operate better in the different host systems. Using fetuin as a model blood glycoprotein donor and lactose as acceptor substrates, we measured enzyme activities at different pH as indicated (Figure 6a-d). Extended incubation periods were required to asses hydrolytic activities for TconTS1 and TconTS2 given their poor sialidase activities compared to the high TS activities (Gbem et al. 2013). Therefore TS activities were run for 30 minutes, while sialidase activities without the acceptor lactose, incubated for 24h. Whereas TconTS1, TconTS3 and TconTS4 had pH optima at around 7 and only low TS activities between pH 9 to 10, the transfer activity of TconTS2 was basically pH-independent between pH 6 and 10. Interestingly, while the TS activity of TconTS4 is highest at pH 7, its sialidase activity has its optimum at pH 9.

Discussion

Two forms of TconTS enzymes purified from polycyclic *T. congolense* cultures showed higher specific activities on synthetic substrates (Tiralongo et al. 2003b) when compared to recombinant TconTS proteins that were expressed in fibroblasts (Koliwer-Brandl et al. 2011). Convincing evidence was provided indicating that the purified proteins from *T. congolense* procyclic cultures possibly contained products from other TconTS genes (Koliwer-Brandl et al. 2011, Gbem et al. 2013). The current study using recombinant proteins showed lack and/or poor activity on synthetic substrates (not shown). We observed that individually TconTS recombinant proteins do not cleave Neu5Ac-MU, the commonly employed substrate in sialidase and trans-sialidase assays. Cleavage of Neu5Ac-MU has been reported in assays involving these enzymes (Engstler et al. 1995, Tiralongo et al. 2003b, Nok and Balogun 2003, Schrader et al. 2003, Schrader et al. 2006, Coustou et al. 2012). These assays were either done with TconTS purified from polycyclic cultures or using blood/serum of infected animals. A mixture of either TconTS1 or TconTS2 with other TconTS resulted in Neu5Ac transfer to acceptor lactose (Figure 3) resulting in formation of 3'SL. This would point to the availability of other forms of TconTS enzymes in the previous reports. More importantly, it illustrates the synergistic effects of these enzymes and an enhanced virulence in infected animals where more than one enzyme is expressed. Secondly, it presents the possibility that when expressed in combination, the repertoire of potential donor substrates available to the parasite is increased possibly supporting a better survival in the host system. cDNA and Western blots analysis on tissues and blood of infected insects and goats respectively have confirmed the expression of more than one TconTS gene (Gbem et al. 2013).

Amino acid composition in the catalytic pockets of TonTS enzymes were reported in the partial sequences of TconTS form 1 and form 2 (Tiralongo et al. 2003a, Tiralongo et al. 2003b) and in TconTS1 variants (Koliwer-Brandl et al. 2011). Recently we reported on the amino acid composition in the catalytic pockets of active TconTS genes; TconTS2, TconTS3 and TconTS4 (Gbem et al. 2013). When TconTS ORFs were aligned with the reference *T. cruzi* TS (Amaya et al. 2004), two residues at consensus positions 293 and 494 were found substituted in TconTS1 and TconTS2, the most active TconTS genes. These residues are conserved in TconTS3 and TconTS4, the TconTS enzymes with low specific activities (Gbem et al. 2013). Structural and catalysis studies (Amaya et al. 2004) showed the residues in question to be essential in interacting with the methylumbelliferyl aglycon or the lactose part of 3'SL. Interactions of the enzymes may lead to formation of heterodimers or heterooligomers making it possible for Neu5Ac-MU to become a substrate. It is possible that other unidentified structural features of the proteins may be responsible for the differences in activity. It has been suggested recently that the lectin domain may play a more significant role in TS activities than previously thought (Gbem et al. 2013). It is also possible that the activity of TconTS enzymes on Neu5Ac-MU may be enhanced by suitable acceptor substrate since TS form 1 purified from polycyclic cultures contained GARP (Tiralongo et al. 2003b). In this vain, it would be interesting to see the effect of individual TconTS gene products on Neu5Ac-MU in the presence of GARP.

In previous studies (Koliwer-Brandl et al. 2011, Gbem et al. 2013), the enzymatic activities of four TconTS gene products using fetuin as a model blood glycoprotein donor substrate were compared. While the use of fetuin as a substrate provided important insights into the biochemical diversity of these enzymes, it was considered nevertheless important to look at a natural medium in which these enzymes operate. For this purpose, serum was employed. Specific activity patterns are similar for both fetuin (Gbem et al. 2013) and serum glycoconjugates in presence of lactose as common acceptor. TconTS2 showed the highest specific activity, followed by TconTS1, TconTS3 and TconTS4 in that order (Table 2, Figure S2 and S3). Except for TconTS4, 3'SL production for the remaining TconTS enzymes is at least 10-fold higher in reactions involving fetuin-bound Sia (Koliwer-Brandl et al. 2011, Gbem et al. 2013) than those in which serum glycoconjugates is the donor substrate. The v_{max} obtained for TconTS1 and TconTS2 from assays with varying lactose concentrations are lower than the specific activities determined by varying the donor substrate, since for technical reasons the donor substrate concentration was non-saturating (0.3- and 0.8-fold of the K_M values for the serum-bound Sia for TconTS1 and TconTS2, respectively).

TconTS1 and TconTS2 have the highest specific activities of the TconTS genes on the substrates considered so far. This however does not preclude the possibility of TconTS3 and TconTS4 being more active on other substrates. Generally, TconTS genes showed lower transfer activities on serum samples (Table 2) when compared to those obtained for fetuin (Gbem et al. 2013), both using as common acceptor. Serum contains a wide array of glycoproteins and/or glycolipids with

different Sia types (Bulai et al. 2002) in addition to other components (Psychogios et al. 2011). The possibility of a complex pattern of sialylation and desialylation is therefore enhanced as well as a more diverse source of Sia. TconTS2 desialylated peaks that are not desialylated by other TconTS enzymes (Figure 5), yet there is 3'SL formation in TconTS1, TconTS3 and TconTS4. This indicates different Sia source from those utilized by TconTS2. Peak desialylation was already pronounced after a 2-hour incubation of serum and lactose with TconTS2 (Figure 5b). Interestingly, no free Sia was detected in the TconTS2 reaction medium up to 8 h (Figure 2c) indicating an efficient Sia transfer to lactose and possibly other acceptors present in serum.

TconTS3 showed an additional product peak (Figure 4a), in addition to the 3'SL peak. The formation of this additional product ,eluting at 8 minutes was favoured. This collaborates our earlier postulations on activities differing on the basis of substrates. Further, when lactose concentration was kept constant and Sia concentration varied, 3'SL formation was only favoured at higher Sia concentration compared to the additional product peak whose formation was favoured at lower serum-Sia concentrations (Figure 4b). It is possible that TconTS3 transfers Sia to different acceptors under differing Sia concentrations and may be employed at different stages of infection in the mammalian life stage; early disease state when Sia concentration is high and late disease stage or advanced anaemia when Sia availability might be low.

TS from *T. cruzi* (Agusti et al. 2007) and *T. congolense* (Engstler et al. 1995) have been shown to transfer hydroxylated sialic acids, *N*-glycolylneuraminic acid, Neu5Gc on to surface acceptors. While Neu5Gc is widely distributed in the animal kingdom, it is absent in the normal tissues of humans (Varki, 2001, Angata and Varki 2002, Sprenger and Duncan, 2012). TconTS transfer both Neu5Ac and Neu5Gc with similar efficiencies (Engstler et al. 1995).

TconTS3 did not show sialidase activity on human serum glycoconjugates. TconTS1 and TconTS2 showed highest specific activities with similar magnitude of Sia release from both substrates. The K_M values for fetuin-bound Sia were similar for TconTS1 and TconTS2, (Table 1). Gbem et al. (2011) reported that in absence of lactose, Sia cleavage from fetuin by TconTS1 and TconTS2 could only be observed at high enzyme concentrations and extended incubation periods with free Sia detected only when 3'SL formation attained 300 µM and 600 µM for TconTS1 and TconTS2, respectively. Similarly, in presence of lactose, free Sia was detected in serum incubated with TconTS1 and TconTS2 only after 3'SL accumulated in the reaction medium and at high enzyme concentrations (Figure 2a-d). However, in absence of an external acceptor, e.g. lactose, sialidase activity for TconTS1 and TconTS2 increased (Table 5). This raises the question of the high amounts of Sia in the blood and serum (Nok and Balogun 2003, Coustou et al. 2012) of animals suffering anaemia from *T. congolense* infections. Koliwer-Brandl et al. (2011) found only partial resialylataion of asialofetuin by TconTS1 variants. Erythrocytes and serum components bearing Sia have evolved to be naturally glycosylated via cellular processing of Sia. These cells and mammalian gylcoconjugates may be poor acceptors of Sias after they have been desialylated. Once parasites

surfaces are sialylated, TS may continue to remove Sia but within an environment of poor acceptors, transfer becomes low.

Trypanosoma are digenetic parasites with life cycles that alternate between two different host systems; the tsetse vector and the mammalian host. Profound differences exist between these two systems, not least the pH as sialidases have rather acid pH optima and are inactive at pH between 9 and 10 (Reuter et al. 1987). The pH at which each enzyme show highest activity was established for TconTS enzymes. Considering the pH optimum for TconTS enzymes, TconTS2 can be expected to transfer Sia efficiently in the fly midgut. It would be interesting to see the impact of a TconTS2 whose activity has been genetically modified to act as a sialidase in the gut of tsetse carrying *Trpanosoma* parasites. Interestingly, the sialidase activity of TconTS4 has a pH optimum around pH 9, in contrast to that for TS activity at pH 7. Nagana is accompanied by severe anaemia, which has been linked to trypanosomal sialidase (Nok and Balogoun 2003). We found that TconTS1 and TconTS3 did not loose their activity even after four weeks at 37ºC (Gbem et al. 2013). As TconS1 activity tends to persist over long periods, this low activity could be compensated over time. This could explain why anaemia persists even at low levels of parasitaemia.

In conclusion, serum glycoconjugates serve as good sources with variable types of Sia, which could be transferred to the *Trypanosoma* parasite surfaces. Interestingly, when lactose acceptor was absented from reaction medium, free Sia accumulated.

Author contribution

TTG purified TconTS proteins, ran TS/sialidase reactions, carried out HPAEC-PAD analysis of all reaction products on both fetuin-bound and serum-bound Sia, analysed data and drafted the manuscript. MW conducted part of experiments on fetuin-bound Sia. JAN supported experimental design and helped with part of HPAEC-PAD data analysis. SK designed, coordinated the study and supported drafting of manuscript.

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Figure legends

Figure 1. Concentration and time dependency of Sia release from fetuin. TconTS enzymes were incubated at 37ºC with 600 µM fetuin-bound Sia. Free Neu5Ac indicating sialidase activity was determined by HPAEC-PAD. **A:** 200 ng enzyme was used for both TconTS1 and TconTS2 while 250 ng enzymes were used for TconTS3 and TconTS4 and incubated at 37ºC over indicated times and 24 h values are given in Table 3. **B:** Varying amounts of TconTS enzymes incubated for 24 h. In all cases, values are means ± standard deviations of at least 3 independent experiments replicated trice.

Figure 2. Concentration and time dependency of TconTS enzymes on human serum glycoconjugates. For concentration dependencies, indicated amounts of recombinant TconTS proteins were incubated with 600 μM serum-bound Sia in presence and absence of 2 mM lactose for 24 h. In the time dependency studies, 200 ng each of TconTS1 and TconTS2, and 250 ng each of TconTS3 and TconTS4 were incubated at 37 °C for the indicated times. Detection of 3'SL represents transfer activity while the presence of Neu5Ac represents sialidase activity. Both were determined using HPAEC-PAD. **A-D**: TconTS1 and TconTS2. **E-H**: TconTS3 and TconTS4. Data points are means ± standard deviations of at least 3 independent experiments, each as triplicates.

Figure 3. TconTS activity on Neu5Ac-MU. TS reactions were setup with 1 mM Neu5Ac-MU and 2 mM lactose as donor and acceptor substrates, respectively, and incubated either as single enzymes or as a mixture (250 ng of each enzyme) and incubated for 24 h at 37 °C as indicated . Neu5Ac and 3'SL was measured using HPAEC-PAD . Bars represent means ± standard deviations of triplicates of 3 independent experiments.

Figure 4. Additional product peak by TconTS3. An additional product peak that elutes at 8 is formed in addition to 3'SL by TconTS3 when incubated with serum-bound Sia in presence of lactose. 250 ng recombinant TconTS3 protein was used against a fixed concentration of one substrate and reactions incubated for 720 mins. **A**: Lactose variation against 600 μM serum-bound Sia. **B**: Serum-bound Sia variation against 2 mM lactose. In all cases, HPAEC-PAD was employed in quantification as described in the Methods section. Data points are means ± standard deviations of at least 3 independent experiments, each as triplicates.

Figure 5. Effects of TconTS1 and tconTS2 on human serum glycoconjugates. 200 ng TconTS1 and TconTS2 protein were incubated with 600 μM serum-bound in presence of 2 mM lactose Sia for 24 h. Letters **a**, **b**, **c** and **d** represent unidentified glycoconjugates peaks while GlcAc represent glucoronic acid, used as internal standard. **A**: TconTS2 showing desialylated peaks. **B**: TconTS1 showing 3'SL formation without desialylation of peaks. Colours represent different incubation times: black, blue, pink, brown and green indicate chromatograms of products obtained after 0, 4, 8, 12, 16 and 24 hours respectively.

Figure 6. pH optima for TconTS enzymes. Reactions to determine pH optima were carried out at indicated pH points as described under Methods using 100 μg fetuin (600 μM fetuin-bound Sia) in presence and absence of lactose. **A**: TconTS1 and **B**: TconTS2: 50 ng TconTS incubated for 30 min to measure transfer activity indicated by 3'SL formation, while 250 ng TconTS incubated with 600 μM fetuin-bound Sia in absence of lactose for 24 hours. **C**: TconTS3 and **D**: TconTS4: 250 ng TconTS incubated for 24 hours in presence and absence of lactose for 1440 mins. All other experimental conditions are as stated in methods. Neu5Ac and 3'SL were measured using HPAEC-PAD as described under Methods. Data points are means ± standard deviations of 3 independent experiments, each as triplicates.

Legends to Supporting Information

Figure S1. Sialidase reaction velocities depending on fetuin-bound Sia concentration. Neu5Ac amounts were determined as described in Methods. v_{max} and K_M shown in Table 1 were calculated from these set of data. **A**: 200 ng TconTS1 and **B**: 200ng TconTS2, both incubated for 2 h with varied fetuin-bound Sia up to 200 mM. **C**: 250 ng TconTS3 incubated for 12 h with varied fetuin-bound Sia up to 200 mM, and **D**: 500ng TconTS4 incubated for 24h with varied fetuin-bound Sia up to 1500 mM. Data points are means ± standard deviations of 3 independent experiments, each as triplicates.

Figure S2. Trans-sialidase reaction velocities depending on serum glycoconjugates concentrations. 3'SL amounts were determined as described in Methods. v_{max} and K_M as shown in Table 2 were calculated from these set of data. **A**: 200 ng TconTS1 incubated with 2 mM lactose and varying serum-bound glycoconjugates for 2 hours. **B**: 200ng TconTS2 incubated with 2 mM lactose and varying serum-bound glycoconjugates for 1 hour. **C**. 250 ng TconTS3 incubated with 2 mM lactose and varying serum-bound glycoconjugates for 12 hours. **D**: 250 ng TconTS4 incubated with 2 mM lactose and varying serum-bound glycoconjugates for 24 hr. Data points are means ± standard deviations of 3 independent experiments, each as triplicates.

Figure S3. Trans-sialidase reaction velocities depending on lactose concentrations. 3'sialyl-lactose amounts were determined as described in methods. v_{max} and K_M as shown in Table 2 were calculated from these set of data. **A**: 200ng TconTS1 was incubated with 600 µM serum glycoconjugates and varying amounts of lactose as indicated for 2 hour. **B**: 200 ng TconTS2 was incubated with 600 µM serum glycoconjugates and varying concentrations of lactose as indicated for 1 hour. **C**: 250 ng TconTS3 was incubated with 600 µM serum glycoconjugates and varying concentrations of lactose as indicated for 12 hours. **D**: 250 ng TconTS4 was incubated with 600 µM serum glycoconjugates and varying concentrations of lactose as indicated for 24 hours. Data points are means ± standard deviations of at least 3 independent experiments, each as triplicates.

Figure S4. Sialidase reaction velocities depending on serum glycoconjugates concentrations. Neu5Ac amounts were determined as described in methods. v_{max} and K_M shown in Table 1 were calculated from these set of data. **A**: 200 ng TconTS1 and **B**: 200ng TconTS2, both incubated for 2 and 1 hour, respectively, with varied serum glycoconjugates concentration without the acceptor lactose. **C**: 250 ng TconTS4 incubated for 24 hour with varied concentrations of serum-bound Sia. Data points are means ± standard deviations of 3 independent experiments, each as triplicates.

Table 1. Kinetic parameters for sialidase activities of TconTS enzymes on fetuin- and serum-bound Sia

TconTS	Fetuin-bound Sia*		Serum-bound Sia*	
	v_{max}** (μmol/(min x mg TS))	K_M** (μM)	v_{max}** (μmol/(min x mg TS))	K_M** (μM)
TconTS1f	85±1.6	20±1.5	63±0.6	155±7.1
TconTS2	99±1.7	30±1.7	105±3.5	412±40
TconTS3	3.2±0.6	137±7.5	-	-
TconTS4	8.1±0.3	441±16	1.3±0.0	1281±146

* 600μM was used as substrate for both fetuin-bound Sia and serum-bound Sia.

**K_M and v_{max} were calculated from Michaelis-Menten kinetics (Figure S1 and S4) by SigmaPlot. Data points are mean ± standard deviations of three independent experiments, each replicated thrice

-= no hydrolytic activity on serum

Table 2. Kinetic parameters of TconTS on sialic acid transfer from serum-bound Sia

	Transfer			
TconTS	Acceptor (lactose)*		Donor substrate (serum-bound Sia)**	
	v_{max}*** (nmol/(min x mg TS))	K_M*** (μM)	v_{max}*** (nmol/(min x mg TS))	K_M*** (μM)
TconTS1f	181±1.5	203±16	315±16	669±83
TconTS2	486±9.3	79±7.1	1005±60	750±104
TconTS3	4.2±1.0	1165±91	5.1±0.1	339±25
TconTS4	2.2±0.1	1321±156	3.2±0.1	259±26

* 600μM serum-bound Sia was used as donor substrate.
**2mM lactose was used as acceptor substrate.
***K_M and v_{max} were calculated from Michaelis-Menten kinetics (Figure S2 and S3) by SigmaPlot.
Data points are mean ± standard deviations of three independent experiments, each as triplicates.

Table 3. Free Sia release from fetuin-bound Sia in absence of lactose

Parameters	TconTS1		TconTS2		TconTS3		TconTS4	
Amount of TconTS used (ng)	200	1000	200	1000	250	1000	250	1000
Sia release (pmol/min., initial velocity)	1.7±0.017		2.2±0.07		0.75±0.004		1.25±0.002	
Total free Sia (nmol) in 24 h*	2.0±0.0.01	12±0.58	2.5±0.03	12±0.62	0.6±0.009	4.2±0.16	1.1±0.00	6.5±0.08

* 30 nmol fetuin-bound Sia (600 μM Sia) was incubated with indicated amounts of various TconTS enzymes

Table 4. 3'SL formation and free sialic acid release from serum-bound Sia by TconTS enzymes

Parameters	TconTS1		TconTS2		TconTS3		TconTS4	
Amount of TconTS used (ng)	200	1000	200	1000	250	1000	250	1000
3'SL formation (pmol/min., initial velocity)*	42±2.5		75±4.32		0.83±0.01		4.0±0.06	
3'SL produced (nmol) in 24 h*	6.0±0.11	6.0±0.23	8.0±0.04	8.0±0.12	0.65±0.004	1.0±0.004	0.8±0.01	1.1±0.001
Free Sia (nmol) in presence of lactose in 24 h	1.0±0.001	1.5±0.01	0.25±0.01	0.25±0.01	n.d.**	n.d.**	0.1±0.00	0.21±0.00
Free Sia (nmol) in absence of lactose in 24 h***	6.5±0.01	6.5±0.01	5.0±0.09	5.0±0.11	n.d.**	0.3±0.02	0.15±0.001	0.45±0.004

* 600 μM serum-bound Sia was used as donor substrate in presence of 2 mM lactose as acceptor
** not detected, below 0.1 nmol
*** 600 μM serum-bound Sia was used as substrate in absence of acceptor

Table 5. Relationship between sialidase and trans-sialidase activities of TconTS enzymes on serum glycoconjugates

TconTS	Trans-sialidase* (nmol/(min x mg TS)	Sialidase (nmol/(min x mg TS)		TS/sialidase	
		- lactose**	+ lactose***	- lactose****	+ lactose
TconTS1f	315±16	63±0.6	1.04±0.001	5	303
TconTS2	1005±60	105±3.5	0.17±0.0	10	5912
TconTS3	5.1±0.1	n.d.*****	n.d.*****	>5	>5
TconTS4	3.2±0.1	1.3±0.0	0.31±0.0001	2.5	10

* TS activity values are given in Table 1

** Sialidase activity values in absence of lactose are given in Table 1

*** Sialidase activities in presence of 2 mM lactose were determined by incubating 1000 ng of indicated TconTS enzymes for 24 h with 600 µM serum-bound Sia. Data points are mean ± standard deviations of 3 independent reactions, each in 3 replicates.

**** sialidase activity values in absence of lactose acceptor.

*****n.d. not determined, below 0.1 nmol Sia

Figure 1

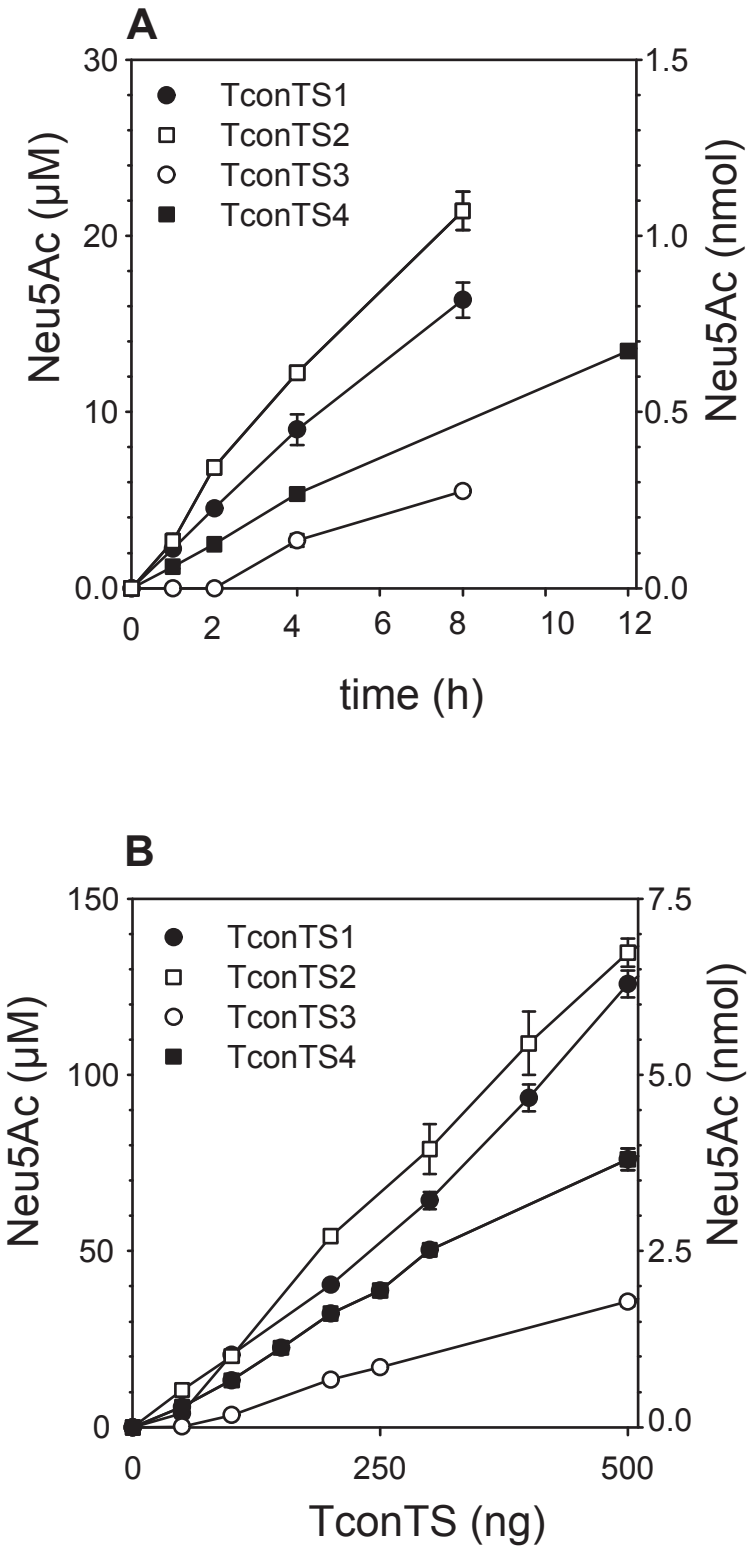

A
30
20
10
0.0
1.5
1.0
0.5
0.0
TconTS1
TconTS2
TconTS3
TconTS4
Neu5Ac (μM)
Neu5Ac (nmol)
0
2
4
6
8
10
12
time (h)
B
150
100
50
0
7.5
5.0
2.5
0.0
TconTS1
TconTS2
TconTS3
TconTS4
Neu5Ac (μM)
Neu5Ac (nmol)
0
250
500
TconTS (ng)

Figure 2

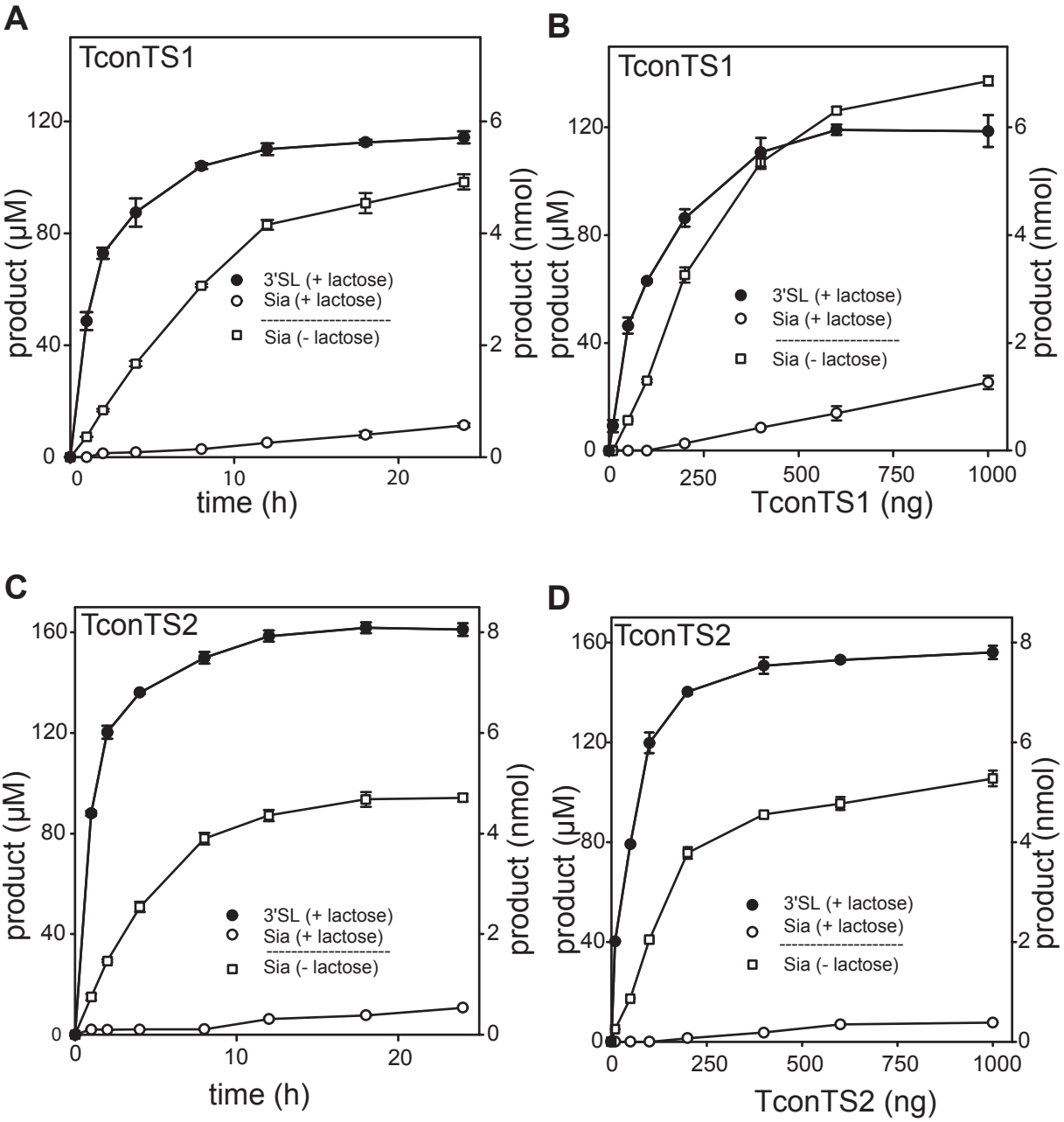

A
TconTS1
product (µM)
product (nmol)
time (h)
3'SL (+ lactose)
Sia (+ lactose)
Sia (- lactose)
B
TconTS1
TconTS1 (ng)
C
TconTS2
D
TconTS2
TconTS2 (ng)

Figure 2

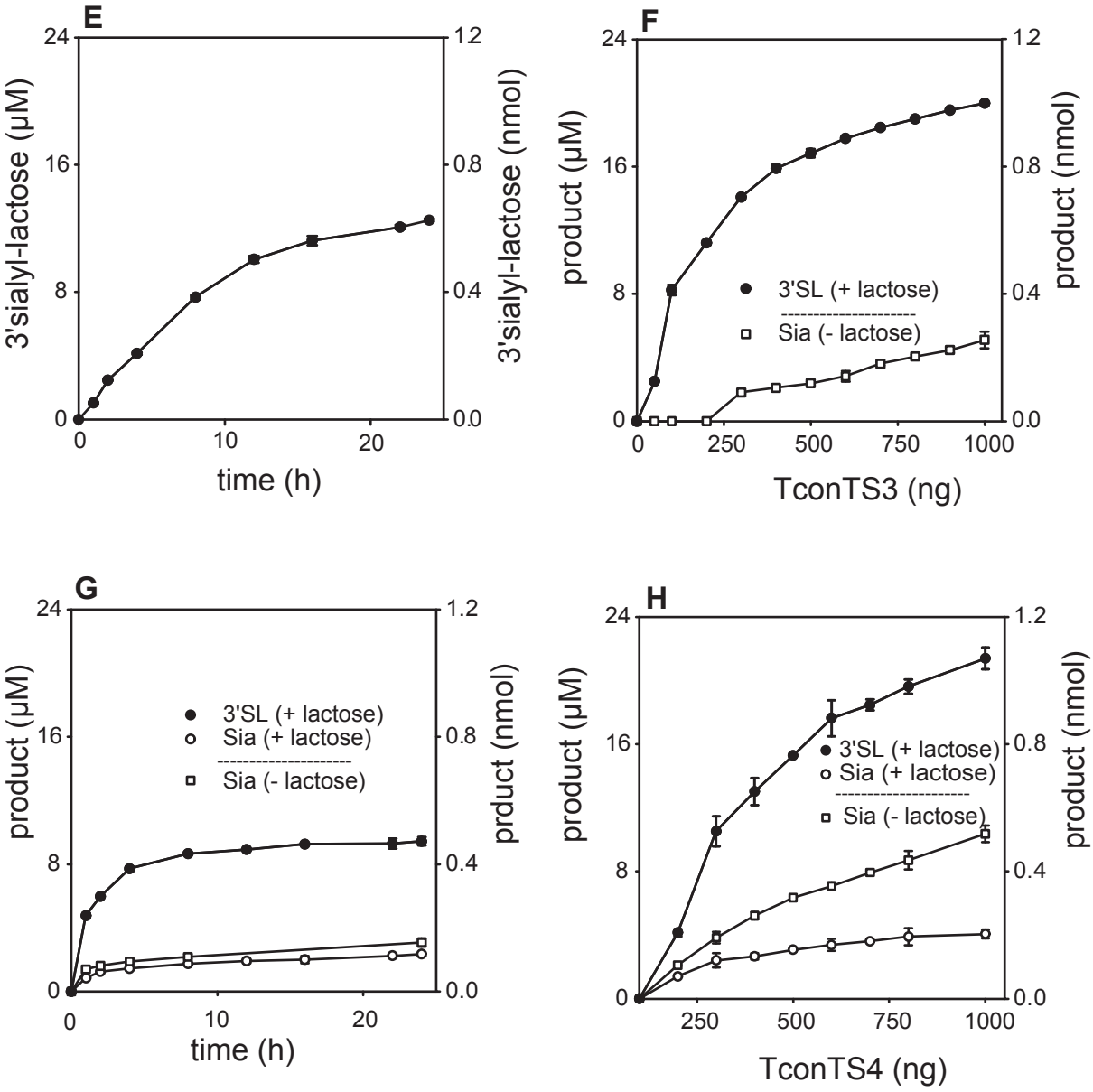

E
3'sialyl-lactose (μM)
3'sialyl-lactose (nmol)
time (h)
F
product (μM)
product (nmol)
3'SL (+ lactose)
Sia (- lactose)
TconTS3 (ng)
G
product (μM)
prduct (nmol)
3'SL (+ lactose)
Sia (+ lactose)
Sia (- lactose)
time (h)
H
product (μM)
product (nmol)
3'SL (+ lactose)
Sia (+ lactose)
Sia (- lactose)
TconTS4 (ng)

Figure 3

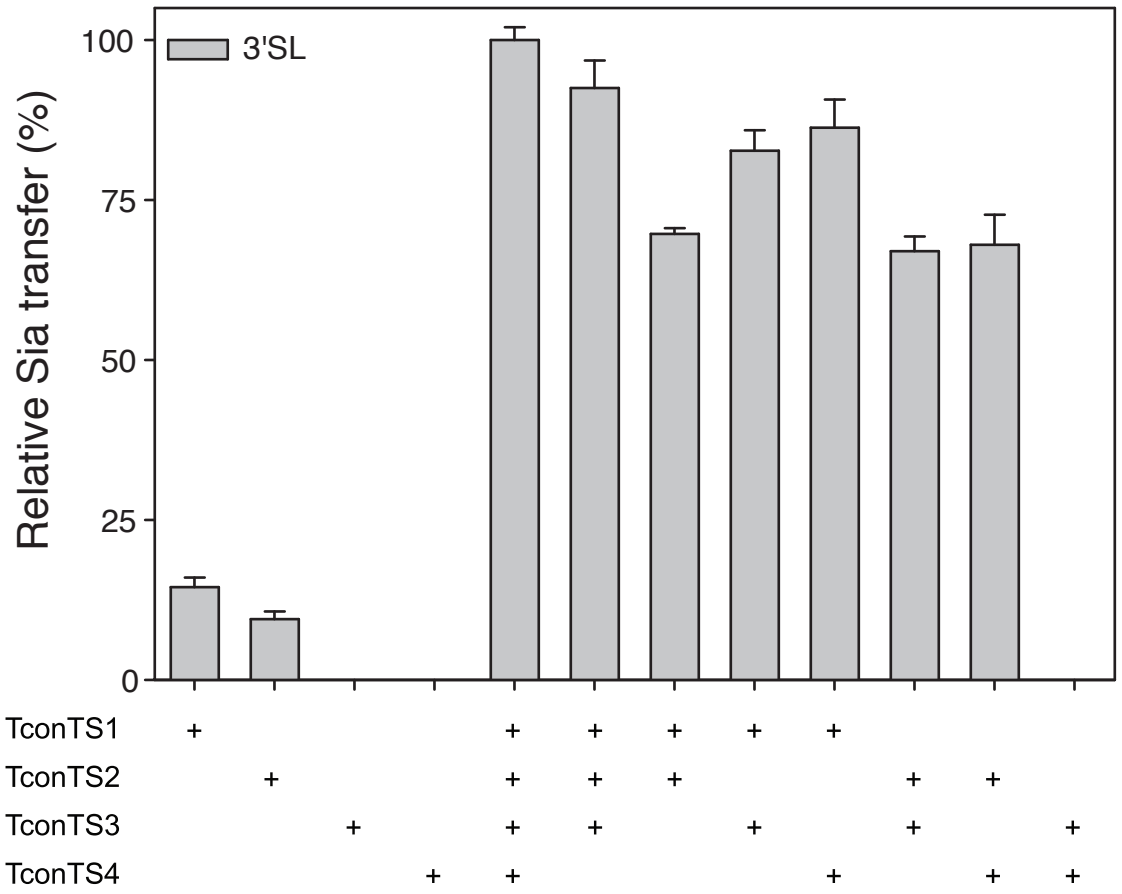

3'SL
Relative Sia transfer (%)
100
75
50
25
0
TconTS1
TconTS2
TconTS3
TconTS4

Figure 4

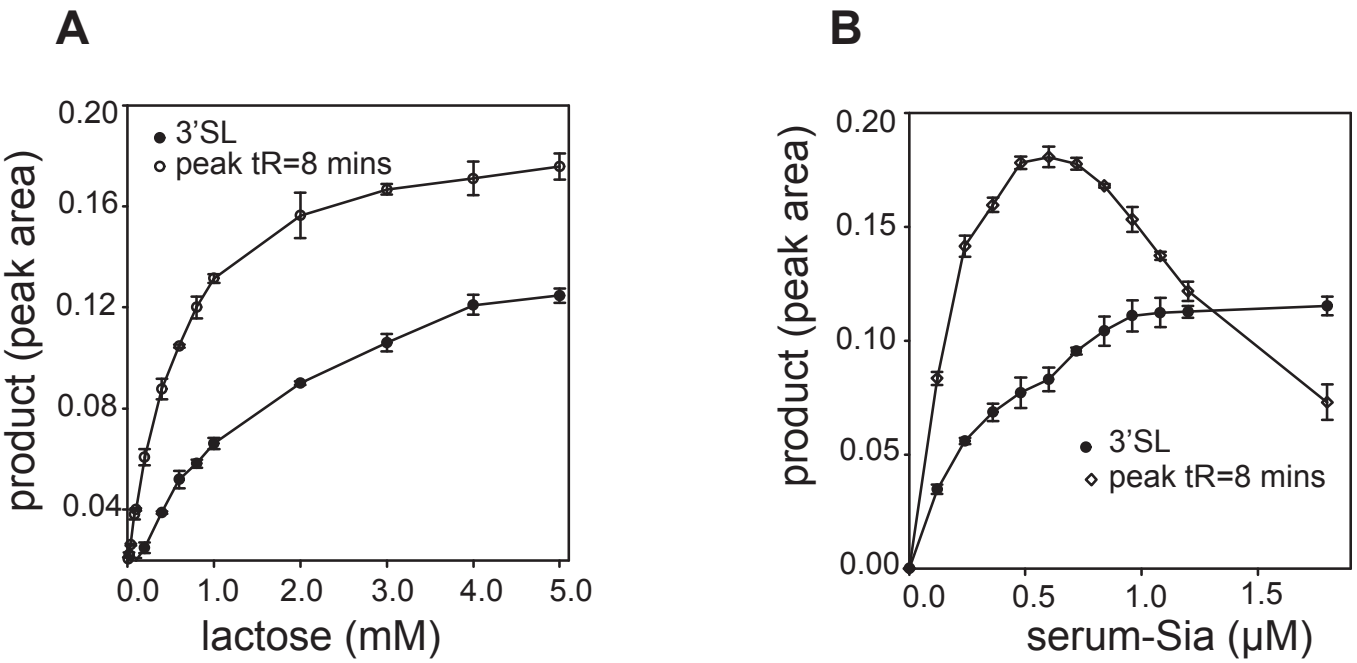
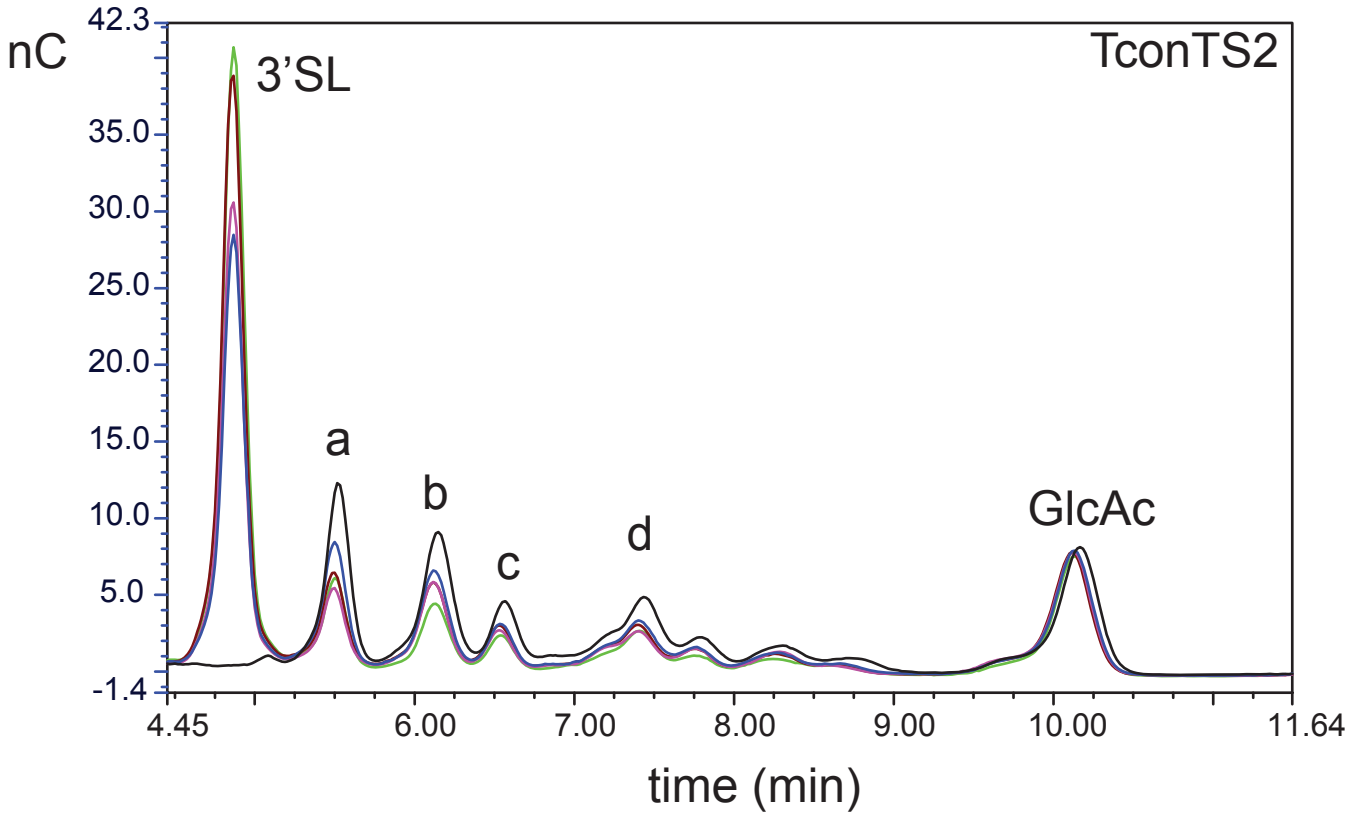

A
B
product (peak area)
lactose (mM)
serum-Sia (μM)
3'SL
peak tR=8 mins
0.20
0.16
0.12
0.08
0.04
0.0
1.0
2.0
3.0
4.0
5.0
0.15
0.10
0.05
0.00
0.5
1.5

Figure 5

A


nC
42.3
35.0
30.0
25.0
20.0
15.0
10.0
5.0
-1.4
3'SL
TconTS2
a
b
c
d
GlcAc
4.45
6.00
7.00
8.00
9.00
10.00
11.64
time (min)

B

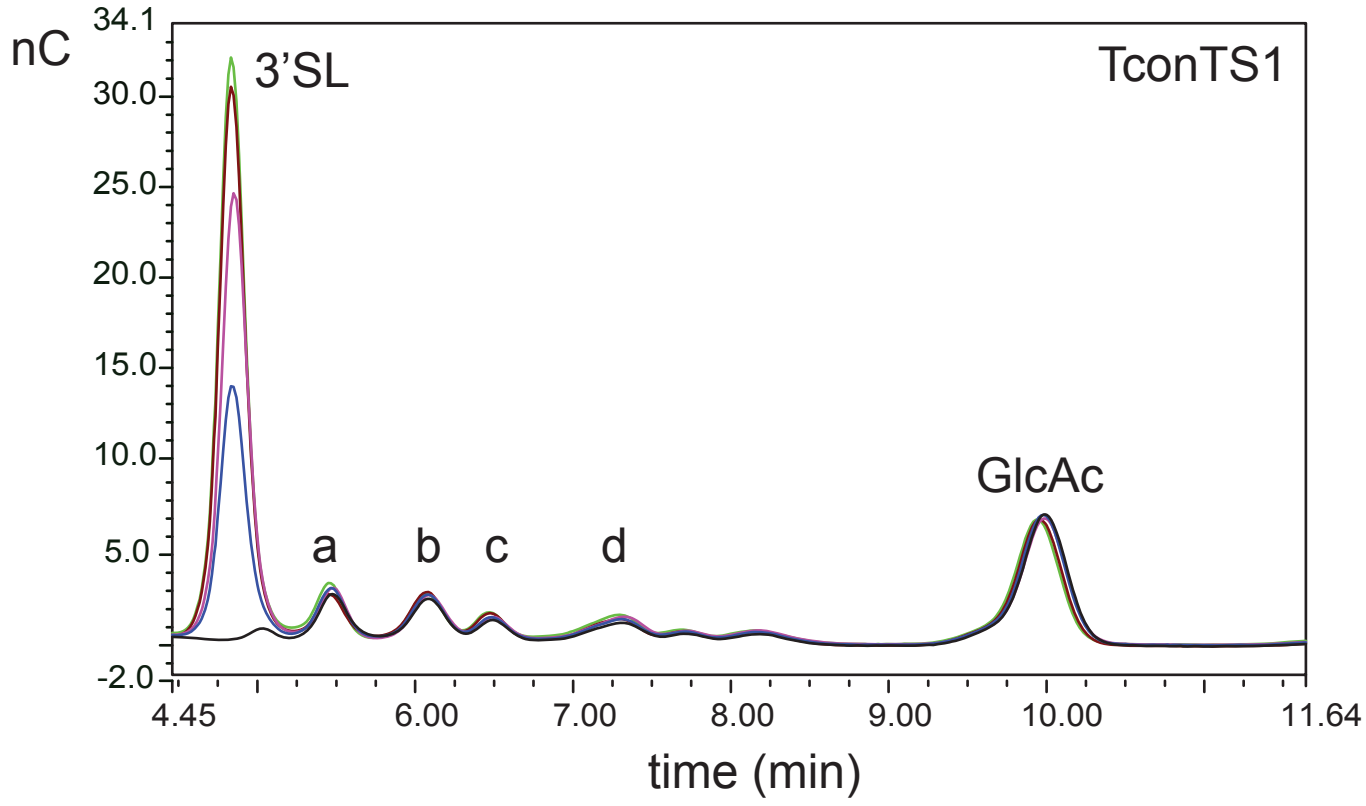
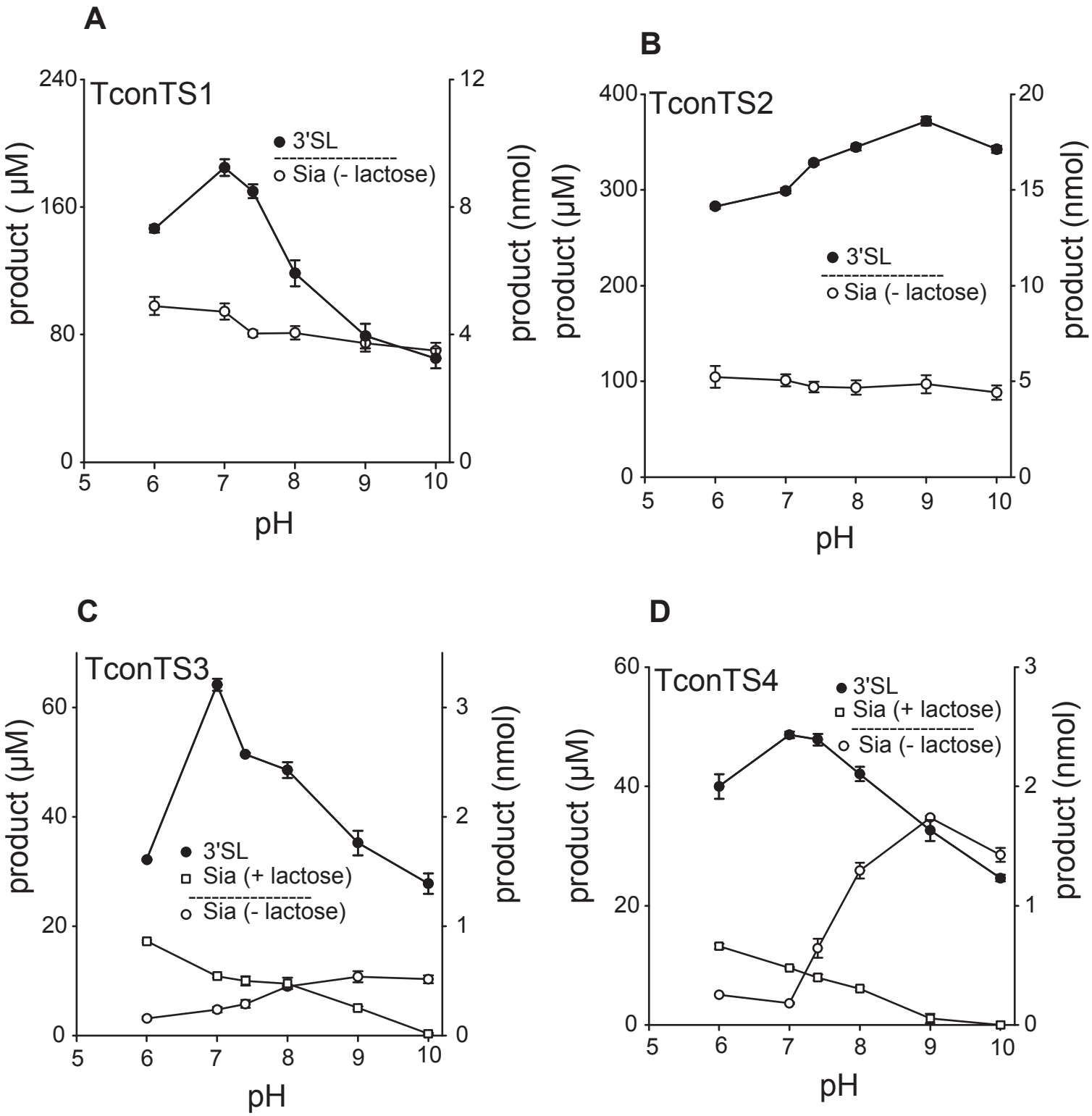

nC
34.1
30.0
25.0
20.0
15.0
10.0
5.0
-2.0
3'SL
TconTS1
a
b
c
d
GlcAc
4.45
6.00
7.00
8.00
9.00
10.00
11.64
time (min)

Figure 6


A
TconTS1
3'SL
Sia (- lactose)
product (µM)
product (nmol)
pH
B
TconTS2
3'SL
Sia (- lactose)
product (µM)
product (nmol)
pH
C
TconTS3
3'SL
Sia (+ lactose)
Sia (- lactose)
product (µM)
product (nmol)
pH
D
TconTS4
3'SL
Sia (+ lactose)
Sia (- lactose)
product (µM)
product (nmol)
pH

5.0.

Summarising Discussion

5.0 General

5.1 TconTS genes from African trypanosomes

5.2 Amino acid residue variations in TS

5.3 Substrates could led to variation in enzyme activity

5.4 TS as target in the fight against AAT

5.5 References

5.0. General

Trans-sialidases (TS) are expressed by parasitic trypanosomes and this special group of enzymes transfer Sialic acids (Sia) in a regio- and stereo-specific manner resulting in α2-3 linkages to underlying Gal or GalNAc. In this work, TS gene products from the animal African parasite, *Trypanosoma congolense* were biochemically characterised. Three distantly related genes with less than 40% similarity to the ones whose products are active were also identified form data bases. Gene products from this group are presumably inactive.

TS was first described in *T. cruzi* and implicated in the pathology of the bloodstream form of the same parasite (Schenkman et al. 1991). It was subsequently described in the procyclic insect forms of the African trypanosomes (Engstler et al. 1993), where it was for long thought to be only stage specifically expressed. In the African trypanosomes, TS has also been linked to the pathogenesis of trypanosomosis. First, it has been shown to be required for establishment of infection in the fly midgut (Nagamune et al. 2004) as transgenic parasites without the ability to express TS showed reduced survival and colonisation rates in the *Glossina* midgut. Of critical importance to survival of the parasite and disease is the establishment of infection in the midgut as fly innate immune responses clear most infections in fly gut (Hu and Aksoy 2006). Recent evidences showed the expression of TconTS4 (TcoTS-D2) and TconTS-Like 2 (TcoTS-like2) genes by the BSFs of infected mice (Coustou et al. 2012). Studies on T*rypanosoma congolense* (Nok and Balogun 2003, Coustou et al. 2012) and *T. vivax* (Guegan et al. 2013) have implicated TS as virulence factors in anaemia in animals suffering those infections. Of note is the fact that TS orthologues are not found in mammals. TS therefore could be a 'weak spot' in the life of the parasite that could be targeted for the fight against trypanosomiasis.

The current study set out with the aim to biochemically characterise TS genes from the most prevalent animal *Trypanosoma* parasite, *T. congolese*. Data mining with partial sequences of TS-forms 1 and 2 (Tiralongo et al. 2003b) as queries on WTSI resulted to full ORFs for the two TS forms. Further searches using complete ORFs resulted in two other related genes and the existence of 3 distantly related members. We classified the former group into TconTS1, TconTS2, TconTS3, TconTS4. The latter distantly related members are collectively referred to as TconTS-like genes and have been named TconTS-like1, TconTS-like2 and TconTS-like3. To make comparisons, we cloned and sequenced genes from from another parasitic African relative, *T. brucei*. Cloned TconTS genes were expressed in mammalian fibroblasts and enzyme activities assayed in order to establish kinetic parameters using different substrates. The findings of the study are presented in the thesis as individual chapters. Each of these chapters contain specific discussions of results presented therein.

5.1. TconTS genes from African trypanosomes

TS genes have a different and diverse occurrence in all known pathogenic trypanosomes (Tables 5.1 and 5.2). TS genes would therefore have evolved as multi-gene family and maintained in the parasites before the split of the continental plates separating for instance the African and American parasites. However, different environmental pressures probably led to the varying degrees of complexity exhibited across trypanosomes. *T. cruzi* has over 1400 TS-like genes (Cazzulo and Frasch 1992, De Pablos and Osuna 2012). Out of these large number, only 12 encode enzymatically active *T. cruzi* trans-sialidase (TcTS) proteins. About half encode for inactive gene products while the remaining half are pseudogenes (Kim et al. 2005). In the African trypanosomes, the TS gene families show up in several copies but with fewer numbers than occur in *T. cruzi*.

T. congolense remains to date the only African trypanosome from which TS genes have been studied in detail (Tiralongo et al. 2003a, Tiralongo et al. 2003b, Koliwer-Brandl et al. 2011, Coustou et al. 2012, Chapters 3 and 4). Of all the TS genes so far identified in African trypanosomes, *T. congolense* (STIB 249) has the highest number with at least 14 active members. In addition, *T. congolense* has 3 other genes (TconTS-like genes) which are considered inactive as they lack most of the critical amino acids required in transfer and hydrolysis activity, though no activity studies have been undertaken. The active genes have been classified into TconTS1, TconTS2, TconTS3 and TconTS4. TconTS1 is a multi-copy gene group with 11 members, all active and share over 96% similarity (Chapter 2). This number could have arisen from active gene rearrangement. In the STIB 249 strain used in this study, only one representative member has been identified for the other TconTS (Chapter 3) but there could be differences between strains as an extra copy, each for TconTS3 and TconTS4 in the IL3000 strain has been reported (Coustou et al. 2012).

So far, 9 TS gene members have been reported for *T. brucei* (Montagna et al. 2002, Montagna, Donelson and Frasch 2006, Nakatani et al. 2011). Two of these genes, TbTS (Montagna et al. 2006, Nakatani et al. 2011) have been enzymatically characterised. In the current study, *T. brucei* TS genes from the genomic DNA of EATRO427 were cloned and sequenced. Validity of gene numbers as well as correct assembly from different reads into contigs listed in databases was confirmed. TbTS1, an orthologue of TconTS1 had at least 3 members. *T. brucei* also retains orthologues for the other groups of TS genes found in *T. congolense* as well as three other gene copies, TconTS like D1, TconTS like D2 and TconTS-like E (Montagna et al. 2006) whose products are presumably inactive due to the non-conservation of the critical amino acids acids.

Data mining from TritrypDB, WSTI and GeneDB identified 6 TS representatives from *T. vivax* (Jackson et al. 2013). Though Guegan et al. (2013) confirmed the existence of these genes by PCR using genomic DNA, peptide evidence could only be obtained for only three of the genes, TvivTS1, TvivTS3 and TvivTS5 in the BSF parasites. Sequence comparison with known TS of

other trypanosomes showed the BSF-expressed genes retained the critical amino acids required for activity and hence presumed to be active (Table 5.1).

Table 5.1. Amino acids in the catalytic domains of TS gene products of animal African trypanosomes involved in enzymatic activities*

T. congolense				*T. brucei*		*T. vivax*			*T.evansi*			
Tcon TS1	Tcon TS2	Tcon TS3	Tcon TS4	TbTS	TbSAC2	Tviv1	Tviv3	Tviv5	TevTS 1	TevTS 2	TevTS 3	TevTS 4
catalysis												
D150	D135	D142	D207	D157	D108	D197	D192	D207	D157	D163	D108	D163
E324	E309	E316	E381	E311	E283	E399	E395	E409	E331	E337	E283	E336
Y438	Y423	Y430	Y493	Y457	Y402	Y510	Y506	Y520	Y457	Y451	Y402	Y450
substrate binding												
R126	R111	R118	R183	R133	R84	R173	R168	R183	R133	R139	R84	R139
R339	R324	R331	R396	R346	R298	R414	R410	R424	R346	R352	R298	R351
R410	R395	R402	R465	R429	L381	R482	R478	R492	R429	R423	R374	R422
R144	R129	R136	R201	R151	R102	R191	R186	R201	R151	R157	R102	R157
D188	D173	D180	D245	D195	D146	D235	D230	D245	D195	D201	D146	D201
Y211	P196	Y203	Y268	Y218	W169	Y258	Y253	Y268	Y218	Y224	W169	Y224
W212	W197	W204	W269	W219	W170	W259	W254	W269	W219	W225	W170	W225
Q289	Q274	Q281	Q364	Q296	Q248	Q364	Q360	Q374	Q296	Q302	Q248	Q301
Y408	W393	W400	Y463	W427	W372	H480	Y476	H490	W427	F421	W372	F420
structure												
A325	A310	S317	A382	A332	S284	S400	S396	S410	A332	S338	S284	S337
P379	P364	P371	P434	P229	I350	P451	P447	P461	P398	P392	P343	P391

*The indicated amino acids have been selected based on structural (Amaya et al. 2004) and mutation (Cremona et al. 1995, Paris et al. 2001) studies with *T. cruzi* TS and on the sequence alignment of TconTS1b with *T. cruzi* (Koliwer-Brandl et al. 2011). *T. vivax* and *T. evansi* genes are mined from WSTI, GeneDB and TritrypDB (Taken from Gbem et al. 2013 with modification by inclusion of the *T. evansi* genes). Colours indicate drastic changes in amino acids.

Using phylogenetic analysis, *T. vivax* genes clustered distinctively away with no orthologues shared among *T. cruzi*, *T. brucei* and *T. congolense* (Chapter 3). This is consistent with a different form of selection pressure operating on *T. vivax* TS genes. *T. vivax* has a different pattern of

development in the tsetse vector, confined only in the cibarium and the mouth parts without extending to the midgut (Moloo and Gray 1989). Evolutionary pressures operating at the level of the vector host may likely be more significant in shaping the development of TS genes.

TS from *T. evansi* (TevTS) have not been studied. There is a report of TS from this parasite (Yakubu et al. 2011) with an NCBI accession number FJ597949. Unfortunately, the PCR primers used in this case were identical to those previously used in amplifying TS from *T. brucei* (Montagna et al. 2002). Equally, the method of species identification is questionable, coupled with the fact that *T. brucei* and *T. evansi* are closely related possibly due to a recent divergence (Gibson et al. 2001) and share between 85 and 99.6% identity in TS genes. Recently, TritrypDB® released the genome sequence of *T. evansi* (Schnaufer et al. unpublished). Taking advantage of this data, *T. evansi* TS genes were mined by using both *T. congolense* and *T. brucei* TS genes as starting queries. A total of 8 genes have been identified without an exact match for FJ597949. Five of these genes conserve the critical amino acids required for activity while the remaining three do not. This later group is presumed inactive and therefore termed TevTS-likes. Phylogenetic analysis showed that except for TconTS-like1, TevTS genes share orthologues with TconTS genes (see Table 5.2). TevTS1 showed two copies, one long (TevSTIB805.7.7540) one short (TevSTIB805.7.7510). Since these share over 98% identity with difference only concentrated at the end of the C-terminal in the short form, it is possible this may be due to wrong assembly of “reads”. Experimental confirmation is required in order to make definite conclusions. However, it if turns out that this indeed is a true gene copy, it would satisfy our previous observations on TS1 gene orthologues possessing multiple copy numbers across the animal African trypanosomes. Interestingly, a short form of TS1 has been reported in *T. brucei* (Montagna et al. 2002). The other TevTS genes, TevTS2, TevTS3 and TevTS4 all showed no evidence for multiple copy numbers from database. Cloning and sequencing would ascertain copy numbers for these genes. *T. evansi* occurs in South America apart from the African continent and this is attributed to the movement of cattle from Africa to South America in the early 19th century (Jones and Davila 2001). Adapting a mechanical mode of transmission as a response to the absence of tsetse flies in South America ensures its survival and transmission. Comparing TS sequences of strains from both continents would offer more insights into the diversity of TS gene from T. *evansi.*

A key question would arise concerning the observation of multiple gene copies in TS1: why the apparent diversity in this family? Recent mass spectrometry and immunoprecipitation with specific anti-sialidase IgG evidences (Coustou et al. 2012) points to the expression of TconTS1 both in the PCF and BSF stages of parasites in mice. Gbem et al. (2013) confirmed the expression of TconTS1 and/or TconTS2 in both the metacyclics forms in insects proboscis as well as BSFs of *T. congolense* experimentally infected goats. TconTS2 has a wide pH optimum (Chapter 3), indicating it could be active both in the insects gut (pH of up to 10) (Dyer et al. 2013) and in mammalian

blood system (pH 7.4). Information on the pH optima of TbTS1 enzyme activity is not available for any of the other African parasites and though TvivTS1 have been identified in the blood of infected mice (Guegan et al. 2013), TviTS genes share no orthologues with any other *Trypanosoma* to warrant meaningful inferences. More studies are needed on TbTS and the recently sequenced TevTS genes. If indeed it turns out that the TbTS and TevTS1 are expressed both in the blood and insects forms, the diversity seen in this orthologue group could be attributed to it being central to the survival of the parasites in both hosts, and certainly would deserve more attention.

5.2. Amino acid residue variations in TS

Mutation studies on TcTS revealed the identity of amino acids required for transfer and hydrolysis activities (Paris et al. 2001), (Amaya et al. 2004), (Paris et al. 2005). While most of these amino acids are conserved across the trypanosomes (Table 5.1), it is nevertheless difficult to make direct comparison of enzyme activities across TS families of African trypanosomes. This is because there are no comprehensive enzyme activity studies for trans-sialidases from *T. brucei*, *T. evansi* and *T. vivax*. Such comparison is only available for *T. congolense*, whose active TS genes have been biochemically characterised (Chapter 2, 3 and 4).

TconTS1 and TconTS2 show higher specific transfer activities than TconTS3 and TconTS4 on fetuin and serum glycoconjugates donor substrates. Comparison of the amino acid sequences of these TconTS genes with that of *T. cruzi* revealed two variations each in the critical amino acid residues involved in substrate binding. These are Y211, Y408 and P192, W393 for TconTS1 and TconTS2, respectively. In *T. cruzi*, these residues are Y199 and W312 and together they form the binding pocket for the acceptor substrate (Amaya et al. 2004, Mitchell et al. 2013). Interestingly, the change in TconTS2 at position 192 where Pro replaces Tyr (Table 5.1) is drastic and yet, TconTS2 exhibited the highest specific transfer activity among all enzymes from *T. congolense*. Phylogenetic analysis have assigned orthologues for TconTS and TbTS genes, using separately the catalytic domain (CD) and lectin domain(LD). Due to high similarity between *T. brucei* and *T. evansi*, phylogenetic analysis unsurprisingly assigned orthologues in a similar way as for *T. brucei* except for TbTS-Like E, where no orthologue is found in *T. evansi* (Table 5.2). The LD grouped the most active TconTS gene together. Identification of orthologues from the other trypanosomes and their subsequent grouping with TconTS genes could be an indication of the activity status of such orthologues. It is important to note here that these genes grouped differently when CD was used (Chapter 3).

It was observed that singly, TconTS genes could not cleave the chromogenic substrate Neu5Ac-MU. However, if either TconTS1 or TconTS2 were mixed with any other TconTS enzyme, these synthetic sialosides were hydrolysed. A synergistic effect has been envisaged. Other unidentified structural features of the proteins may be responsible for this effect.

Table 5.3. Relationships between trans-sialidase like genes from *T. congolense* and *T. evansi*

T. congolense				*T. brucei*			*T. evansi* *		
Names	**Coustou et al. 2012**	**Enzyme activity****	**% similarity to *T. evansi***	**Names**	**GeneID (GeneDB)**	**Enzyme activity***	**Names**	**GeneID (TritrypDB)**	**% similarity to *T. brucei***
TconTS1***	TcoTS-A	TS [1], TS / SA [2,3]	56	TbTS	Tb927.7.6850	TS	TevTS1	TevSTIB805.7.7540	99.2
				TbTSsh	Tb927.7.6830		TevTSsh****	TevSTIB805.7.7510	99.1
TconTS2	TcoTS-C	TS / SA [2,3]	46	TbSA C [4]	Tb927.8.7340	SA[4]	TevTS2	TevSTIB805.5.690	99.6
				TbSA C2 [5]	Tb927.8.7350	TS [5], SA [5]			
TconTS3	TcoTS-B1 TcoTS-B2	TS [2,3]	53	TbSA C [4]	Tb927.8.7340	SA[4]	TevTS3	TevSTIB805.8.7690 TevSTIB805.8.7700	96.4
				TbSA C2 ([5]	Tb927.8.7350	TS [5], SA [5]			
TconTS4	TcoTS-D2 TcoTS-D1	TS / SA [2,3]	54	TbSA B [4]	Tb927.5.640		TevTS4	TevSTIB805.7.8230	83.5
				TbSA B2 [5]	Tb927.7.7480				
TconTS-like1	TcoTS-like3	presumed inactive		TbTS-like E [4]	Tb927.5.440	presumed inactive	TevTS-Like1		
TconTS-like2	TcoTS-like2	presumed inactive		TbTS-like D2 [4]	Tb11.01.3240	presumed inactive	TevTS-Like2	TevSTIB805.11.01.11790	98.8
TconTS-like3	TcoTS-like1	presumed inactive		bTS-like D1 [4]	Tb927.2.5280	presumed inactive	TevTS-Like3	TevSTIB805.2.2980	99.4

*Data mined from TritrypDB; genome sequence and annotation (Schnaufer et al. unpublished), enzyme activities yet to be studied

**Enzyme activity references

***11 copy members Ref. [1]

****TevTS short form

[1]Koliwer-Brandl et al. 2011

[2]Gbem et al. 2013

[3]Gbem et al. submitted manuscript / Results: Chapter 4

[4]Montagna et al. (2004)

[5]Nakatani et al. (2011)

The observation that TconTS1 and TconTS2 grouped together when LD domains were used in phylogenetic analysis led to the the suggestion that the lectin domain may play a more significant role in TS activities than previously thought. Preliminary results from experiments with swapped domains lend credence to this observation (Chapter 3). Equally, a mouse monoclonal antibody raised against purified native TconTS protein (Tiralongo et al. 2003b) binds to both TconTS1 and TconTS2. Epitope mapping identified the epitope as been located in the lectin domain of both enzymes (Chapter 3). In the past, emphasis on TS enzyme activities have been on the catalytic domain that harbours amino acid residues involved in substrate binding, conformational changes and catalysis. The lectin domain hardly attracted any attention. Detailed experiments will be necessary to fully ascertain this observation, but, if finally proven, the impact of this finding on rational drug design and other avenues in the fight against the pathogenesis of African trypanosomosis would be enhanced.

5.3. Substrates could led to variation in enzyme activity

Expression of TconTS genes in the mammalian fibroblasts CHO_{Lec1} cells represents the first time TS genes were expressed in fibroblasts. Comparing with reports from literature, specific activities have been observed to be much lower for proteins expressed in bacteria than those expressed in fibroblasts (Chapter 3). Poor specific activities were observed when fibroblasts-expressed proteins were incubated with synthetic substrates, compared to natural substrates (Chapter 2 & 3). Specific activities were highest for TconTS1 and TconTS2 when fetuin and serum glycoconjugates were used as donor and lactose as acceptor. In both cases, free Neu5Ac became detectable only when 3'SL accumulated in the reaction. In absence of lactose however, sialidase activity was observed for TconTS1, TconTS2, TconTS4 but none for TconTS3 on serum samples. Lactose has been shown to be a suitable acceptor for the transferred Sia (Chapters 2 & 3,). Similar observations have been made with TbTS enzymes (Engstler et al. 1993).

Specific activities for TconTS enzymes on natural substrates were higher for fetuin-bound Sia than serum glycoconjugates with lactose as acceptor substrate (Table 5.3). Serum presents several donor and acceptor possibilities. A complex pattern of Sia removal and transfer is possible.

This means lactose may not be the only acceptor present as opposed to a single substrate like fetuin. This could lead to an underestimation of sialylation by such a method as used in the study. TconTS1 and TconTS2 transferred between 50 and 60% Sia from fetuin (Chapter 3), and between 20 and 27% from serum glycoconjugates (Chapter 4). More Sia is therefore transferred from fetuin than from serum by the two enzymes, with TconTS2 transferring higher Sia in both cases. For both enzymes, maximum free Sia released in presence of lactose was not more than 1.5 nmol. Sia release by TconTS1 in absence of lactose is comparable to amounts of 3'SL formed when lactose is present while TconS2 released slightly less Sia than 3'SL formed (Chapter 4). While this

Table 5.3. Specific activities of Sia transfer from fetuin- and serum-bound Sia by TconTS enzymes

TconTS	Donor (nmol/(min x mg TS)		ratio	Acceptor lactose (nmol/(min x mg TS)		ratio
	fetuin-bound Sia*[1]	serum-bound Sia*[2]		with fetuin-bound Sia**[1]	with serum-bound Sia**[2]	
TconTS1	7900±300	315±16	25	4300±100	181±1.5	24
TconTS2	17,600±130	1005±60	18	17,850±130	486±9.3	37
TconTS3	170±20	5.1±0.1	33	56.7±1.4	4.2±1.0	14
TconTS4	6.7±0.2	3.2±0.1	2.1	7.5±0.2	2.2±0.1	3.4

* 600 μM fetuin- and serum-bound Sia was used as donor substrate
** 2 mM lactose used as acceptor
[1]data from Results: Chapter 3
[2] data from Results: Chapter 4

difference may not be relevant, it is possible that these TconTS enzymes access different Sia on serum glycoconjugates thereby giving the parasite access to a variety of Sia in the host and hence better survival. BSF parasites require Sia in order to escape lysis by trypanolytic factors present in serum (Vanhamme et al. 2003). Incubating serum glycoconjugates with a mixture of the two enzymes would reveal whether or not they use one or different Sia types.

Sialidase activity of TconTS4 on fetuin was suppressed when increasing concentrations of lactose were used (Chapter 3). The presence of lactose, for instance by direct infusion, could lower parasites sialylation, with lactose as the preferred acceptor. Loss of Sia from parasites' surfaces could also occur. The sum total effect of these processes is clearance of parasites from the host body system. Indeed, infusion of lactose in sheep suffering experimental *T. congolense* infections suppressed anaemia (Chapter 3, personal communication). More studies are required for a detailed understanding of acceptor binding towards inhibition of TconTS by use of acceptors.

The finding that individual TconTS genes do not cleave the widely used Neu5Ac-MU substrate except when mixed with other TconTS (section 5.2 above) is significant, even towards standardising activity tests. Although this may be due to absence of a suitable acceptor substrate as reports supporting cleavage of Neu5Ac-MU by TS enzymes possibly contained natural acceptors like GARP (Tiralongo et al. 2003b, Schrader et al. 2003, Schrader et al. 2006), further studies are needed for example in presence of GARP to make conclusive statements on this finding. Use of Neu5Ac-MU is wide spread and seems to be the preferred method, but may not be the most suitable method at least in testing for activities of recombinant TconTS genes.

There is no obvious link between trypanosomiasis and synthetic substrates' cleavage by TS enzymes. By extrapolation however, one could assume a sort of synergy that might arise from combined expression of these gene products in mammalian blood. In this vain, cleavage of Sia from hitherto difficult linkages or donors could be achieved, with enhanced virulence as a result. It is also reasonable to assume that combined expression of different TS genes provides the broader repertoire of potential Sia sources available to the parasite leading to a better survival of the parasites in the host. Serum and blood contain several glycoproteins and/or glycolipids. The possibility of a complex pattern of sialylation and desialylation is envisaged and has been observed for serum and equally revealed an interesting pattern of sialylation at varying serum-bound Sia concentrations (Chapter 4). The parasite is equipped with different acceptors on its surface. It is tempting to postulate that the parasites deploy specific set of acceptors with better efficiencies at given Sia concentration or stages of infection.

5.4. TS as target in the fight against AAT

It has been established that SA /TS genes of *T. congolense* (Nok and Balogun 2003, Coustou et al. 2012) and those of *T. vivax* (Guegan et al. 2013) are involved in anaemia, a major clinical sign for

trypanosomiasis caused by these parasites. mRNA evidence from insect vector tissue and blood of infected goats showed that apart from TconTS1, TconTS2 and TconTS4 are also expressed in the mammalian blood (Gbem et al. 2013). However, as monoclonal antibodies are not available for TconTS2, TconTS3 and TconTS4, presence of various gene products at protein levels could not be confirmed. No study to the best of our knowledge has reported on whether or not the BSF forms of *T. brucei* and *T. evansi* also express these genes. However, the expression of common orthologues shared among *T. congolense*, *T. brucei* and *T. evansi* in blood would have the same beneficial effect(s) to these parasites. In both *T. congolese* and *T. vivax*, the expressed enzymes have been implicated as virulence factors in anaemia resulting from *Trypanosoma* infections.

Apart from the active TS genes, TS-like genes are retained between *T. congolense*, *T. brucei* (Guegan et al. 2013, Jackson et al. 2013, Chapter 3) and *T. evansi* as common orthologues (Table 5.2). The obvious question would be why retaining inactive genes? Interestingly, TconTS-like2 (TcoTS-like 2 in Coustou et al. 2012) is reported to be expressed in the blood of infected mice and showed protection against *T. congolense* when used as a DNA vaccine (Coustou et al. 2012). Could the expression of the inactive TS-like genes in mammals be a “decoy” by the parasite to avoid immune responses such as complement lysis? In *T. cruzi*, the C-terminally located SAPA is a gene domain found both in the active and inactive TcTS (Pereira et al. 1991). SAPA increases the half-life of TS in the blood (Buscaglia et al. 1999) and is also thought to serve as a decoy by TS to evade the host immune system (Frasch, 1994). SAPA is unrelated to TS-like proteins and it is absent in TS and TS-like genes of African trypanosomes. The expression of TS-like genes may serve a similar purpose. In *T. cruzi* however, a T/C transition resulting in Tyr342-His replacement differentiates active from inactive trans-sialidase (Cremona et al. 1995). While enzymatically inactive, these genes retain the substrate binding, hence lectin-like ability (Cremona et al. 1995), (Todeschini et al. 2002a, Todeschini et al. 2004). Todeschini et al. (2002b) used an inactive TcTS and observed stimulation of host's T-lymphocytes. Recently, crystallographic and enzyme kinetics studies showed residual hydrolytic activities retained in inactive TcTS (Oppezzo et al. 2011). It would be interesting to study expression profiles of TS-like genes in the BSFs of African trypanosomes by quantitative RT-PCR experiments and their possible role in the pathogenesis of the African trypanosomes.

Several compounds have been used as inhibitors of TS mainly in *T. cruzi* (reviewed in Chapter 1). These have met with varying degrees of success but till date, no potent inhibitor has been reported. Gene knockout is obviously unattractive due to the presence of several TS copies/gene members. RNA*i* while suppressing activities in *T. brucei* did not lead to total inhibition of gene expression (Montagna et al. 2006). The reason for this is obvious as closely related genes with over 80% sequence identity have been identified for TbSA B and TbSA C (Nakatani et al. 2011). TS are shed in the blood of the mammalian hosts and elicit several antibody responses (Dc-Rubin

and Schenkman 2012). Recently, Buschiazzo et al. (2012) reported a mouse monoclonal Ab, mAb13G9 showing strong inhibition of parasite surface sialylation of *T. cruzi*. This finding is interesting especially in the African trypanosomes as fewer TS genes have been reported. Vaccination of animals with recombinant genes should lead to immune response studies and a possible screening and selection of a potent neutralising antibody against TS.

There have been ongoing efforts to employ tsetse gut symbiont in the fight against trypanosomiasis. In line with this, naturally occurring tsetse symbionts like *Wolbachia* sp, *Sodalis* sp and *Wigglesworthia* sp thought to influence *Trypanosoma* infections in tsetse have been studied (Wamwiri et al. 2013). Does any of the naturally occurring midgut tsetse flora express sialidases which may act as a competitor to any TS expressed in fly midgut? Such a sialidase would essentially desialylate glycoconjugates from mammalian blood and parasites and prevent establishment of infection in vector gut. So far, there has been no such report. What however is known is that endosymbionts are not universally present (Wamwiri et al. 2013) in the over 30 extant *Glossina* spp (Krafsur 2009) all capable of transmitting trypanosomes. In light of this, a transgenic endosymbiont carrying an active sialidase gene may be a more reasonable approach. However, such a sialidase should be highly active and at such pH conditions as prevalent in tsetse midgut (Dyer et al. 2013). TconTS2 is the TconTS with the highest specific activity. Moreover, its pH optima equally meet the pH conditions in the fly midgut (Chapter 4) and should be considered for genetic manipulation to enhance its sialidase activity.

In conclusion, this study characterised *T. congolense* TS by utilising recombinant gene products made from the genomic DNA of the organism. All TconTS genes transfer Sia with varying specific activities, TconTS1 and TconTS2 showing the highest specific activities. Activity studies on blood glycoconjugates reveal different preferences for Sia by TconTS enzymes with high sialidase activity for TconTS1 and TconTS2 in absence of preferred acceptors, possibly accounting for the high amount of free Sia observed in blood and serum of infected animals. TconTS2 gene shows a robust pH optima that could warrant it exhibit activity both in the vector and the mammalian host. Phylogenetic analysis employing different domains of TS genes from sequenced and mined data reveal orthologues across the *Trypanosoma* genus, clustering TconTS gene products with higher specific activities in together when lectin domain were used. The multi-copy nature of the TS1 gene group seems to be a common feature in parasitic trypanosomes.

5.5. References

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6.0.

Outlook

6.0 Outlook

6.0 Outlook

Apart from infecting animals, atypical cases of human (a-HT) infections with animal trypanosomes (Truc et al. 2013) have been documented. This portends great worry with the spate of drug resistance coupled with the little or no possibility of new drugs on the horizon. TS clearly presents a weak-spot and should constitute an attractive target for alternative arsenals.

The role of the lectin domain in pathogenesis of trypanosomiasis needs further investigation. Domain exchanges, followed by enzyme activity studies of fibroblasts expressed 'chimera TconTS' should provide immediate insight into the role of lectin domain in activity.

The multi-copy nature of TS1 gene group seems a common feature of the animal African trypanosomes. The variability in this gene group may be central in the survival of these parasites. Studies are required on the other parasitic animal African trypanosomes to decipher the true situation of this gene group. Equally, the inactive TconTS-like genes occur as orthologues in African trypanosomes that establish infections in the vector host. This suggests a role in the pathogenesis for these genes and needs further investigation.

We have shown by mRNA experiments that TconTS genes are expressed in the blood of mammalian hosts. However, since monoclonal antibodies are not yet available for TconTS2, TconTS3 and TconTS4, difficulty still remains as as to which these TconTS gene products are present in the blood. While a mouse monoclonal Ab is available, this Ab reacts with TconTS2 thereby making it impossible at the moment to say with certainty which of these TconTS proteins is expressed in the blood of infected animals. There is need to raise monoclonals which are specific for each TconTS protein.

Tsetse vector harbour endosymbionts. The idea of an endosymbiont expressing sialidase that could counter TS activities in the gut of the insect host has been muted. Unfortunately, the distribution of endosymbionts is not catholic in the tsetse gut, placing a limitation on such a potential approach. Transgenic endosymbiont carrying an active sialidase gene may be a more reasonable approach. TconTS2 is highly active even at pH conditions prevalent in tsetse midgut, and should therefore be considered for genetic manipulation to enhance its sialidase activity towards creating a common transgenic endosymbiont.

Appendix

List of publications

Curriculum vitae

List of Publication

Koliwer-Brandl, H., T. T. Gbem, M. Waespy, O. Reichert, P. Mandel, E. Drebitz, F. Dietz & S. Kelm (2011) Biochemical characterization of trans-sialidase TS1 variants from *Trypanosoma congolense*. *BMC Biochem,* 12**,** 39.

Gbem, T.T., M. Waespy, B. Hesse, F. Dietz, J. Smith, G.D. Chechet, J.A. Nok., S. Kelm (2013) Biochemical diversity in the *Trypansoma congolense* trans-sialidase family. *PLOS Neglt Trop Disease,* 7(12): e2549. Doi: 10.1371/journal.pntd.0002549.

Gbem, T.T., Waespy, M., Nok, J.A., S. Kelm (2013) Enzymatic activities of trans-sialidases from T*rypanosoma congolense* on blood glycoconjugate (manuscript)

Curriculum vitae

Biodata

Born in Kwande, Benue State, Nigeria, married, with children

Educational qualification

1. Bsc Biology (1993); Ahmadu Bello University, Zaria, Nigeria
2. MSc, BMB (2008); Universität Bremen, Germany
3. PhD (in view) since December, 2008

Work

1. Assistant Lecturer, Ahmadu Bello University (1998 - 2005)