Trans-Sialidase from *Trypanosoma congolense* – Isolation, Characterisation and Molecular Biology

Dissertation

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von

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Statement of Originality

I declare that this thesis has not been submitted in any form for another degree at any other university. The material discussed in this thesis is my own work, unless otherwise stated. Information derived from the literature or unpublished work of others has been acknowledged in the text and a list of references provided.

Evelin Tiralongo Bremen, den 16.12.2002

Abbreviations

aa	amino acid
AIDS	acquired immunodeficiency syndrome
4-amino-Neu2en5Ac	5-*N*-acetyl-2,3-didehydro-2,4-dideoxy-4-amino-neuraminic acid
Bis/Tris	2,2-Bis-(hydroxymethyl)-2,2´,2´´-nitrilotriethanol
bp	base pair
BSM	bovine submandibular gland mucin
CNS	central nervous system
CMP-Neu5Ac	cytidine-5`-monophosphate *N*-acetylneuraminic acid
Da	dalton
DDT	dichlorodiphenyltrichloroethane
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide 5`-triphosphate
dUTP	deoxyuridine 5`-triphosphate
DTT	dithiothreitol
E.coli	*Escherichia coli*
ESM	equine submandibular gland mucin
FCS	fetal calf serum
GARP	glutamic acid-alanine-rich protein
GPI	glycosylphoshatidylinositol
4-guanidino-Neu2en5Ac	5-*N*-acetyl-2,3-didehydro-2,4-dideoxy-4-guanidinyl-neuraminic acid
HIV	human immunodeficiency virus
H_2O_2	hydrogen peroxide
IEF	isoelectric focusing
IL-6	interleukin 6
Ig	immunoglobulin
IPTG	isopropylthiogalactoside
Kdn	deaminoneuraminic acid
K_M	Michaelis-Menten constant

LNnT	lacto-*N*-neotetraose
LNT	lacto-*N*-tetraose
mAb	monoclonal antibody
MAG	myelin-associated glycoprotein
mRNA	messenger ribonucleic acid
MU	4-methylumbelliferone
MUGal	2`(4-methylumbelliferyl)galactoside
MULac	2`(4-methylumbelliferyl)lactoside
MUNeu5Ac	2`(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid
Neu2en5Ac	5-*N*-acetyl-2-deoxy-2,3-didehydro-neuraminic acid
Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
NO	nitrogen monoxide
PARP	procyclic acidic repetitive protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RT	reverse transcription
SA	sialidase
SAPA	shed acute phase antigen
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
α2,3-SL	sialyllactose (Neu5Acα2,3-lactose)
Siglecs	sialic acid recognising Ig-like lectins
STIB	Swiss Tropical Institute Basel
TBS	tris-buffered saline
T.b.br.	*Trypanosoma brucei brucei**
T.con.	*Trypanosoma congolense**
*T.con.*TS1	*T.con.*trans-sialidase sequence 1 (long)
*T.con.*TS2	*T.con.*trans-sialidase sequence 2 (short)
T.cr.	*Trypanosoma cruzi**
T.r.	*Trypanosoma rangeli**
TS	trans-sialidase
TS-form 1	high molecular weight TS form

TS-form 2	low molecular weight TS form
Tris	2-amino-2(hydroxymethyl)-1,3-propanediol
V_{max}	maximum velocity
VSG	variant surface glycoprotein
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
🗐	see section

*This form of abbreviation is based on that introduced by Montagna *et al.* (2002), *Eur. J. Biochem.* 269, 1-10.

Abbreviations and symbols for amino acids

Amino acid	Three-letter abbreviation	One-letter symbol	Amino acid	Three-letter abbreviation	One-letter symbol
Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic acid	Asp	D	Phenylalanine	Phe	F
Cystein	Cys	C	Proline	Pro	P
Glutamine	Gln	Q	Serine	Ser	S
Glutamic acid	Glu	E	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

Nomenclature of bases, nucleosides and nucleotides

Base	Deoxyribonucleoside	Deoxyribonucleotide
Adenine (A)	Deoxyadenosine	Deoxyadenylate (dAMP)
Guanine (G)	Deoxyguanosine	Deoxyguanylate (dGMP)
Thymine (T)	Deoxythymidine	Deoxythymidilate (dTMP)
Cytosine (C)	Deoxycytidine	Deoxycytidylate (dCMP)

Additionally the following abbreviations were used:
R = A or G M = C or A K = G or T V = A, C or G
Y = T or C S = G or C N = A, G, T or C

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CHAPTER 1

General Introduction

1.1. Sialic acids

Beside proteins, nucleic acids and lipids carbohydrates are one of the four major classes of biomolecules,. Carbohydrates are aldehyde or ketone compounds with multiple hydroxyl groups. Because of their large molecular variety they exhibit a great number of functions. Carbohydrates serve as energy stores, fuels and metabolic intermediates, they form part of the structural framework of DNA and RNA, and are structural elements in the cell walls of bacteria and plants, as well as in the exoskeletons of arthropods. Eukaryotic cells are surrounded by a glycocalyx, which consists of carbohydrate chains (glycans) linked to proteins and lipids of the cell membrane. Within the group of glycoconjugates containing glycans a group of carbohydrates exist which are referred to as sialic acids.

The name sialic acid originates from the Greek word “sialos”, meaning saliva, because the mucins of saliva reveal a high content of these compounds. This name was first coined in 1957 by Blix, Gottschalk and Klenk [1], however, sialic acid is also referred to as neuraminic acid. The term neuraminic acid was first used by Klenk [2] because of the discovery of a sialic acid-containing glycolipid fraction, later identified as ganglioside, from brain [3].

1.1.1. Structural diversity of sialic acids

Since the 1930`s more than 40 naturally occurring derivatives of the nine carbon sugar neuraminic acid (5-Amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid; Neu) have been found. The unsubstituted form, neuraminic acid (Neu), does not exist in its free form in nature. Usually the amino group is acetylated leading to *N*-acetylneuraminic acid (5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid; Neu5Ac (Fig. 1), the most widespread form of sialic acid [4]. Substituting one of the hydrogens in the methyl moiety of the acetyl group by a hydroxyl group yields *N*-glycolylneuraminic acid (5-hydroxyacetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid; Neu5Gc)

(Fig. 1). Substitution of the amino group by a hydroxyl at position 5 of Neu leads to the loss of the amino group resulting in deaminoneuraminic acid (2-keto-3-deoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid; Kdn) (Fig. 1) [4].

Sialic acids can undergo further modifications at any one of the four hydroxyl groups located at C-4, -7, -8, -9. These groups can be methylated or form esters, such as acetyl, lactyl, sulphate or phosphate esters. An introduction of a double bond between C-2 and C-3 has also been described [4]. Sialic acids are large molecules and the carboxyl group at position 1 provides a negative charge under physiological conditions, thus characterising it as a strong organic acid (pk 2.2) [5].

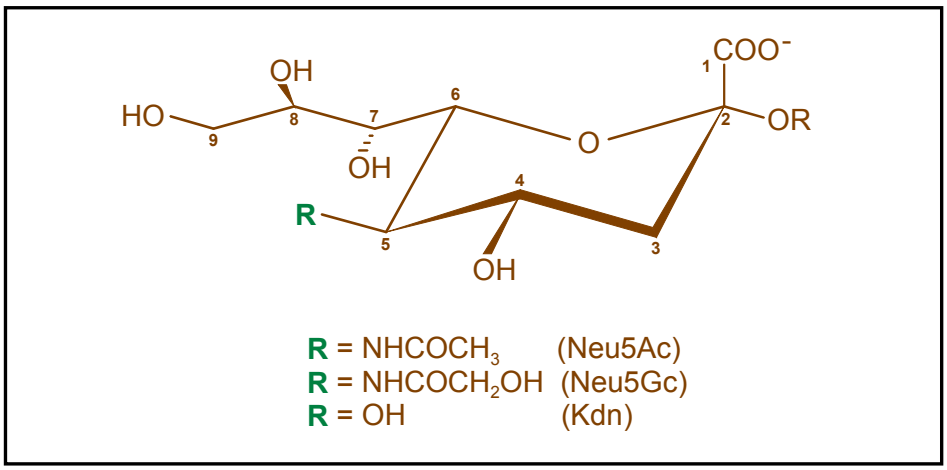

Fig.1: Structure of three important sialic acids

1.1.2. Distribution of sialic acids

Generally, sialic acids are found at the terminal position of glycan chains present on glycoproteins, glycolipids and oligosaccharides, or represent polysaccharides. They are either α-2,3 or α-2,6 glycosidically linked to D-galactose or *N*-acetyl-D-galactosamine, α-2,6-linked to *N*-acetyl-D-glucosamine or α-2,8 linked to another sialic acid molecule forming polysialic acids [4].

Interestingly, sialic acids do not exist in plants and higher fungi [6]. However, Neu5Ac has been found in deuterostoma from the echinoderms upwards to humans [6;7], whereas their existence in the protostomate lineage is rare and restricted to certain developmental stages of some insects [8-10]. In addition, sialic acids have been found in some protozoa [11], viruses and bacteria [4], even though the sialic acids of viral and trypanosomal glycoconjugates seems to originate from host cell-glycoconjugates [12-14].

Similar to Neu5Ac, Neu5Gc has been found sporadically in protozoa [11] and in protostoma [15], however, Neu5Gc could not be detected in bacteria and viruses [16]. Neu5Gc occurs frequently in deuterostoma, especially in primitive marine invertebrates like echinoderms, where it represents the major sialic acid derivative [17]. Neu5Gc has also been detected in humans, but not only in particular cancers as was previous reported [18]. Instead, Neu5Gc has now been shown to occur in a number of normal and fetal human tissues [19], where trace amounts may originate from dietary source [20;21].

Kdn was first described in eggs of the rainbow trout [22]. Since then Kdn-containing glycans have been found in different organisms ranging from bacteria to lower vertebrates, including amphibians and fish, as well as in mammalian cells and tissues [23].

1.1.3. Biological roles of sialic acids

Because of their structural diversity sialic acids have been implicated in a vast array of biological processes. Due to their negative charge, sialic acids are involved in binding and transport of positively charged molecules (e.g. Ca^{2+}) [24], as well as in attraction and repulsion processes between cells and molecules. The repulsive forces acting between their negative charges stabilise the correct conformation of glycoproteins [25] and are important for the lubricative and protective function of mucins, found in saliva and on epithelial cells [4]. Moreover, the repulsive effects of negatively charged sialic acids hinder aggregation of erythrocytes [26].

Sialic acids play an important role in specific recognition processes. That is, they are necessary components of receptors for many endogenous substances like cytokines and other hormones. Likewise, many pathogenic agents such as bacteria (e.g. *Escherichia coli* [27;28], *Heliobacter pylori* [29]), viruses (e.g. Influenza viruses [30]), toxins (e.g. cholera-toxin [31]) and protozoa (e.g. *Trypanosoma cruzi* [32]) bind to host cells *via* sialic acid-containing receptors. Additionally, sialic acid recognising proteins, selectins, which recognise sialylated glycan structures (e.g. sialyl-Lewis^x and sialyl-Lewis^a) on the surface of leucocytes, play an important role in the initial stage of adhesion of leucocytes to endothelia prior to their evasion into the lymphatic tissue [33].

Furthermore, "Siglecs", a group of sialic acid-recognising Ig-like lectins, can recognise sialic acid with a far greater specificity than selectins. Until a few years ago only four Siglecs were known (sialoadhesin; CD22; myelin-associated glycoprotein, MAG; CD33). However, a further six human CD33-related Siglecs with features of inhibitory receptors have been identified [34]. Sialoadhesin, found on macrophages from murine bone marrow, CD22, expressed on B cells, and CD33, including its six relatives, expressed by discrete subsets of leukocytes are implicated in the development and trafficking of leucocytes in the lymphatic tissue, as well as in the regulation of the immune system [34;35]. MAG, on the other hand, is expressed by myelinating glial cells in the central and peripheral nervous system mediating cell-cell interactions between myelinating glial cells and neurons [36].

Sialic acids can also mask specific cellular recognition sites, as has been observed for erythrocytes and other blood cells, as well as serum glycoproteins, where the addition of sialic acid to the sub-terminal galactose impedes the binding of galactose-specific receptors of macrophages and hepatocytes hindering the degradation of those molecules [4;37;38]. The same masking effect can, on the other hand, help to hide antigenic sites on bacteria (e.g. *Neisseria gonorrhoeae* [39]), protozoa (e.g. *Trypanosoma cruzi* [32]) and tumor cells [40] from the host immune system.

1.2. Sialidase, Sialyltransferase and Trans-sialidase

1.2.1. Sialidases

Sialidase (neuraminidase, *N*-Acetylneuraminosyl-glycohydrolase, EC 3.2.1.18, SA), the key enzyme of sialic acid catabolism, hydrolyses glycosidic linkages between sialic acid and the penultimate sugar of the glycan chains of glycoconjugates (Fig. 2) [12]. SA, corresponding to the occurrence of sialic acids, has been described in deuterostoma from echinoderms upwards to humans [4]. Additionally, viruses, protozoa, bacteria and fungi have been found to express SA, although these organism mostly lack sialic acids [4]. Viral, bacterial and mammalian SA have been studied extensively and some have been characterised biochemically and genetically [41;42], however, SA has not been found in plants [43].

The role of SA as pathogenic factors is controversial. Certainly, SA increases the impact of microbial species by cleaving terminal sialic acid residues from host cell glycoconjugates. With that SA can facilitate their propagation and invasion of host tissue, as was shown for *Clostridium perfringens* and *Bacterioides fragiles* [44]. Furthermore, by demasking subterminal host cell structures receptors for parasites and toxins become available, as shown for cholera-toxin [45]. Additionally, SA on the surface of Influenza A and B virus cleaves sialic acid residues from the protective mucus layer of the host respiratory apparatus allowing the virus to spread [46]. This knowledge was exploited for the development of an anti-influenza drug which consists of a modified sialic acid that strongly inhibits SA [47].

In contrast, SA are commonly found in non-pathogenic organisms where they are involved in the carbohydrate catabolism of glycoproteins and glycolipids (lysosomal SA). Two human diseases, sialidosis and galactosialidosis, are associated with a defect or deficiency of lysosomal SA. During sialidosis, also referred to as mucolipidosis I, there is an accumulation and excessive urinary excretion of sialyloligosaccharides with patients

exhibiting congential, neurological and bone abnormalities [48]. In galactosialidosis, referred to as mucolipidosis II, there is a combined deficiency of SA and β-galactosidase, leading to the same symptoms as shown for sialidosis [48]. Moreover, a ganglioside-specific SA involved in the catabolism of gangliosides in lysosomes, plasma membrane and myelin has been described. With this, ganglioside-specific SA activity of the plasma membrane was found to control proliferation and differentiation on neuroblastoma cells [49].

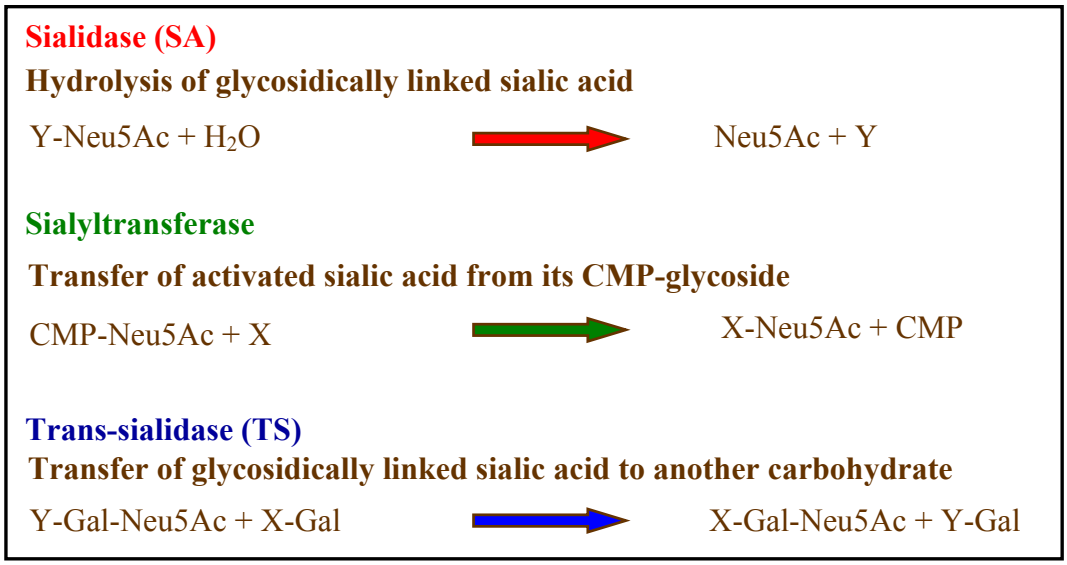

Fig. 2: Comparison of sialidase, sialyltransferase and trans-sialidase

1.2.2. Sialyltransferases

Sialyltransferases are a family of glycosyltransferases that transfer sialic acid from CMP-activated sialic acid to carbohydrate groups of glycoconjugates (Fig. 2). Sialyltransferases form either α2,3; α2,6; α2,8 or α2,9 linkages between sialic acid and an appropriate acceptor molecule [4]. Sialyltransferases from mammals, which are located in the Golgi apparatus, and from some bacterial species have been extensively studied. Presently, the amino acid sequences for at least 15 distinct members of the sialyltransferase family are available [4]. Moreover, sialyltransferase activity is not only increased in many tumors, but also varies in their linkage specificity, leading to a higher degree and different mode of sialylation, when compared to normal tissue [4].

1.2.3. Trans-sialidases

Trans-sialidases (TS) combine the features of SA and sialyltransferases. TS catalyse the transfer of, preferably, α2,3-carbohydrate-linked sialic acids to another carbohydrate forming a new α2,3-glycosidic linkage to galactose or *N*-acetylgalactosamine (Fig. 2 and 3) [4]. Unlike sialyltransferases, which require CMP-Neu5Ac as the monosaccharide donor, TS is able to transfer sialic acids from a variety of sialyl-α-galactose donor molecules. In the absence of an appropriate acceptor TS acts like a SA, similar to viral, bacterial, mammalian and trypanosomal SA, hydrolysing glycosidically linked sialic acids. However, TS is more efficient in transferring than hydrolysing terminal sialic acid [50;51].

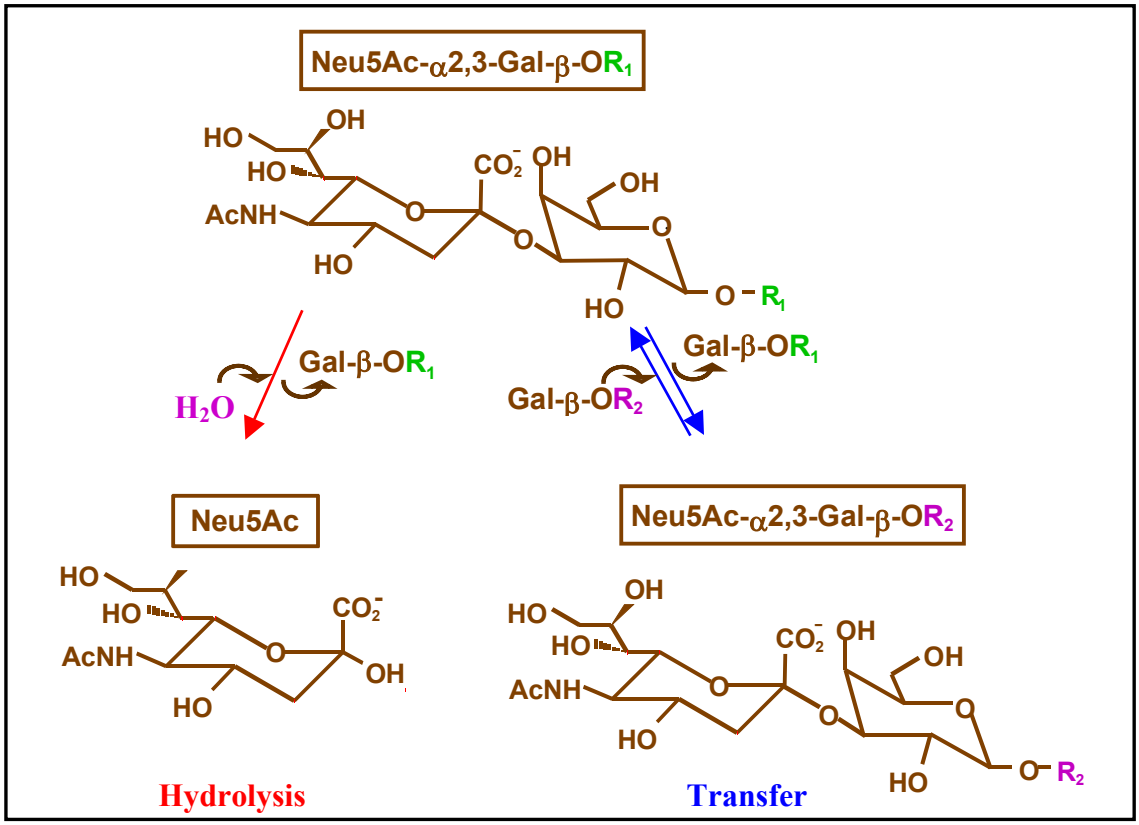

Fig. 3: SA and transfer activities displayed by trans-sialidases

TS was first described in American and, subsequently, in African trypanosomes [13;14;52]. Moreover, single reports on TS activity in *Endotrypanum* species, a parasite of

forest dwelling tree sloths [53], as well as in *Corynebacterium diphtheriae* [54] and in human plasma [55] have been published.

1.3. Trypanosomes

Trypanosomes are parasitic flagellates that belong to the order kinetoplastida, so called because of the large DNA-containing structure, the kinetoplast, found at the base of the flagellum [56]. The kinetoplast protozoa represents one of the earliest extant groups of eukaryotes containing mitochondria [57]. Two suborders exist within the kinetoplastids, the Bodonina and the Trypanosomatina. There are approximately eight genera within the family Trypanosomatidae (Table 1).

Table 1: Taxonomy of Kinetoplastid Protozoa

Order	Suborder	Family	Genus
Kinetoplastida	Bodonina	Bodonidae	Bodo Ichtyobodo
		Cryptobiidae	Cryptobia Trypanoplasma
	Trypanosomatina	Trypanosomatidae	Trypanosoma Leishmania Endotrypanum Crithidia Blastocrithidia Leptomonas Herpetomonas Phytomonas

TS was first described in the American trypanosome *Trypanosoma cruzi* (Fig. 4) [13] and since then studied thoroughly [58]. Similarly, TS has also been found in African trypanosomes like *Trypanosoma brucei gambiense, Trypanosoma brucei rhodensiense, Trypanosoma brucei brucei* and *Trypanosoma congolense* (Fig. 4) [13;14;14;52].

Interestingly, TS does not occur in all trypanosoma species, such as *Trypanosoma evansi* and *Trypanosoma equiperdum,* and *Trypanosoma rangeli* only expresses SA activity, but no TS (Fig. 4) [14;59;60].

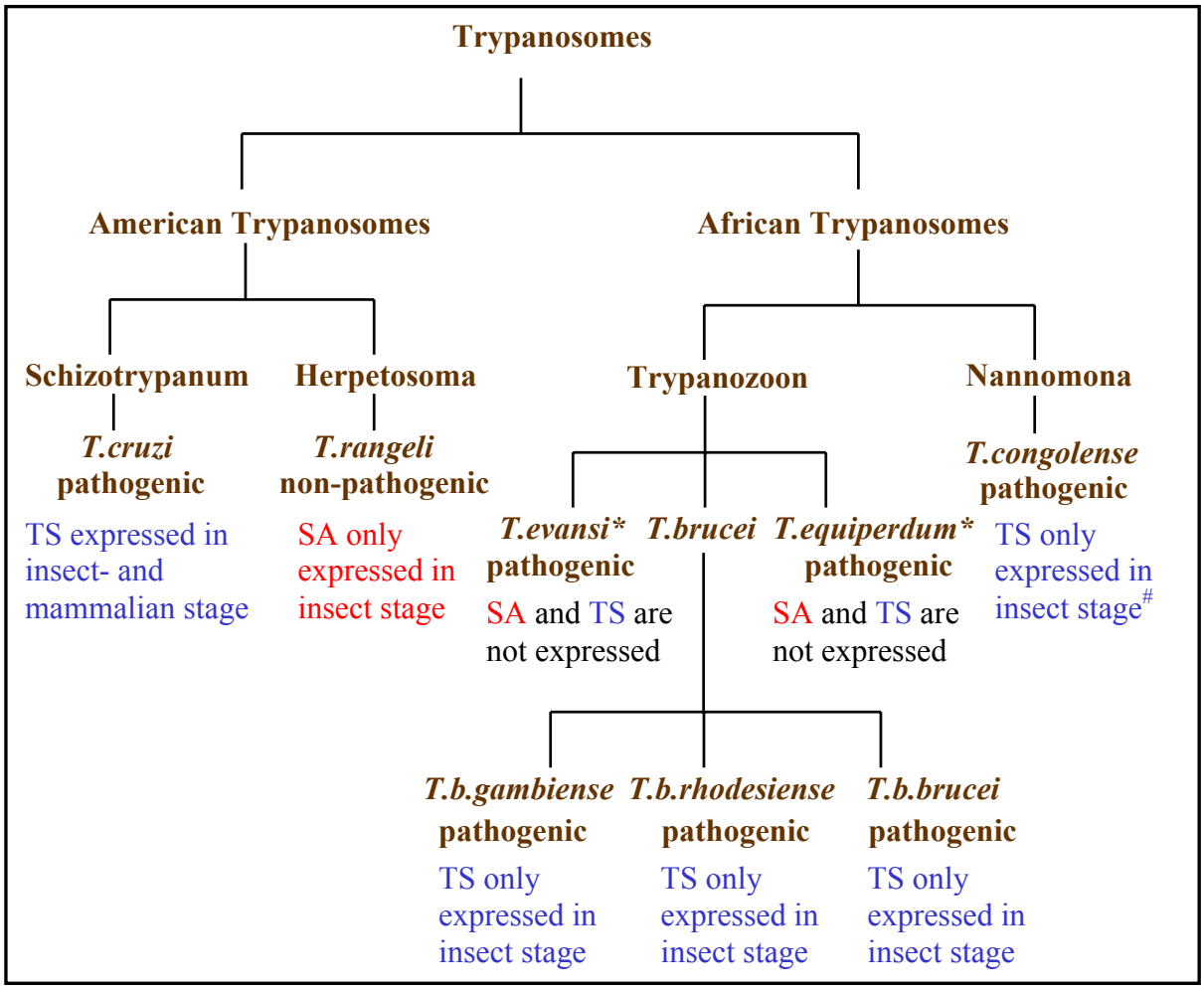

Fig. 4: Overview of trypanosoma species mentioned in the text
* only mammalian stage, # 1.3.3. (life cycle)

1.3.1. Diseases caused by trypanosomes

Trypanosomes have been around for more than 300 million years and are ubiquitous parasites of insects, plants, birds, fish, amphibians and mammals. Fortunately, only a few species of trypanosomes are pathogenic [61].

Trypanosoma cruzi, the etiologic agent of Chagas disease (American trypanosomiasis), is responsible for a chronic debilitating, incurable disease afflicting millions of people in Latin and South America [62]. In Africa the parasites *Trypanosoma brucei*

gambiense and *Trypanosoma brucei rhodesiense* are the causes of the West and East African human sleeping sickness, respectively (African Trypanosomiasis) (Fig. 5A) [61]. According to the World Health Organisation in certain provinces of Angola and Southern Sudan sleeping sickness has become the first or second greatest cause of mortality, ahead of HIV/AIDS. Nagana, the trypanosomiasis in African ruminants (Fig. 5B), is caused by three trypanosome species *Trypanosoma vivax*, *Trypanosoma brucei brucei* and *Trypanosoma congolense* [61]. The disease occurs over about one third of the African continent and it is estimated that nearly one third of Africa's cattle and more than twice as many small ruminants are at risk of infection. Because of the human suffering, and as a constraint on development in Africa, trypanosomiasis in livestock ranks alongside the major human parasitic diseases [63].

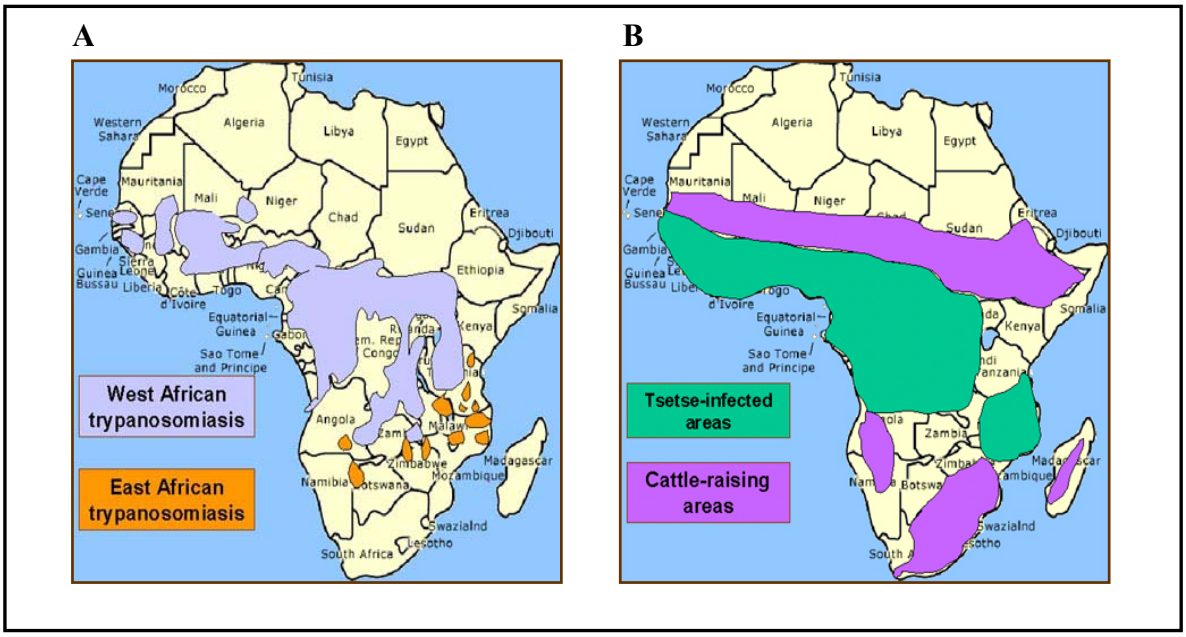

Fig. 5: A, Distribution of West (*T. b. gambiense*) and East (*T. b. rhodesiense*) African sleeping sickness. B, Distribution of tsestse fly, which corresponds to the occurrence of Nagana (green) and Nagana-free, cattle raising areas (purple)

Both, American and African trypanosomiasis exhibit similar symptoms and pathogenicity. Usually the diseases begin with unspecific symptoms such as headaches, chills, fever and bone and muscle pain, leading to the characteristic swelling of the lymph nodes.

The second phase of the diseases starts when the parasites cross the blood brain barrier and infest the central nervous system (CNS). Characteristic symptoms like confusion, sensory disturbance (e.g. disruption of the sleep cycle) and poor coordination appear. This chronic phase is mainly seen in adults and lasts for several years, however, during this period the gradual loss of muscle tone leads to death due to heart failure [64]. Similar symptoms to that observed for American and African trypanosomiasis, like anemia and loss of weight, occur in cattle suffering from Nagana, which stands for "loss of spirit" in the Zulu language [65].

1.3.2. Treatment of American and African trypanosomiasis and Nagana

Presently there is no potent vaccine against trypanosomes available and alternatives to the chemotherapy for the treatment of Chagas disease and human sleeping sickness are urgently needed. Currently available drugs are active in acute or short term chronic infections, but have very low antiparasitic activity against the prevalent chronic form of the disease, and toxic side effects are frequently encountered [66]. This is due to the fact that the anti-parasitic activity of the available medicine (nitrofurans and nitroimidazoles) is inextricable to mammalian host toxicity [66].

Only a couple of drugs have been licensed to treat the diseases (reviewed in [64;67;68]). Two of the compounds, pentamidine and suramin, are used prior to CNS involvement. The arsenic-based drug, melarsoprol, which is in use since 1949, is applied once parasites are established in the CNS. The alternative, eflornithine, is better tolerated but difficult to administer, as well as being only effective against late stage disease caused by *T. b. gambiense*, whereas ineffective against *T. b. rhodesiense*. Another drug, nifurtimox, is a cheap, orally administered drug not yet fully validated for the treatment of human African trypanosomiasis, but already employed in trials against melarsoprol-refractory late stage disease [67]. Currently, efforts are being made to establish an oral form of eflornithine for the treatment of *T. b. gambiense* [68], as well as to finalise the development of a new triazole

derivative with selective effect on *T. cruzi*`s own sterol biosynthesis by inhibiting the sterol C14α-demethylase of the parasite [66].

Chemotherapy of Nagana has been reliant for over 40 years on diminazene, isometamidium and homidium. Due to the intensive use and structural similarities of these drugs, trypanosomes have developed multiple drug resistance in Ethiopia, Kenya, Somalia and many other African countries. However, recently, it has been found that anti-trypanosome cysteine proteinase antibodies may modulate the trypanosome-induced pathology in cattle [65]. The treatment of Nagana has mainly been focussed on the reduction of the tsetse fly vector, only capable of flying short distances, by spraying with the pesticide DDT or clearing of bush in order to produce bush-free belts to isolate the area. Other methods of control include removing reservoir host from the area and breeding resistant stock animals [69;70].

1.3.3. Life cycle of trypanosomes

Trypanosomes are successful parasites which manage to survive in the vector and to escape the host`s immune response. Different trypanosoma species utilise different vectors and hosts, that is, the American trypanosome *T. cruzi* is transmitted by *Triatoma infestans*, whereas the African trypanosomes *T. b. brucei* and *T. congolense* are transmitted by *Glossina* spp. [62;69]. Moreover, trypanosomes show variations in their life cycles. Therefore, in the following section the particular life cycle of *T. congolense* will be discussed in detail (Fig. 6). This cycle is very similar to the life cycle of *T. b. brucei* [61], but different to *T. cruzi* [62].

The infection starts when trypanosomes reach the skin of the vertebrate host due to the bite of an infected tsetse fly (*Glossina* spp.). Parasites move from the site of infection to the draining lymphatic vessels and blood stream. The parasites proliferate within the bloodstream and later invade other tissues including the CNS of the host [64]. *T. congolense* exist in the vertebrate host as infective, metacyclic forms located in the blood stream (Fig. 6). The surface

coat of bloodstream forms of the trypanosomes consists of VSG`s (Variable Surface Glycoproteins), which are encoded by probably 1000 VSG genes [61].

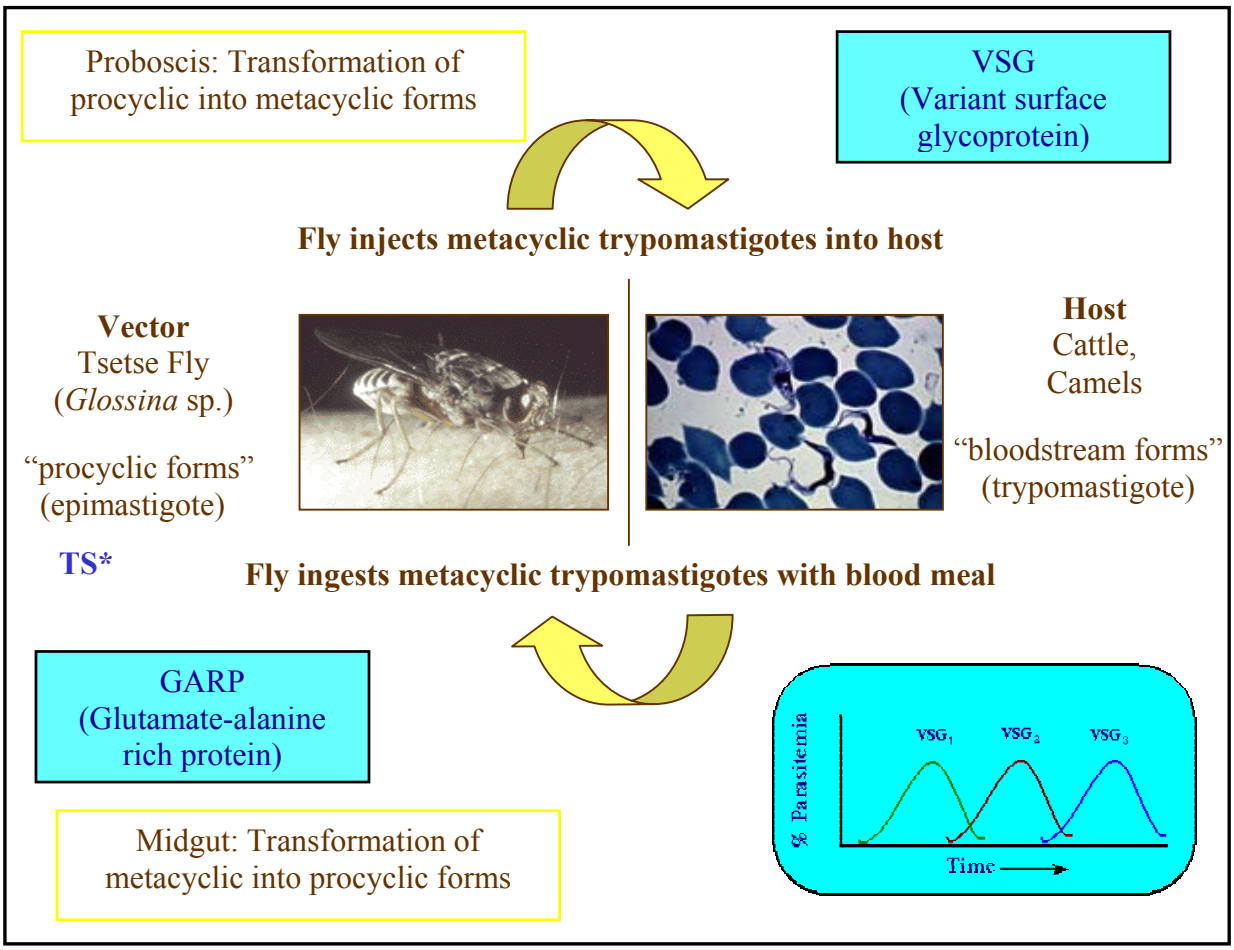

Fig. 6: Life cycle of *Trypanosoma congolense*
***** TS is only expressed in the insect stage

If, for example, some trypanosomes possess VSG "1" on their surface, and the immune system raises antibodies against all of the population`s antigens, as a result most of the parasites die. A few trypanosomes, however, change their coat by expressing VSG "2". Due to the expression of the VSG "2" these parasites survive and give rise to a new population. By the time the host raises antibodies against the new VSG coat (VSG "2"), some of the trypanosomes change their surface coat again and survive [71]. The changes in VSG expression allow *T. congolense* to elude the immune response [72]. Like the African

trypanosomes *T. congolense* and *T. b. brucei*, *T. cruzi* escapes from the host immune response, but not by changing the coat, instead, by hiding inside cells [56].

With the blood meal metacyclic trypomastigotes get transmitted from an infected host to the tsetse fly (Fig. 6). The trypanosomes reach the midgut of the fly, loose the VSG and transform into non-infective, procyclic forms [61]. The procyclic forms are covered by GARP (glutamic acid-alanine-rich protein), which is the major surface glycoprotein of the insect stage of *T. congolense* [73]. Subsequently, the trypanosomes move to the proboscis of the tsetse fly and replicate (Fig. 6). Following replication, they start to express VSG again and transform into infective, metacyclic forms. With the bite of an infected tsetse fly the trypanosomes reach the skin of the vertebrate host from where the infection of the host starts again [64].

Interestingly, the expression of TS is developmentally regulated. Only during its insect stage *T. congolense* produces TS [14]. This is similar to *T. b. brucei* [14], but different to *T. cruzi*, which is the only species that expresses TS in both, its insect (procyclic form) and mammalian stage (bloodstream form) [62]. Trypanosomes are unable to synthesize sialic acids [11], instead they utilize TS to transfer sialic acid from the environment onto trypanosomal surface molecules (see next section). With that TS is believed to play a role for the survival of the parasites inside the vector and, in the case of *T. cruzi*, also in the host.

1.4. Function of Trans-sialidase

In the African species *T. b. br*ucei and *T. congolense*, where TS is only expressed in the procyclic insect stage, the enzyme is used to sialylate the major cell surface glycoprotein of the parasites (e.g. *T. b. brucei,* PARP; *T. congolense,* GARP) in the vector (tsetse fly) [14]. Thus, a negatively charged glycocalyx is formed, which is believed to protect the parasites from digestive conditions in the fly gut and enables them to interact with epithelial cells

[52;74]. In the case of *T.cruzi*, TS is employed to acquire sialic acid from mammalian host glycoconjugates to sialylate mucin-like acceptor molecules in the parasite plasma membrane [32]. Furthermore, TS sialylates host cell glycoconjugates to generate receptors, which are used for parasite adherence and subsequent entry into host cells [58].

Since *T. cruzi* TS was the first TS described, with the recombinant enzyme now being available, the function of TS has mainly been studied for *T. cruzi* TS. It has been shown, that killing of trypanosomes mediated by the lytic antibodies anti-(α-Gal) is specifically decreased by parasite surface coat sialylation. Sialylation does not affect survival of *T. cruzi* either at low pH or in the presence of H_2O_2, but increases survival in the presence of agents that generate NO [75]. Furthermore, antibodies that inhibit *T. cruzi* TS activity reduced mammalian cell invasion *in vitro* [76], as well as TS from *T. cruzi* induces apoptosis in cells from the immune system *in vivo* [77].

Additionally, it has recently been reported that *T.cruzi* TS itself directs neuronal differentiation in PC12 cells [78], stimulates IL-6 secretion from normal human endothelial cells [79], as well as potentiating T cell activation through antigen-presenting cells [80]. These results suggest that TS drives the polyclonal lymphocyte activation in acute *T. cruzi* infection, a phenomenon contributing to the pathogenesis of Chagas` disease [80].

Given that trypanosomiasis has reached epidemic proportions in some countries, the development of various TS inhibitors could not only serve in combating trypanosomes inside the host, in the case of *T.cr*uzi, but also inside the vector, in the case of *T. b. br*ucei and *T. congolense.*

1.5. Application of Trans-sialidase

Investigating TS is of major scientific significance not only because of its involvement in the pathogenicity of trypanosmes, but also because of its biotechnological capability. It was

demonstrated in the past that the synthesis of glycosidic linkages can be achieved using glycosidases and glycosyltransferases and that enzyme-catalysed formation of glycosidic linkages offers, in comparison to classical chemical methods, several advantages (e.g. performance of the synthetic step in aqueous solutions, avoidance of intermediate purification) [81].

TS is able to transfer Neu5Ac in a stereo- and regio-specific manner and because of this can be utilised for the synthesis of a variety of biologically relevant structures of the type Neu5Acα2,3Galβ1-R. Using an α2,3-specific TS from *T. cruzi* a variety of *N*-linked oligosaccharides have been synthesised [82-84]. Additionally, it was demonstrated, that the introduction of β-galactosidase in the TS catalysed sialylation improved the yields of the desired sialylated products [85]. Other articles reported the sialylation of T- and T_N- antigen (T: Galβ1,3GalNAcα-O-Ser/Thr, T_N: GalNAcα-O-Ser/Thr) using bacterial sialidases [86] and human and mouse recombinant sialyltransferases [87]. T (Thomsen-Friedenreich), sialyl-T, T_N (Thomsen noveau) and sialyl-T_N are known to be tumor associated carbohydrate antigens [88-91] and have been detected on the surface of common human malignant tumors [92]. Therefore studies to design synthetic and semi-synthetic oligosaccharide epitopes for immunological testing, as well as for the development of synthetic carbohydrate based anticancer vaccines have been increased [93;94].

A further application for TS could be the sialylation of human milk compounds. As an example an enzymatic approach for the complete synthesis of the trisaccharide 3`sialyl-*N*-acetyllactosamine combining *Bacillus circulans* β-galactosidase and *T. cruzi* TS has been described [95]. As a major constituent of human milk, this trisaccharide, as well as other sialylated oligosaccharides (e.g. sialylated lacto-*N*-tetraose and lacto-*N*-neotetraose) play an important role in the immune response of infants against bacterial and viral infections in the gastrointestinal and urinary tract [96-100].

Moreover, Vetere *et al.* (1997) reported on the synthesis of Neu5Acα2,3Gal β1,4-xylosyl-p-nitrophenylβ(1-R) as a potential inhibitor of human skin fibroblast glycosaminoglycan biosynthesis using *Eschericha coli* β-galactosidase and *T. cruzi* TS [101]. Similarly, a recent publication has reported on the use of TS as a potential tool for sialylation of glycoconjugates in the baculovirus-insect cells system [102].

Since the clearance of asialoglycoconjugates represents a problem during therapeutic administration of recombinant glycoproteins, the modification of the oligosaccharides by TS can be used as a powerful tool to delineate the function of carbohydrates in glycoproteins and to engineer, for example glycoprotein/-peptide hormones with a longer half-life and/or higher bioactivity [37].

1.6. State of Research

So far, only two TS have been studied in detail, the American *T. cruzi* TS [50;103;104] and the African *T. b. brucei* TS [52;105;106]. Both have been found to generate multimeric forms (Table 2), however, the reason for the generation of multimeric aggregates of TS, as well as their composition have not been studied. Additional properties of native *T. cruzi* and *T. b. brucei* TS are summarised in Table 2.

With regard to substrate specificity *T. cruzi* TS and *T. b. brucei* TS exhibit a preference for α2,3-linked sialic acid [50;106]. This is in common with most of the usual SA [4]. Sialic acids in α2,6- linkage, on the other hand, do not serve as donor substrates for *T. cruzi* [107], however *T. b. brucei* can utilise α2,6- linked sialic acid, but at an 8 fold lower rate in comparison to α2,3-linked sialic acid [106]. Only ß-linked galactose and *N*-acetylgalactosamine residues present on a variety of oligosaccharides and glycoconjugates are acceptors for both TS, with β1,4-linked galactose-containing acceptors being preferred over

β1,3-linked and β1,6-linked-galactose [107]. Interestingly, *T. cruzi* and *T. b. brucei* TS are not inhibited by Neu2en5Ac and *N*-(4-nitrophenyl)oxamic acid [106;107], which are known inhibitors of bacterial sialidases [4]. Further information concerning the characterisation of native *T. cruzi* and *T. b. brucei* TS is outlined in Chapter 2.

Table 2: Properties of *T. cruzi* and *T. b. brucei* TS

Properties	*T. cruzi* TS[1]	*T. b. brucei* TS[2]
Expression	metacyclic and procyclic	procyclic
Membrane association	GPI anchor	GPI anchor
Molecular weight		
multimeric forms	metacyclic stage: >400 kDa procyclic stage: none	two major activity peaks: 660 and 180 kDa
monomeric forms	metacyclic stage: 100-220 kDa procyclic stage: 90 kDa	between 60-80 kDa
Antibody reactivity	cultivation and stage-specific Ab recognition	no cross reactivity with antiserum and mAb against *T. cruzi* TS

[1]findings reported in [58;62] [2]findings reported in [52;106]

T. cruzi TS is encoded by approximately 140 genes [108;109], at least some of which occur in tandem arrays [110] and on multiple chromosomes [111]. Many of these genes have been found to code for inactive proteins [112]. In contrast, a recent publication reporting on the gene sequence of *T. b. brucei* TS postulates that in this African trypanosome TS genes are present in a low copy number (minimum two) [51]. Generally, the N-terminal domains of *T. cruzi* and *T. b. brucei* TS share up to 30 % identity with bacterial SA [113], possessing the conserved motifs (Asp boxes) described in bacterial, viral and trypanosomal SA [113], as well as the model of *T.cr.*TS displays a β-propeller topology [114] similar to that of bacterial and viral SA [115-117].

The SA expressed by *T. rangeli*, a non-pathogenic relative of *T. cruzi*, has been isolated and characterized biochemically [59] and genetically [118]. So far, no crystal structure for TS exists. However, the crystal structure of *T. rangeli* SA has been used to model the structure of *T. cruzi* TS [114]. Subsequently, a comparison between the crystal structure of *T. rangeli* SA and the model of *T. cruzi* TS has been carried out [114], however, neither the exact mechanism of the transfer reaction nor the reasons why TS is more efficient in transferring than hydrolysing terminal sialic acid are understood. Nevertheless, some active site residues have been shown to be critical for transfer activity [51;114;119;120]. Detailed information concerning these active site residues, as well as further molecular biological data concerning TS are outlined in Chapter 3.

1.7. Project Objectives

Due to the involvement of TS in the pathogenicity of trypanosomes, as well as its important biotechnological capability investigating TS has become increasingly attractive over the past decade.

The primary aim of this study was to purify and characterise TS from another African trypanosome, *T. congolense*, with the subsequent intention of obtaining sequence information for *T. congolense* TS. Sequence information for TS from an additional African trypanosoma species would help to further elucidate the mechanism of TS, as well as confirm or reveal findings about essential residues required for TS transfer activity. This information would enhance the opportunity to develop high potential, structure-based TS inhibitors.

Furthermore, the purification of the native *T. congolense* TS would allow the comparison of its properties with those exhibited by *T. cruzi* and *T. b. brucei* TS. Such information, especially concerning substrate specificities, would be necessary to evaluate the usefulness of *T. congolense* TS for the sialylation of biologically active compounds.

Moreover, as described in previous sections, *T. cruzi* TS consists of a polymorphic family which possess active and inactive members, as well as during purification *T. cruzi* and *T. b. brucei* TS have been shown to form multimeric aggregates. With that in mind, this study was also interested in the identification of different possible members of the *T. congolense* TS family, as well as in the composition and importance of multimeric aggregates of *T. congolense* TS.

In Chapter 2 the purification and characterisation of two *T. congolense* TS forms, as well as the production of specific anti-*T. congolense* TS mAb are described. Additionally, Chapter 3 outlines the elucidation of two partial *T. congolense* TS gene sequences. Both chapters represent manuscripts submitted for publication, whereas Chapter 4 combines unpublished data relevant to TS purification and molecular biology.

1.8. References

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CHAPTER 2

Publication:

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

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Declaration of my contribution to the publication

The experiments stated in this manuscript were designed, processed and interpreted by me, except for the production, partial detection (ELISA test) and isotyping of the monoclonal antibody which was done by Dr H. Lange and Dr H. Lemke and the substrate specificity studies which were performed by Dr S. Schrader. I have written the publication in collaboration with all co-authors. However, in the section "Experimental Procedures" the single part concerning the monoclonal antibody production was added by Dr H. Lange.

2.1. Abstract

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

2.2. Introduction

The flagellated protozoa, trypanosomes, the agents of several diseases, express a unique type of glycosyltransferase, called trans-sialidase (TS)[1], which is believed to play an important role in maintaining pathogenicity of the parasites [1;2]. Unlike typical sialyltransferases, which require CMP-activated sialic acid as the monosaccharide donor [3], TS catalyses the transfer of, preferably, α2,3-carbohydrate-linked sialic acids to another carbohydrate forming a new α2,3-glycosidic linkage to galactose or *N*-acetylgalactosamine. In the absence of an appropriate acceptor TS acts like a sialidase (SA), similar to viral, bacterial, mammalian and trypanosomal SA, hydrolyzing glycosidically linked sialic acids [1;2].

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

Trypanosomes are unable to synthesize sialic acids, instead they utilize TS to transfer sialic acid from the environment onto trypanosomal surface molecules. In the case of *T.cr*., TS is employed to acquire sialic acid from mammalian host glycoconjugates to sialylate mucin-like acceptor molecules in the parasite plasma membrane [8]. Furthermore, TS sialylates host cell glycoconjugates to generate receptors, which are used for parasite adherence and subsequent entry into host cells [2]. In the African species *T.b.br*. and *T.con*., where TS is only expressed in the procyclic insect stage, the enzyme is used to sialylate the

major cell surface glycoprotein of the parasites (e.g. PARP, GARP) in the vector (tsetse fly). Thus, a negatively charged glycocalyx is formed, which is believed to protect the parasites from digestive conditions in the fly gut, or from substances present in the fly`s blood meal, and enables them to interact with epithelial cells [6;9]. Additionally, it has recently been reported, that *T.cr.*TS itself directs neuronal differentiation in PC12 cells [10], stimulates IL-6 secretion from normal human endothelial cells [11], as well as potentiating T cell activation through antigen-presenting cells [12].

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

To date, only two trypanosomal TS have been studied in detail, the American *T.cr.*TS [15-17] and the African *T.b.br.*TS [6;7;18], with different genes encoding *T.cr.*TS [19-21] and *T.b.br.*TS [22] being identified and analyzed. Furthermore, the SA expressed by *Trypanosoma rangeli* (*T.r.*), a non-pathogenic relative of *T.cr.*, has been isolated and characterized biochemically [23] and genetically [24]. Although a comparison of the crystal structure of *T.r.*SA with a modeled structure of *T.cr.*TS has been carried out [25], neither the exact mechanism of the transfer reaction nor the reasons why TS is more efficient in transferring than hydrolyzing terminal sialic acid are understood.

Since native and recombinant enzymes can differ in their glycosylation, antibody specificity and biochemical properties, it is important that the native enzyme be purified and characterized, with the subsequent aim of obtaining sequence information. This is especially important, as several genes encoding TS/SA enzymes, or even silent genes may exist in trypanosomes, as has been shown for *T.cr.* [26]. Here we describe the purification and characterization of two TS forms from the African trypanosome *T.con.* and their identification

using micro-sequencing. Moreover, we report on the production of monoclonal antibodies raised against both enzyme forms and their subsequent use in purification. Additionally, we present characterization studies which reveal significant differences between both TS forms concerning their transfer to SA ratios and catalytic efficiencies using various acceptor substrates.

2.3. Experimental Procedures

2.3.1. Materials

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

2.3.2. Substances

2`(4-methylumbelliferyl)lactoside (MULac) was provided by Dr T. Yoshino (Tokyo, Japan), 4-amino-Neu2en5Ac and 4-guanidino-Neu2en5Ac by Dr M. von Itzstein (Gold Coast, Australia), suramin was a gift from Dr P. Nickel (Bonn, Germany) and recombinant *T.cr.*TS and *T.b.br.*TS were a gift from Dr A.C.C. Frasch (Buenos Aires, Argentina). Neu5Acα2,3-lactose (α2,3-SL) and Neu5Acα2,6-lactose were isolated from cow colostrum according to Veh *et al.* (1981) [27]. Neu5Acα2,3-*N*-acetyl-lactosamine was purchased from Dextra-Laboratories (Reading, UK) and fetuin from ICN. Sialyl-oligosaccharides from bovine and

human milk, as well as glycomacropeptide and apolactoferrin were provided by Milupa GmbH & Co. KG (Friedrichsdorf, Germany). Sialyl-Lewis^x, *N*-acetyllactosamine, lacto-*N*-biose I, lacto-*N*-neotetraose, lacto-*N*-tetraose, lactose, lactitol, mannose, galactose, glucose, maltose, galactose-α1,4-galactose and Neu5Ac were obtained from Calbiochem-Novabiochem GmbH (Bad Soden, Germany). Chondroitin sulfate A, heparan sulfate, dextran sulfate, heparin (high and low molecular weight), Neu5Ac, Neu2en5Ac, 2`(4-methylumbelliferyl)galactoside (MUGal) and *N*-(4-nitrophenyl)oxamic acid were purchased from Sigma.

2.3.3. Antibodies

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

2.3.4. Cultivation

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

2.3.5. Assays

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

SA activity was routinely tested in the presence of 1 mM MUNeu5Ac in 20 mM Bis/Tris buffer, pH 7.0 [29]. The reaction mixture was incubated for 120 min at 37 °C in black 96-well-plates (Microfluor, Dynex, U.S.A.). By the addition of 0.08 M glycine/NaOH buffer, pH 10, the reaction was terminated and the fluorescence of MU released measured immediately at an excitation and emission wavelength of 365 nm and 450 nm, respectively. The instrument was calibrated with MU standard solutions. One unit of SA activity equals one μmol of MU released per minute, which is equivalent to 1 μmol of sialic acid released per minute.

TS activity was routinely tested using the non-radioactive assay described by Schrader *et al.*[2]. Briefly, TS activity was monitored by incubating 25 μl of enzyme solution in 50 mM Bis/Tris buffer, pH 7.0, containing 1 mM α2,3-SL as the donor and 0.5 mM MUGal as the acceptor in a final volume of 50 μl at 37°C for 2 h. The reaction was terminated by the addition of ice cold water and, subsequently, applied to mini-columns containing Q-Sepharose FF. After washing, the sialylated product was eluted with 1 M HCl, hydrolyzed at 95°C for 45 min and cooled on ice. The samples were neutralized, adjusted to pH 10 and MU released was measured as stated above. One unit of TS activity equals one μmol of MU released per minute, which is equivalent to 1 μmol of sialic acid transferred per minute. During the course of this study the TS test described above was modified by applying the assay principle to a 96-well-plate format. Because of its enhanced throughput all TS tests for

mAb screening, as well as kinetic experiments were performed using the 96-well-plate assay (Schrader *et al.*)[2].

Protein concentration was determined using either the BCA protein assay kit from Pierce (Cologne, Germany) or the Bio-Rad protein assay [30] from Bio-Rad (Munich, Germany), as described by the manufacturer. All assays were performed in 96-well-plates employing BSA as the standard, and photometric determination were performed using a 96-well-plate photometer (Tecan Sunrise, Tecan Deutschland GmbH).

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

2.3.6. Separation and purification of the two TS forms

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

The separation of two major TS activity peaks was provided by chromatography on Q-Sepharose FF. The concentrated culture supernatant was applied to a column (2 x 20 cm) of Q-Sepharose FF, equilibrated with 20 mM Bis/Tris buffer, pH 7.0, at a flow rate of 0.6 ml/min. Following extensive washing bound TS activities were eluted using a 600 ml continuous NaCl gradient (0 to 0.8 M) in 20 mM Bis/Tris buffer, pH 7.0. Fractions of 6 ml were collected and analyzed for transfer and SA activity. A larger Q-Sepharose column could

not be employed due to poor separation of the two TS forms. Therefore, several Q-Sepharose runs were performed using the column size stated above, with separated TS-form 1 and TS-form 2 following each run being combined and further purified individually by isoelectric focusing (IEF).

Isoelectric focusing was carried out in a 16 ml Rotor cell (Rotofor Preparative Isoelectric Focusing Cell, Biorad) using ampholytes which provided a pH range between pH 4-6 (Biolyte pH 4-6, Biorad). The buffer contained in the collected fractions was immediately exchanged, fractions concentrated and activity determined. Active fractions were pooled and further purified by gel filtration chromatography.

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

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

BALB/c (H-2^d) mice, obtained from Harlan/Winkelmann (Borchen, Germany) and reared under conventional conditions, were used for the production of monoclonal antibodies (mAb). Female BALB/c mice 6 weeks of age were injected three times intraperitoneally with 25 µg of the partially purified TS forms adsorbed to 2 mg $Al(OH)_3$ (Imject ®Alum, Pierce, Rockford, USA). Three days after the last injection spleen cells of one mouse was fused with non-secretor Ag8.653 myeloma cells [32] by the conventional polyethylene glycol-mediated fusion technique [33]. After fusion, cells were plated in 288 wells of 24-well hybridoma

plates (Greiner, Nürtingen, Germany) in RPMI 1640 supplemented with 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycine, 100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine. The medium was further supplemented with 10 % conditioned medium from the J774 cell line. Wells containing antigen-specific IgG-secreting hybridomas were identified *via* ELISA using mouse-IgG-specific antiserum and an enzyme immunoassay (TS activity binding assay). Clones in positive wells were subcloned and reanalysed.

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

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

2.3.8. Immunoaffinity chromatography

Purified anti-*T.con.*TS (mAb 7/23, 24 mg) were incubated for 2 h with rProtein A-Sepharose FF (5 ml) and equilibrated with binding buffer (20 mM Na_2HPO_4, NaH_2PO_4, pH 7.0). Following washing with 70 ml of binding buffer, the matrix was further washed with cross-linker buffer (0.2 M triethylamine, pH 8.5) and, subsequently, incubated with 10 ml of cross-linker reagent (20 mM dimethyl pimelimidate in cross-linker buffer) at room temperature for 1 h. The cross-linking reaction was terminated by washing the column with 70 ml of 0.2 M ethanolamine, pH 9, followed by 70 ml of binding buffer and 70 ml of Na citrate buffer, pH 3.0. A flow rate of 1-1.25 ml/min was used at all stages of matrix preparation. The column was washed with binding buffer prior to immunoaffinity chromatography.

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

2.3.9. Micro-sequencing

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

2.3.10. Kinetic studies

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

2.3.11. Donor and acceptor substrate specificities and inhibitor studies

A number of glycoconjugates, as well as mono- and oligosaccharides (🗐 2.6., Table 4) were assayed as potential donors using the TS assay described. Known viral and bacterial SA inhibitors, as well as salts (NaCl, KCl), cations (20 mM Ca^{2+}, Mg^{2+}, Mn^{2+}; 5 mM Cu^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}) and other compounds including anti-*T.con.*TS mAb (0-20 µg/ml) were assayed for their ability to inhibit TS activity using essentially the standard TS assay described, except additives were pre-incubated in the assay mixture for 30 min at room temperature prior to starting the reaction. Potential TS acceptors (🗐 2.6., Table 4) were assayed in a similar manner to that described for potential inhibitors.

2.3.12. SDS-Polyacrylamide Gel Electrophoresis (PAGE) and immunoblot analyses

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

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

2.4. Results

2.4.1. Cultivation

In axenic cultures the trypanosomes grow as flagellate forms called epimastigotes, which correspond to the forms found in the guts of blood-sucking vectors (procyclic or insect forms) [37]. In contrast to the African trypanosome *T.b.br.*, where TS activity was found to be only membrane-bound [6;7], TS activity in procyclic forms of *T.con.* was detected in the culture supernatant and membrane-bound [5]. During the cultivation of procyclic forms of *T.con.* in FCS/hemin-containing media the cell number increased during 3 to 4 days from 1 x 10^6 cells/ml to 7 x 10^6 cells/ml, whereas the parasites died within 3 to 4 days when cultivated in FCS/hemin-free media. However, in both cases the parasites did produce enzymatic activity during cultivation. When cultivating the parasites in FCS/hemin-containing media 84 % of transfer activity and 97 % of SA activity were measured in the culture supernatant. In contrast, cultivation of the trypanosomes in FCS/hemin-free media resulted in only 25 % of transfer activity and 60 % of SA activity being observed in the culture supernatant. In both cases the residual activity was bound to the cell pellet. However, in the culture supernatant derived from the cultivation without FCS/hemin the specific enzymatic activity was 4 times higher than that seen in the culture supernatant obtained from FCS/hemin-containing media. Therefore, and because of the quenching effect of hemin on enzyme activity assays, parasites were cultivated in FCS/hemin-free media.

2.4.2. Separation and purification of two TS forms from *T. congolense*

The separation and partial purification of the two TS forms, summarized in Table 1 (🗐 2.6.), was achieved by employing a combination of anion exchange chromatography, isoelectric focusing, gel filtration and, subsequently, immunoaffinity chromatography. Ion

exchange chromatography was chosen as a first step in the purification cascade, mainly because of its high capacity, but also due to its ability to sufficiently separate the two enzyme forms (2.6., Fig. 1A). Activity-positive fractions eluting at a salt concentration higher than 0.3 M were combined and referred to as TS-form 1, whereas TS active fractions eluting at a salt concentration below 0.3 M were combined and called TS-form 2. As can be seen in Table 1 (2.6.), following Q-Sepharose a difference in the transfer to SA activity ratios for both TS forms could already be observed. That is, TS-form 1 and TS-form 2 exhibited a transfer to SA activity ratio of 17 and 2.4, respectively. Even though no significant enrichment of transfer and SA activity was obtained, this first chromatography step provided effective separation of TS-form 1 and TS-form 2.

Following ion exchange chromatography each form was treated independently. Further purification of TS-form 2 was achieved by IEF (2.6., Table 1), however IEF was not particularly effective at further enriching TS-form 1, instead only leading to a loss of TS activity, and was therefore not used in the purification of TS-form 1. The isoelectric points obtained for TS-form 1 and 2 were found to be pH 4-5 and pH 5-6.5, respectively (2.6., Fig. 1C).

The two TS forms were further purified independently using gel filtration on Sephadex-G150 SF. As shown in Fig. 1B and Fig. 1D (2.6.), the molecular weights of TS-form 1 and 2 were found to be 350-600 and 130-180 kDa, respectively. The transfer activities of TS-form 1 and form 2 were purified by 30- and 150-fold, respectively, but interestingly in the case of TS-form 1 only very low amounts of SA activity could be detected (2.6., Table 1). This shows that after complete separation and partial purification of the two TS forms both possessed very different transfer to SA activity ratios (8000 for TS-form 1; 45 for TS-form 2). The SDS-gel in Fig. 2 (2.6.) depicts the protein pattern during the various stages of purification. For both TS forms a clear enrichment of a 90 kDa band on SDS-PAGE under

reduced conditions was observed (2.6., Fig. 2, lane 1-3, 5-7). However, a number of contaminating proteins still remained. Therefore a further specific purification step was sort.

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

Both partially purified TS forms were used for mAb production, with a combination of ELISA and the TS binding test, described under Experimental Procedures, being employed for the detection of TS-specific antibodies. Clone 7/23 was found, using the TS binding test, to reduce TS activity in a TS containing sample by 75 %. TS activity present on the Dynabeads was also detected for clone 7/23. Monoclonal Ab 7/23 was found to belong to the subclass IgG2b. Additionally, the V region of mAb 7/23 sequenced was analyzed (V_L sequence: AY198310; V_H sequence: AY198309). After resolving the concentrated supernatant, as well as the two separated TS forms on SDS-PAGE, immunoblotting with anti-*T.con.*TS mAb (mAb 7/23) led to the staining of a single protein band at about 90 kDa, which had been shown to be enriched after purification of the two TS forms (2.6., Fig. 2 and 3A). In contrast, anti-*T.con.*TS mAb did not cross react with the 70 kDa band representing recombinant *T.cr.*TS and the 80 kDa band representing *T.b.br.*TS (2.6., Fig. 3B). Furthermore, immunoblotting analysis revealed that employing anti-*T.cr.*TS antiserum a 70 kDa protein band representing recombinant *T.cr.*TS was detected, whereas the 90 kDa protein band of the two *T.con.*TS forms was not (2.6., Fig. 3C). These results are very similar to that seen for antiserum and mAb raised against *T.cr.*TS which showed no cross-reactivity with *T.b.br.*TS [6].

2.4.4. Immunoaffinity chromatography

Immunoaffinity chromatography employing the anti-*T.con.*TS mAb 7/23 was used for further purification of the two TS forms. For both forms the majority of TS activity was eluted using 20 mM Na citrate buffer, pH 3.0. The transfer activities of TS-form 1 and TS-form 2 were enriched by 1071- and 4200-fold, respectively, whereas the SA activity which could only be measured for TS-form 2, was enriched by approximately 28-fold. The two purified TS forms migrated with an apparent molecular weight of 90 kDa on SDS-PAGE (🗐 2.6., Fig. 2, lane 4 and 8). However, TS-form 1 which was purified to apparent homogeneity provided a far greater recovery and was therefore used for micro-sequencing.

2.4.5. Micro-sequencing

Following the purification scheme outlined in Table 1 (🗐 2.6.) the 90 kDa protein of TS-form 1 (🗐 2.6., Fig. 2, indicated with an arrow) was excised following SDS-PAGE and peptides were analyzed after in-gel trypsin digestion. Subsequently, a peptide with the amino acid sequence VVDPTVVAK, in which the mass data showed a high similarity with the mass data of a peptide from *T.b.br.*TS, was obtained. Additionally, a BLAST database search revealed that the observed peptide showed 100 % sequence identity with a peptide from *T.b.br.*TS (aa 193–201), as well as with a peptide from one of two *T.con.*TS gene sequences (*T.con.*TS1) obtained using a PCR-based cloning approach[3] (🗐 2.6., Table 2). With that, the 90 kDa protein of TS-form 1, as well as indirectly through immunoblot analysis the 90 kDa protein of TS-form 2, were identified as a TS. In contrast, the analyzed peptide did not show 100 % sequence identity with peptides from *T.r.*SA, *T.cr.*TS and a second *T.con.*TS sequence[3] (*T.con.*TS2) (🗐 2.6., Table 2).

2.4.6. Immunoblotting with anti-*T.congolense* GARP monoclonal antibodies

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

Concentrated culture supernatant, and TS forms at various stages of purification, were analyzed by immunoblotting with anti-*T.con*. GARP mAb. Under reducing conditions GARP migrates as a 28-32 kDa protein band on SDS-PAGE [39] and as can be seen in Fig. 4 (🗐 2.6.) a protein band at about 30 kDa was detected. Surprisingly, GARP was only detected in the concentrated culture supernatant and in two purification stages of the higher molecular weight TS form, TS-form 1, with the intensity of the signal increasing proportionally with specific TS activity (🗐 2.6., Fig. 4). This points towards an association between TS-form 1 and GARP under the mild purification conditions used in Q-Sepharose FF and Sephadex-G150 SF chromatography. However, immunoblot analysis of the immunoaffinity-purified TS-form 1 was unable to detect GARP (🗐 2.6., Fig. 4), indicating that the final purification step disrupted the possible association between GARP and TS-form 1. This may have been due to the intensive washing steps (1 M NaCl and 20 mM Na citrate, pH 4.5) used during immunoaffinity chromatography. No bands reacting with anti-*T.con*. GARP mAb were observed in all TS-form 2 samples investigated (🗐 2.6., Fig. 4).

2.4.7. Kinetic studies

Kinetic studies were performed using various donor and acceptor substrates generally employed to determine SA and transfer activities. As can be seen in Table 3 (🗐 2.6.), when

using MUGal as the acceptor, TS-form 1 and 2 bind the donor substrate α2,3-SL with very similar affinities. The K_M values for α2,3-SL of 0.3 mM and 0.2 mM for TS-form 1 and 2, respectively, are in good agreement with that reported for native *T.b.br.*TS (K_M : 0.5 mM) [7]. Both TS forms were found to prefer MULac over MUGal as the acceptor, however, the catalytic efficiency (expressed as app. V_{max}/K_M) is three to four times higher for both acceptors in the case of TS-form 1. TS-form 2 was also found to bind MUNeu5Ac, the donor substrate routinely used to measure the hydrolyzing reaction, with high affinity. A K_M value of 0.09 mM is very similar to that reported for the native *T.b.br.*TS (K_M 0.16 mM) [7].

Kinetic parameters for the hydrolyzing reaction could not be determined in the case of TS-form 1, because of insufficient SA activity. Interestingly, in comparison to TS-form 1 which possessed predominately transfer activity, TS-form 2 was found to hydrolyze sialic acid from MUNeu5Ac 5 times more efficiently than transferring sialic acid from the donor α2,3-SL to GalMU (🗐 2.6., Table 3). These results suggest that not only do the two TS forms consist of different transfer to SA activity ratios, but additionally that TS-form 2 hydrolyzes sialic acid more efficiently than TS-form 1. On the other hand TS-form 2, which is more efficient in transferring sialic acid, behaves similar to the previously reported native and recombinant *T.cr.*TS and *T.b.br.*TS [7;17;22;40].

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

2.4.8. Donor and acceptor substrate specificities

A number of sialoglycoconjugates were tested as potential sialic acid donors for TS form 1 and 2 from *T. con.* (🗐 2.6., Table 4A). Both TS forms revealed no differences in their donor substrate specificities, with the exception of apolactoferrin. As has been previously

observed for *T.cr.*TS [17;41], *T.b.br.*TS [7] and crude *T.con.*TS [5] both isolated *T.con.*TS forms preferably catalyze the transfer of α2,3-linked sialic acid (α2,3-SL and Neu5Acα2,3-*N*-acetyl-lactosamine). Sialic acids in α2,6 linkage also serve as reasonable donor substrates (2.6., Table 4A), which differs from the findings reported for *T.cr.*TS [41]. However, the results are similar to those reported for *T.b.br.*TS which can also utilise α2,6- linked sialic acid, but at an lower rate in comparison to *T.con.*TS [7]. However, as previously reported for the crude *T.con.*TS [5], fetuin served as a good donor substrate with a high transfer rate being observed for both *T.con.*TS forms. The presence of a fucose near the terminal galactose residue (sialyl-Lewis^x) resulted in a decrease in *T.con.*TS activity. This has also been shown for *T.cr.*TS [17] and *T.b.br.*TS [6]. Moreover, sialyl-oligosaccharides from bovine and human milk, as well as the κ-casein glycomacropeptide, known to inhibit bacterial and viral adhesion [42], proved to be excellent sialic acid donors for both TS forms (2.6., Table 4A).

Various substrates were tested for their ability to act as sialic acid acceptors for *T.con.*TS (2.6., Table 4B). Both TS forms were found to possess a similar acceptor preference, however, some acceptor substrate specificity differences were observed between the two forms. In agreement with that reported for *T.cr.*TS [41], β1,4-linked galactose-containing substrates were better acceptors for both *T.con.*TS forms than β1,3-linked galactose-containing substrates (2.6., Table 4B). Lactose and its derivatives serve as good acceptor substrates. In agreement with earlier studies on *T.b.br.*TS [5;7], monosaccharides did not act as sialic acid acceptors for the *T.con.*TS forms, apart from a slight effect using galactose (2.6., Table 4B). Parodi *et al*. (1992) [20] reported that maltose and cellobiose can be sialylated by *T.cr.*TS, however, they did not serve as sialic acid acceptors for both *T.con.*TS forms, even at a concentration of 5 mM. Moreover, the lipooligosaccharides LNnT and LNT (lacto-*N*-tetraose and lacto-*N*-neotetraose), which prevent the adherence of bacteria to epithelial cells, showed good acceptor properties for both TS forms (2.6., Table 4B).

This is of importance because the sialylation of these biological relevant structures increases their survival in blood serum [43].

However, for all acceptor substrates tested TS-form 1 was found to utilize the various acceptors at about 2-fold higher rate. This mirrors the kinetic results summarized in Table 3 (🗐 2.6.), where it is shown that TS-form 1 utilized the various acceptors with a greater efficiency in comparison to TS-form 2.

2.4.9. Inhibitor studies

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

In contrast, the synthetic SA inhibitor *N*-(4-nitrophenyl)oxamic acid inhibited SA activity by 25 % at a concentration of 2 mM, but transfer activity by only 5 % and 10 % for TS-form 1 and 2, respectively. Furthermore, the anti-malaria drug suramin which has previously been shown to be a strong inhibitor of ganglioside SA from human brain tissue (IC_{50} 7 µg/ml) [46], exhibited a slight (17 %) inhibitory effect on the SA activity of TS-form 2 at a concentration of 25 µg/ml, whereas the transfer activity of the two TS forms was not effected.

Anti-*T.con.*TS mAb was found to only slightly inhibit (20 % at 100 µg/ml) the transfer activity of both enzyme forms and had no effect on the SA activity of TS-form 2. Glycosaminoglycans, like heparan sulfate and chondroitin sulfate A, as well as dextran sulfate

are known inhibitors of mammalian sialidases [47], but they were found to have no inhibitory effect, neither on the transfer activity of TS-form 1 and 2, nor on the SA activity of TS-form 2 at a concentration of 25 mg/ml.

The addition of 1 M NaCl or 1 M KCl resulted in the reduction of SA and transfer activities by greater than 50 %, however, full activity could be restored after desalting. Moreover, the addition of 5 mM Co^{2+}, Zn^{2+} and Fe^{2+} inhibited SA and transfer activities of both TS forms by 20-40 %, whereas the addition of 20 mM Ca^{2+}, Mg^{2+} and Mn^{2+} had no effect on SA and transfer activities of both TS forms. This confirms earlier findings that TS are not activated by Ca^{2+} [5;18] as opposed to most viral and bacterial SA that require Ca^{2+} for full activity [48].

2.5. Discussion

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

Cultivation in FCS-containing media resulted in the majority of the enzyme activity being detected in the culture supernatant, whereas when cultivated in FCS-free media *T.con.*TS activity was found to be mainly membrane-associated. It is unclear if *T.con.*

produces a membrane-bound TS, which is GPI anchored and released into the medium due to the action of the parasites` own proteases and phosholipases, or if two different *T.con.*TS species exist, one soluble and one membrane-bound, which are expressed depending on cultivation conditions. Since the GPI anchor has no influence on the enzymatic activity [52] and soluble proteins are easier to purify, we isolated *T.con.*TS from concentrated culture supernatant using FCS-free media because the specific activity of TS was increased.

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

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

In the case of *T.con.* we were able to proof, using micro-sequencing, that the isolated 90 kDa protein of TS-form 2 is indeed a TS. Interestingly, in both *T.con.*TS forms a 90 kDa protein band was detected using the anti-*T.con.*TS mAb 7/23, indicating that TS-form 2, which was not micro-sequenced, could also be identified as TS. However, further investigations are necessary to determine whether the 90 kDa proteins from both forms

represent monomers with the same primary structure and perhaps different folding, or are products of different TS genes, since the anti-*T.con.*TS mAb 7/23 might recognise a common epitope or tertiary structure of both TS forms.

The finding that under mild conditions GARP is co-purified with TS-form 1 seems plausible for a number of reasons. Firstly, GARP is the natural substrate of *T.con*.TS. Secondly, protein complexes concerning various SA [54] and protein-protein interactions involving other glutamic acid rich proteins, also referred to as GARP`s, have been reported [55;56]. Moreover, the possibility that the trypanosomal GARP can mediate or facilitate the formation of oligomers of TS-form 1 is supported by the finding that the interaction between the cGMP-gated channel and peripherin-2 proteins of the rod photoreceptor outer segment of vertebrates are mediated by a glutamic acid rich protein [56]. At this stage, it is unclear whether an association between trypanosomal GARP and TS-form 1 could account for the differences in transfer and SA activity observed for TS-form 1 and 2. However, Poetsch *et al.* (2001) also observed an interaction between mammalian phosphodiesterase and mammalian GARP which led the authors to speculate that this association may play a role in modulating phosphodiesterase activity [56].

The fact that only TS-form 1 is co-purified with GARP may be due to the possibility that only one of the two TS forms, TS-form 1, can interact with GARP. Considering that TS-form 1 has been shown to utilize various acceptor substrates with a higher catalytic efficiency, it is possible that that GARP stabilizes TS-form 1 or that the 90 kDa protein bands of TS-form 1 and 2 actually represent monomers of identical molecular weight, but are encoded by two different genes, from which one codes for a protein with a slightly enhanced acceptor binding capacity (TS-form 1). This notion is sustained by recent findings identifying two different *T.con.*TS gene sequences, which share only 50 % identity with each other[3]. The isolated *T.con.*TS forms were found to have the same donor and acceptor substrate preferences, however, interestingly TS-form 1 utilized the acceptor substrates more efficiently than TS-

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

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

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

Studies on the enzymatic transfer of sialic acid catalyzed by TS, particular substrate specificities, will enhance its biotechnological applications. That is, TS could potentially be utilized for the sialylation of the T- and T_N- antigens, as has been shown using bacterial SA [58] and human and mouse recombinant sialyltransferases [59]. The sialylation of recombinant glycoproteins, as well as human milk compounds like LNT and LNnT could also conceivably be carried out utilizing TS.

Given that trypanosomiasis has reached epidemic magnitude in some countries, one should consider methods to control not only the disease, but also its transmission stage inside the vector [57]. Efforts have been made to establish bush-free belts, in order to reduce the spread of the tsetse fly. Taking this into consideration, the development of various TS inhibitors could not only serve in combating trypanosomes inside the host, in the case of *T.cr*., but also inside the vector, in the case of *T.b.br*. and *T.con.*.

2.6. Figures and Tables

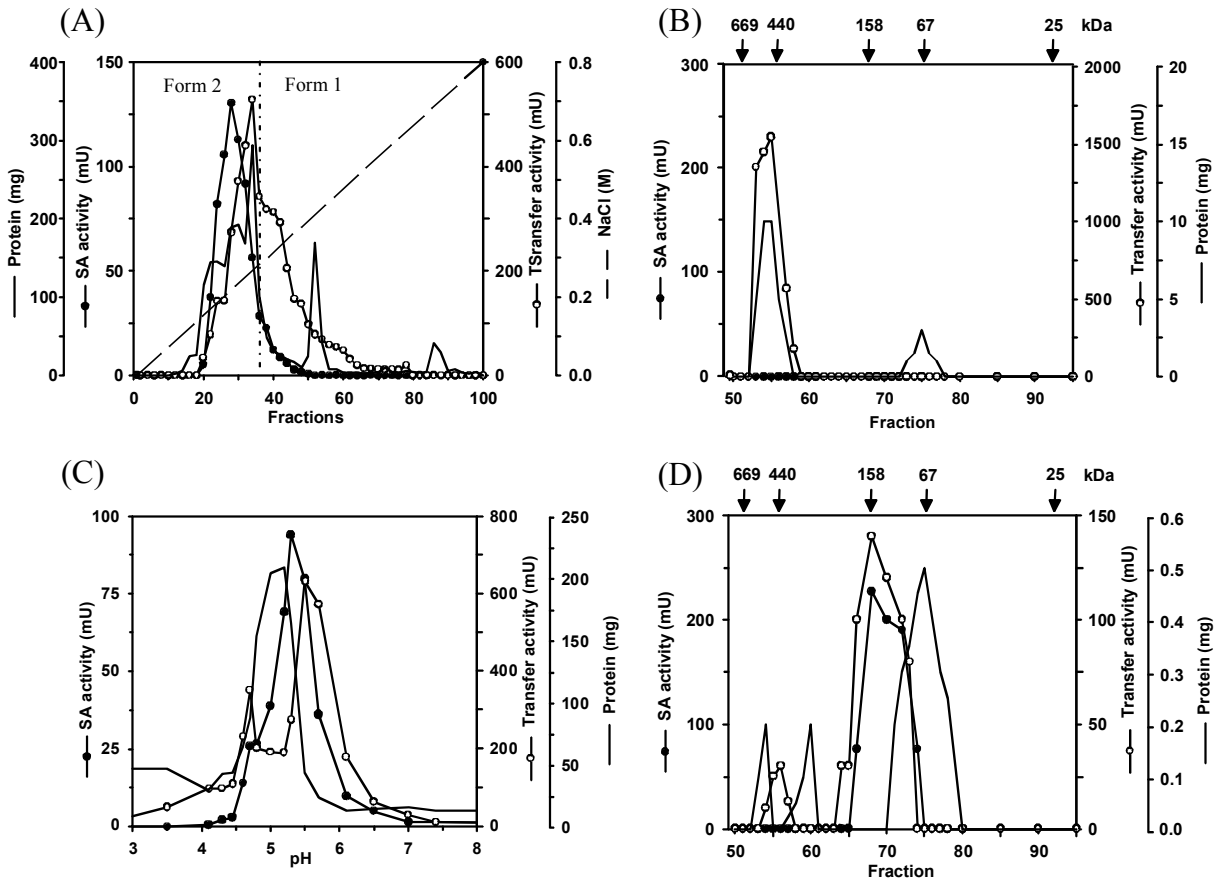

Fig. 1: Elution profiles of the various chromatography steps used during the purification of *T.con.*TS forms. Details of conditions for sample application and elution are given under Experimental Procedures. (A) Ion exchange chromatography on Q-Sepharose FF of concentrated *T.con.* culture supernatant.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

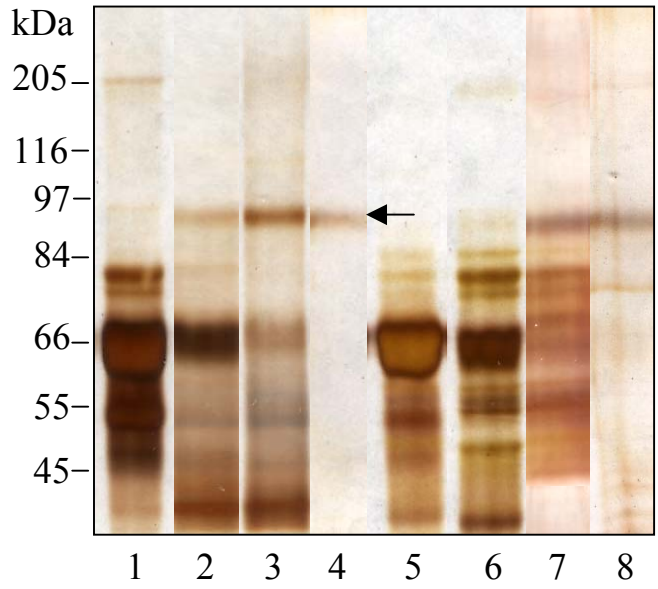

Fig. 2: Silver-stained SDS-PAGE showing the various stages in the purification of *T.con.*TS form 1 and 2. Two to four µg of protein was applied to each well. Lane 1: concentrated culture supernatant, lane 2: TS-form 1 following Q-Sepharose FF, lane 3: TS-form 1 following gel filtration, lane 4: TS-form 1 following immunoaffinity chromatography, lane 5: TS-form 2 following Q-Sepharose FF, lane 6: TS-form 2 following IEF, lane 7: TS-form 2 following gel filtration, lane 8: TS-form 2 following immunoaffinity chromatography. The arrow indicates the protein band which was used for micro-sequencing.

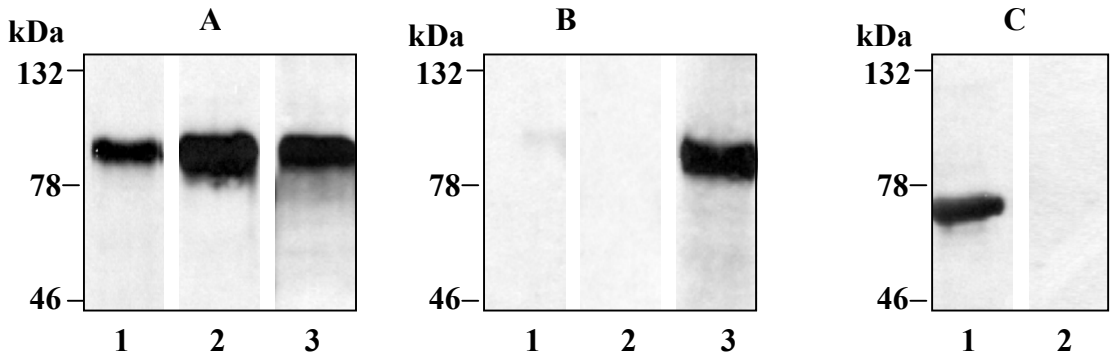

Fig. 3: Immunoblots of *T.con.*TS forms with anti-*T.con.*TS mAb (mAb 7/23) and anti-*T.cr.*TS antiserum following SDS-PAGE. A: Immunostaining of *T.con.*TS with anti-*T.con.*TS mAb 7/23. Lane 1: concentrated culture supernatant, lane 2: TS-form 1, lane 3: TS-form 2. B: Immunostaining of recombinant *T.cr.*TS (lane 1), recombinant *T.b.br.*TS (lane 2) and *T.con.*TS (lane 3) with *T.con.*TS mAb 7/23. C: Immunostaining of recombinant *T.cr.*TS (lane 1) and *T.con.*TS (lane 2) with anti-*T.cr.*TS antiserum.

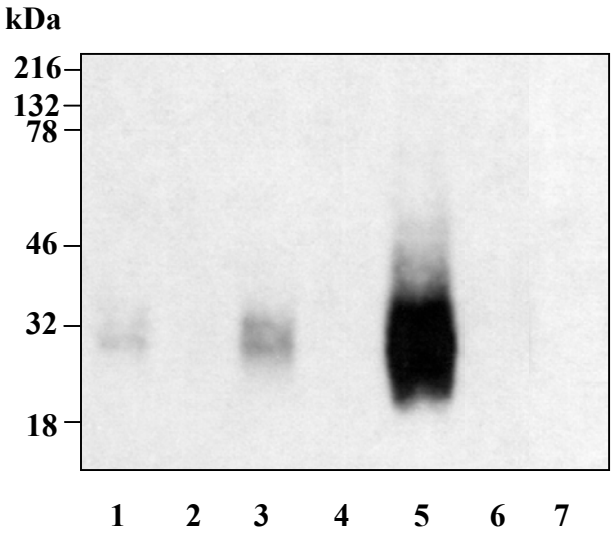

Fig. 4: Immunoblots of *T.con.*TS with anti-*T.con*. GARP mAb following SDS-PAGE. Lane 1: concentrated culture supernatant, lane 2: TS-form 2 following Q-Sepharose FF, lane 3: TS-form 1 following Q-Sepharose FF, lane 4: TS-form 2 following gel filtration, lane 5: TS-form 1 following gel filtration, lane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

Table 1: Summary of the purification of both TS forms of *T. con.*

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

[a] One unit of activity equals one µmol of MU released per minute, which is equivalent to 1 µmol of sialic acid released or transferred per minute.
[b] Conc. Cult S/N: concentrated culture supernatant

Table 2: Comparison of the peptide sequence VVDPTVVAK derived from a 90 kDa protein representing TS-form 1 following immunoaffinity purification with the sequences of known trypanosomal SA and TS including the newly identified *T.con.*TS sequences[3]. The italicized sequences show 100 % identity with the found peptide. The GenBank accession numbers of the sequences are stated in parentheses.

Enzymes	Peptide sequence
*T.r.*SA (U83180)	VMDATVIVK
*T.cr.*TS (D50685)	VVDPTVIVK
*T.b.br.*TS (AF310232)	*VVDPTVVAK*
*T.con.*TS1[3] (AJ535487)	*VVDPTVVAK*
*T.con.*TS2[3] (AJ535488)	VVDPTVVVK

Table 3: Kinetic parameters for both *T.con.*TS forms. Apparent V_{max} and K_M values were calculated from the Michaelis-Menten curve. Apparent V_{max}/K_M values represent the catalytic efficiency.

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

[1] n.d.: not determined

Table 4: Substrate specificity of both *T.con*.TS forms.

Substrates	Concentration [a]	Form 1	Form 2
A) Donor		Relative transfer activity (%)[b]	
Sialylα2,3-*N*-acetyllactoseamine; Neu5Acα2,3Galβ1,4GlcNAc	1 mM	111	115
Sialylα2,3-lactose; Neu5Acα2,3Galβ1,4Glc	1 mM	100	100
	0.5 mM	90	90
	0.25 mM	70	70
Sialylα2,6-lactose; Neu5Acα2,6Galβ1,4Glc	1mM	31	26
Sialyl-Lewis [x]; Neu5Acα2,3Galβ1,4[Fucα1,3]GlcNacβ1,3Galβ1,4Glc	0.25 mM	11	11
Fetuin	0.5 mM	78	78
Sialyloligosaccharides, bovine milk	0.5 mM	79	70
Sialyloligosaccharides, human milk	0.5 mM	70	66
Glycomacropeptide	0.5 mM	77	71
Apolactoferrin	0.5 mM	45	20
B) Acceptor		Relative transfer activity (decrease in %)	
N-acetylactoseamine; Galβ1,4GlcNAc	1 mM	49	24
Lacto-*N*-biose I; Galβ1,3GlcNAc	1 mM	40	13
Lacto-*N*-neotetraose; Galβ1,4GlcNAcβ1,3Galβ1,4 Glc	1 mM	56	39
Lacto-*N*-tetraose; Galβ1,3GlcNAcβ1,3Galβ1,4 Glc	1 mM	31	12
Lactose; Galβ1,4Glc	1 mM	45	24
Lactitol	1 mM	61	35
Galactose-β1,4-galactose; Galβ1,4Gal	1 mM	37	15
Galactose	5 mM	14	7
Glucose	5 mM	0	0
Mannose	5 mM	0	0
Maltose	5 mM	0	0

A) Relative transfer rate from sialic acid-containing compounds onto 0.5 mM MUGal. B) Relative transfer rate given as percent reduction in the synthesis of MUGalNeu5Ac. For details see "Experimental Procedures" (2.3.). [a]The concentration of potential donors is stated as the concentration of bound sialic acid. [b]The same total activity was used for both TS forms in all assays.

2.7. Abbreviations, Footnotes and Acknowledgements

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[1] FCS, fetal calf serum; GARP, glutamic acid-alanine-rich protein; IEF, isoelectric focusing; mAb, monoclonal antibody; MU, 4-methylumbelliferone; MUGal, 2`(4-methylumbelliferyl)galactoside; MULac, 2`(4-methylumbelliferyl)lactoside; MUNeu5Ac, 2`(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid; Neu5Ac, *N*-acetylneuraminic acid; Neu2en5Ac, 5-*N*-acetyl-2-deoxy-2,3-didehydro-neuraminic acid; PARP, procyclic acidic repetitive protein; SA, sialidase; α2,3-SL, sialyllactose (Neu5Acα2,3-lactose); *T.b.br.*, *Trypanosoma brucei brucei*; *T.con.*, *Trypanosoma congolense*; *T.cr.*, *Trypanosoma cruzi*; *T.r.*, *Trypanosoma rangeli*; TS, trans-sialidase

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

[3] Evelin Tiralongo, Ilka Martensen, Joachim Grötzinger, Joe Tiralongo and Roland Schauer, Trans-sialidase conserves most of the critical active site residues found in other trans-sialidases, submitted to the Journal "Molecular and Biochemical Parasitology" (🗐 Chapter 3)

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CHAPTER 3

Publication:

Trans-sialidase from *Trypanosoma congolense* conserves most of the critical active site residues found in other trans-sialidases

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Running title: Sequence information for *Trypanosoma congolense* trans-sialidases

Declaration of my contribution to the publication

With the exception of the modelling of the N-terminal domain of *T. congolense* TS1, which was performed in collaboration with Dr J. Grötzinger, I have planned, processed and analysed the experiments stated in this publication. The manuscript was written by me in discussion with all co-authors. However, in the section "Experimental Procedures" the single part concerning the N-terminal modelling of *T. congolense* TS1 was added by Dr J. Grötzinger.

3.1. Abstract

Trypanosoma congolense is the agent of Nagana, the trypanosomiasis in African ruminants. Trypanosomes express an enzyme called trans-sialidase, which is believed to play an important role in maintaining pathogenicity of the parasites. Thus far, only two complete trans-sialidase sequences have been characterised, one from the American trypanosome *T. cruzi* and one from the African trypanosome *T. brucei brucei*. Although a structure of *T. cruzi* trans-sialidase has been modelled by comparison with the crystal structure of *T. rangeli* sialidase, the exact mechanism of the transfer reaction is still not understood. The availability of further trans-sialidase sequences will ensure a better understanding of how transfer activity can be achieved and will provide the opportunity to develop highly specific, structure based trans-sialidase inhibitors. Utilising a PCR-based approach two different trans-sialidase gene copies from *T. congolense* were identified, which share only 50 % identity with each other, but show significant similarity with known viral, bacterial and trypanosomal sialidases and trans-sialidases. The longer sequence (*T.con.*TS1) shares 56 % identity with the primary sequence of *T. b. brucei* trans-sialidase and 43 % with *T. cruzi* trans-sialidase, whereas the shorter sequence (*T.con.*TS2) exhibits 46 % identity to *T. b. brucei* trans-sialidase, but 45 % with *T. cruzi* trans-sialidase. In both partial sequences most of the critical active site residues common to other trypanosomal sialidases and trans-sialidases are conserved. This was further illustrated by modelling the active site of *T.con.*TS1. Moreover, a peptide sequence derived from the native, active trans-sialidase from *T. congolense* was found within *T.con.*TS1.

3.2. Introduction

Trypanosomes, such as *T. cruzi* (*T.cr*.) [1], *T. brucei brucei* (*T.b.br*.) [2;3], *T. congolense* (*T.con*.) [4] and other protozoan parasites like *Endotrypanum* sp. [5] express a unique type of glycosyltransferase, called trans-sialidase (TS). Unlike typical sialyltransferases, which require CMP-activated sialic acids as the monosaccharide donor [6], TS catalyses the transfer of, preferably, α2,3-carbohydrate-linked sialic acid to another carbohydrate forming a new α2,3-glycosidic linkage to galactose or *N*-acetylgalactosamine. In the absence of an appropriate acceptor TS acts like a sialidase (SA), similar to viral, bacterial and trypanosomal SA, hydrolysing glycosidically linked sialic acid [7;8].

Trypanosomes are unable to synthesise sialic acids, instead they utilise TS to transfer sialic acid from the environment onto trypanosomal surface molecules. This has been found to play a major role for the survival and pathogenicity of the parasites inside the vector and the host [8]. In the case of *T.cr.,* TS is employed to acquire sialic acid from mammalian host glycoconjugates to sialylate mucin-like acceptor molecules in the parasites plasma membrane. Furthermore, TS sialylates host cell glycoconjugates to generate receptors, which are used for parasite adherence and subsequent entry into host cells [8]. In the African species, *T.b.br.* and *T.con.,* TS is only expressed in the procyclic insect stage, sialylating the major cell surface glycoproteins of the parasites in the vector. Thus, a negatively charged glycocalyx is formed which is believed to protect the parasites from digestive conditions in the fly gut and may enable them to interact with epithelial cells [2;9]. Additionally, it has recently been reported that *T.cr.*TS itself directs neuronal differentiation in PC12 cells [10], stimulates IL-6 secretion from normal human endothelial cells [11], as well as potentiating T cell activation through antigen-presenting cells [12].

Thus far, only two complete TS sequences have been studied, one from an American (*T.cr.*) and one from an African (*T.b.br.*) trypanosome. Although a comparison of the

modelled structure of *T.cr.*TS with the crystal structure of a SA from the closely related trypanosome *T. rangeli* (*T.r.*) has been carried out [13], neither the exact mechanism of the transfer reaction nor the structural features of the TS protein that support its efficient sugar transfer are understood. Moreover, the reasons why TS is more efficient in transferring than hydrolysing terminal sialic acid have remained unclear.

However, the available crystal structure of *T.r.*SA and the homology model of *T.cr.*TS show several distinct structural features. That is, *T.r.*SA and *T.cr.*TS possess an N-terminal domain (~ 380 amino acids, including Asp boxes and FRIP region) which share up to 30 % identity with bacterial SA [14], as well as displaying a β-propeller topology [15] similar to that of bacterial and viral SA. The N-terminus is connected *via* an α helix with a C-terminal domain having the characteristic β-barrel topology of plant lectins [13]. Within the C-terminal domain 150 amino acids are found which exhibit no similarity to any known sequence. This is followed by residues building a fibronectin type III domain (Fn3 domain) [16]. The *T.r.*SA ends with a second α-helix, which in *T.cr.*TS is followed by a long C-terminal tandem repeat of 12 amino acids (SAPA repeats, shed acute phase antigen repeats) and finally by a glycosylphosphatidylinositol (GPI) anchor. The SAPA repeats and GPI anchor are not required for transfer activity [17].

Because of the low overall level of amino acid identity (20-30 %) between bacterial and trypanosomal enzymes, any cross genera sequence comparisons, employed to identify essential amino acid residues or subdomains involved in the sialic acid transfer, seem not to be very useful. Here we report on the identification of two partial TS gene sequences from the African trypanosome *T. con.,* and their comparison with the primary structure of known viral, bacterial and trypanosomal SA and TS. Additionally, we have modelled the active site of one of the *T.con.*TS gene sequences on the basis of the crystal structure of the *T.r.*SA in complex with an inhibitor [13].

Investigating TS has become increasingly attractive over the last years not only because of its involvement in trypanosomal pathogenicity, but also because of its biotechnological importance. That is, TS is a unique enzyme that, because of its ability to transfer Neu5Ac in a stereo- and regio-specific manner, can be utilised to synthesise a variety of biologically relevant structures of the type Neu5Acα2,3Galβ1-R [18-21]. By providing sequence information of TS from an additional African trypanosoma species, this study will help to further elucidate the mechanism of TS, as well as confirm or reveal findings about essential residues required for the transfer activity of TS. Furthermore, the comparison of these additional TS sequences will provide the opportunity to develop high potential, structure based TS inhibitors.

3.3. Experimental Procedures

3.3.1. Reagents and general methods

All chemicals were analytical grade or higher and purchased from Biomol (Hamburg, Germany), Merck (Darmstadt, Germany) or Sigma (Deisenhofen, Germany). Oligonucleotide primers were synthesised by Eurogentec (Seraing, Belgium). *Taq* polymerase and dNTPs were also purchased from Eurogentec. The "Sawady mid range PCR-system" was obtained from Peqlab (Erlangen, Germany). Restriction enzymes were purchased from Invitrogen (Karlsruhe, Germany) and pGEM®-T Vector System I was acquired from Promega (Mannheim, Germany). PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and extracted from agarose gels using the QIAquick extraction kit (Qiagen). Plasmid DNA was isolated with the QIAprep plasmid miniprep kit (Qiagen). All sequencing analyses were performed by MWG Biotech (Ebersberg, Germany). Sequence

alignments were achieved using the program Clustal W from the HUSAR package, and shaded with Genedoc.

3.3.2. Trypanosomes

Procyclic forms of *T. congolense* (STIB 249; kindly provided by Dr Retro Brun from the Swiss Tropical Institute in Basel) were cultivated axenically in SM/SDM 79 medium, containing 10 % fetal calf serum and 0.001 % hemin [22].

3.3.3. DNA isolation

After three days of cultivation procyclic forms of trypanosomes were harvested *via* centrifugation and total DNA was isolated using a conventional phenol/chloroform extraction with proteinase K [23]. Briefly, a cell pellet obtained from a 200 ml culture, was resuspended in 500 µl of PBS buffer, pH 7.2, and centrifuged for 30 seconds. The supernatant was removed and 500 µl EB buffer (Qiagen) containing of 0.2 mg/ml proteinase K and 0.04 mg/ml RNAse were added. After an overnight incubation at 37 °C a phenol/chloroform extraction was performed. By adding 3 M sodium acetate (1/10 of the volume) and 100 % ethanol (2.5 times the volume), the DNA was precipitated from the aqueous phase and removed with the aid of a glass staff. After drying, the DNA was resuspended in an appropriate amount of TE buffer (10 mM Tris / 1 mM EDTA, pH 7.5-8.0).

3.3.4. PCR with degenerate primers

Within an alignment of the nucleotide sequences of *T.cr.*TS, *T.b.br.*TS and *T.r.*SA conserved or similar regions near the N-terminus, in the middle and near the C-terminus of

the sequences were established and degenerate primers deduced from those areas (🗐 3.6., Table I). These primers were used in all possible combinations in the following PCR reaction. A reaction mixture containing 1 µl (1-2 µg) of genomic DNA, 25 pmol of each primer, 40 nmol dNTPs (10 nmol each) and 5 µl high yield reaction buffer in a final volume of 49 µl was prepared. Subsequently, a hot-start PCR in a "T Gradient Thermocycler" (Biometra GmbH, Göttingen, Germany) was performed and after the addition of one unit of *Taq* polymerase at 72 °C the following program was applied: 30 x (30 s 95 °C, 45 s 46 °C/48 °C/50 °C, 2 min 72 °C), 7 min 72 °C. Depending on the primer combination, three different annealing temperatures (see above) were used.

3.3.5. Cloning of PCR products

PCR reactions were analysed using a 0.7 % agarose gel stained with ethidium bromide. PCR products possessing the expected size were extracted from the gel, purified, introduced into the pGEM®-T Vector by T/A cloning as described by the manufacturer and, subsequently, sequenced.

3.3.6. PCR with specific primers

Specific primers were synthesised according to the two novel sequences of putative *T.con.*TS. The primer sequences were as follows:

For *T.con.*TS 1 (long): 5`-GGTGGGAGAACGTGGAAGAG-3`(TconTS1Ps);

For *T.con.*TS 1 (long): 5`-GAAGCGCTAGCACCACCTGG-3`(TconTS1Pas);

For *T.con.*TS 2 (short): 5`-CACTTGTTGAGATAGACGGCG-3`(TconTS2Ps);

For *T.con.*TS 2 (short): 5`-CACAGTTATGGCAATTGAGCTAC-3`(TconTS2Pas)

Subsequently, a hot-start PCR was performed utilising the primers TconTS1Ps/TconTS1Pas in one and TconTS2Ps/TconTS2Pas in another PCR reaction. Apart from the 10 x buffer (high specificity) and an annealing temperature of 58 °C all other reagents and reaction conditions were as described above.

3.3.7. Modelling of the N-terminal domain of *T. congolense* TS1

The crystal structure of *T.r.*SA-Neu2en5Ac complex [13] served as a template for the three dimensional model of the *T.con.*TS1. Based on the alignment (🗐 3.6., Fig. 4) amino acid residues were exchanged in the template. Insertions and deletions in *T.con.*TS1 were modeled using a database approach included in the software package WHATIF [24]. The database was searched for a peptide sequence of the appropriate length, which was fitted to the template. All loops were selected from the database so as to give a minimum root means square (rms) distance between the ends of the loops. Loops with unfavorable backbone angles or van der Waals clashes were excluded. In a last step the three-dimensional structural model was energy-minimized using the steepest descent algorithm implemented in the GROMOS force field [25]. For graphical representation the Ribbons program [26] was used.

3.4. Results

3.4.1. PCR with degenerate primers

An alignment of the nucleotide sequences of *T.cr.*TS, *T.b.br.*TS and *T.r.*SA was performed in order to design degenerate primers to amplify *T.con.*TS from genomic DNA. As described under “Experimental Procedures” (🗐 3.3.4.), conserved or similar regions near the N-terminus, in the middle and near the C-terminus of the sequences were chosen for the

design of degenerate primers. In total 47 PCR reactions using all possible primer combinations were performed. Figure 1 (🗐 3.6.) shows the location of the deduced primers (panel A) and the size of the expected products depending on the primer combination used (panel B). Arrows indicate PCR products that possessed significant similarity to other TS. PCR products obtained with other primer combinations showed no similarity to known TS or SA.

After comparing the TS-like PCR products with each other, it became obvious that six of the overlapping PCR products (No. 4, 18, 20, 22, 26 and 34) must be derived from one gene sequence, whereas another two overlapping PCR products (1 and 7), which showed significant differences to the first 6, probably originate from another TS gene. Therefore, the appropriate PCR products were assembled resulting in two different partial gene sequences of *T.con.*TS (🗐 3.6., Fig. 2).

3.4.2. PCR with specific primers

In order to check whether the two partial gene sequences can be specifically amplified from genomic DNA, a set of specific primers for each gene sequence was designed. After performing PCR reactions with the specific primers, one product for each reaction was observed (🗐 3.6., Fig. 3). The two products were purified and subsequently sequenced. In both cases the sequence information obtained from the assembled PCR products was confirmed with an identity value of 98 %.

3.4.3. Comparison of the two partial *T. congolense* TS genes with each other

One of the partial sequences, referred to as *T.con.*TS1 (long, AJ535487), consisted of 1491 bp (497 aa), the other, referred to as *T.con.*TS2 (short, AJ535488), was composed of 831

bp (277 aa). Both deduced amino acid sequences share only 50 % identity (57 % similarity) with each other (3.6., Fig. 2). However, the amino acid sequence of *T.con.*TS1 (long) shows 56 % identity (67 % similarity) to the primary sequence of *T.b.br.*TS, 43 % identity (54 % similarity) to *T.cr.*TS and 40 % identity (53 % similarity) to *T.r.*SA. In contrast, the amino acid sequence of *T.con.*TS2 (short) exhibits only 46 % identity (63 % similarity) to *T.b.br.*TS, but 45 % identity (64 % similarity) to *T.cr.*TS and 42 % identity (63 % similarity) to *T.r.*SA.

3.4.4. Conserved motifs found in the two partial *T. congolense* TS and in viral, bacterial and trypanosomal sialidase and trans-sialidase genes

Although both partial *T.con.*TS gene sequences differ significantly from each other, both primary structures show similarity to known bacterial, viral and trypanosomal SA and TS (3.6., Fig. 4). That is, both *T.con.*TS gene sequences contain three copies of a conserved consensus sequence, called Asp box (3.6., Fig. 4, Box 2-4), which contains aspartate at a central position in a stretch of 8 amino acids (S-X-D-X-G-X-T-W) that is repeated three to five times in sialidase sequences [13;14;27]. Another conserved motif near the N-terminus termed FRIP region (3.6., Fig. 4, Box 1), which is common to bacterial and trypanosomal SA and TS, was also found in *T.con.*TS2, but not in *T.con.*TS1 because the PCR product does not cover this region. The motif LYCLHE (3.6., Fig. 4, D) common to all known trypanosomal TS, was also found in *T.con.*TS1, but not in *T.con.*TS2, again due to partial sequence information. Furthermore, the stretch of amino acids ISRVIGNS (3.6., Fig. 4, B) and VPVMLITHP (3.6., Fig. 4, C) have now been found to occur in all African TS genes so far studied.

3.4.5. Conservation of critical active site residues in the *T. congolense* TS gene sequences

The conservation of critical active site residues are presented with regards to their postulated function. Unless otherwise stated, the residues were numbered according to *T.r.*SA and *T.cr.*TS sequences shown in Fig. 4 and Table II (🗐 3.6.) and the corresponding residue numbers for all other sequences are outlined in Fig. 4 or Table II (🗐 3.6.). The residues are illustrated in Fig. 5B (🗐 3.6.).

In the two *T.con.* TS gene sequences reported here all of the critical active site amino acid residues required for sialic acid binding in viral [28;29] and bacterial SA [30] are conserved (🗐 3.6., Table II, Fig. 4). These preserved residues include an arginine triad (R36, R246, R315), which binds the carboxylate group common to all sialic acid derivatives, a glutamic acid (E358), that stabilises the position of one of the triad arginines and a negatively charged group (D60), which approaches the bound sialic acid from the solvent side and is believed to act as a possible proton donor in the hydrolytic reaction [31]. Additionally, two essential residues (E231, Y343) exist, which are close to the sialic acid C1-C2 bond and therefore are implicated in stabilising an oxocarbonium ion transition state intermediate [29;30]. The conservation of most of these essential amino acid residues in the newly elucidated *T.con.*TS sequences was confirmed, but due to partial sequence information two residues (R36, D60) in *T.con.*TS1 and three (R315, Y343 and E358) in the shorter sequence *T.con.*TS2 remain to be investigated (🗐 3.6., Table II, Fig. 4).

Additionally, in bacterial and viral SA a hydrophobic pocket which accommodates the *N*-acetyl group of sialic acid has been described [29;30]. In the crystal structure of the trypanosomal SA from *T.r.* the amino acid residues forming that pocket have been identified as M96, F114, W121 and I177 [13]. Although differences between these residues in *T.r.*SA

and in trypanosomal TS, including *T.con.*TS1 and *T.con.*TS2 occur, the substitutions conserve the hydrophobic character of the pocket (🗐 3.6., Table II, Fig.4).

Other amino acid residues conserved in the active site of bacterial SA [30] and in all trypanosomal SA and TS [13;32], including partially in *T.con.*TS1 and completely in *T.con.*TS2, are an arginine (R54) and aspartic acid (D97), which form strong hydrogen bonds with the O4 of sialic acids (🗐 3.6., Table II, Fig. 4). In comparison to bacterial and trypanosomal SA and TS this interaction with the O4 of sialic acid is weaker in the viral enzymes (which only occurs through aspartic acid) and allows *N*-acetyl-4-*O*-acetylneuraminyl-2,3-lactose to be hydrolysed, whereas the bulky 4-*O*-acetyl group hinders binding to the bacterial and trypanosomal SA and TS [33]. This can explain why the potent viral sialidase inhibitor, 5-*N*-acetyl-2,3-didehydro-2,4-dideoxy-4-guanidinyl-neuraminic acid [34], which has a substitution at this site, does not inhibit bacterial and trypanosomal SA and TS, including *T.con.*TS (E. Tiralongo *et al.*, submitted, 🗐 2.4.9.).

A comparison of the crystal structure of *T.r.*SA with a model structure of *T.cr.*TS emphasises the crucial role of a few amino acid residues within the substrate-binding cleft in modulating the transfer activity [13]. An aromatic residue (Y120 in *T.cr.*TS) and a shallow depression generated by Y249, P284 and W313 (in *T.cr.*TS) are believed to define a distinct binding site specific for the terminal β-galactose acceptor [13]. Site directed mutagenisis showed that the substitution Y120S, as well as P284Q led to a significant decrease or complete loss of transfer activity in *T.cr.*TS and *T.b.br.*TS [32;35;36]. The sequence of *T.con.*TS1 not only contains the essential amino acid Y120, but also P284, emphasising the possible impact of these residues. For the shorter sequence, *T.con.*TS2, P284 was present, whereas the important tyrosine residue (Y120) was substituted with a proline (🗐 3.6., Table II, Fig. 4). It cannot be excluded that this amino acid substitution effects transfer activity.

Additionally, it has been reported that the double mutant (Y249G/P284Q) of *T.cr.*TS looses its TS activity [36]. This must be due to the single mutation P284Q, since at position

Y249 diverse amino acids can be found in both *T.con.*TS genes, as well as in *T.cr.*TS and *T.b.br.*TS (🗐 3.6., Table II, Fig. 4). The finding that the active recombinant *T.b.br.*TS exhibits the amino acid exchange Y249G [32] further supports this notion.

It has been suggested that an exposed aromatic residue (W313) is responsible for the high specificity towards α2,3-linked sialic acid glycoconjugates displayed by trypanosomal SA and TS [13;36], as well as by *Salmonella thyphimurium* [37] and *Macrobdella decora* [38] SA. It has been shown that this tryptophan makes up part of a loop insertion that is missing in other microbial SA that exhibit a broader specificity [36]. Moreover, in *T.cr.*TS, as well as in *T.b.br.*TS, the mutation W to A led to the complete loss of transfer activity, independent of which regioisomer of sialyllactose was used [32;36]. In *T.con.*TS1 the residue W313 is replaced by the amino acid tyrosine. This substitution with another aromatic amino acid would probably not effect the function of the enzyme in the same way as the aliphatic alanine. In *T.con.*TS2 the analogous position to W313 was not part of the amino acid sequence deduced from the PCR product (🗐 3.6., Table II, Fig. 4).

Furthermore, another amino acid, P232, was found to be necessary for full transfer activity in *T.cr.*TS, since a P232A mutation resulted in reduced TS activity [39]. However, in the gene sequence of *T.b.br*.TS the residue P232 is substituted by an alanine, nevertheless the recombinant enzyme was shown to be active [32]. In both *T.con.*TS genes P232 is also substituted by an alanine (🗐 3.6., Table II, Fig. 4). Since the same substitution was detected in the active recombinant *T.b.br*.TS, this amino acid exchange does not seem to have an effect on the enzymatic activity and might be common to all TS of African trypanosomes.

The functional role of another amino acid exchange, V180 (in *T.r.*SA) to alanine in *T.cr.*TS, *T.b.br.*TS and *T.con.*TS1 has not yet been assessed. Interestingly, in *T.con.*TS2 this substitution does not exist (🗐 3.6., Table II, Fig. 4).

3.4.6. Model of the N-terminal domain of *T. congolense* TS1

Due to the high similarity of both *T.con.*TS to other trypanosomal TS, as well as to *T.r.*SA from which a crystal structure exists, the N-terminal domain of the longer partial sequence, *T.con.*TS1, was modelled based on the crystal structure of *T.r.*SA complexed with its inhibitor Neu2en5Ac [13]. In order to be able to complete the *T.con.*TS1 model at the N-terminus the first 63 amino acids were taken from the *T.r.*SA sequence (🗐 3.6., Fig. 4). In the model presented here, the N-terminal domain of *T.con.*TS1 revealed a β-barrel propeller topology (🗐 3.6., Fig. 5A) similar to that of bacterial, viral and trypanosomal SA, as well as TS [13;15]. Furthermore, the conserved Asp boxes were found to occur in the loops connecting the third and fourth β-strands of the first, third and fourth β-sheets (🗐 3.6., Fig. 5A), as described for *T.r.*SA [13].

Moreover, the model of the N-terminal domain of *T.con.*TS1 also provided the ability to examine the three dimensional settings of the critical active site residues in complex with Neu2en5Ac. Figure 5B (🗐 3.6.) reveals that all important active site residues occupy similar positions to that observed in *T.r.*SA and the modelled *T.cr.*TS [13]. As can be seen the sialic acid analogue Neu2en5Ac (🗐 3.6., Fig. 5B, yellow) fits in the active site, with the important interactions to residues involved in sialic acid binding (🗐 3.6., Fig. 5B, red including V34, Y52) being possible. Furthermore, amino acid residues shown in blue (🗐 3.6., Fig. 5B, except V34, Y52) may provide an acceptor binding site, since they are conserved within *T.cr.*TS, *T.b.br.*TS and *T.con.*TS1, but not in *T.r.*SA.

3.4.7. Comparison of the two partial T. congolense TS gene sequences with peptide sequences derived from the analysis of the native, active *T. congolense* enzyme

Following purification to homogeneity, the native, active enzyme from *T.con.* was investigated by mass spectrometry after trypsin digest. Several peptides were sequenced, with the amino acid sequence of one major peptide, VVDPTVVAK, being obtained (E. Tiralongo *et al.*, submitted, 2.4.5.). This exact peptide sequence was found in *T.b.br.*TS, as well as in the partial sequences of *T.con.*TS1 (3.6., Fig. 4, A). However, in *T.con.*TS2 the amino acid at the analogous position is valine.

3.5. Discussion

In *T.cr.* the TS family is encoded by approximately 140 genes [40], many of them coding for inactive proteins. In contrast, a recent publication [32] reporting on the gene sequence of *T.b.br.*TS postulates that in this African trypanosome TS genes are present in a low copy number (minimum two). In agreement with this, our experiments identified two different partial TS gene sequences from the African trypanosome *T.con.*, suggesting that the number of gene copies is lower in African trypanosomes. Interestingly, the two partial sequences of *T.con.*TS share only 50 % identity with each other. This is unique among TS gene copies described so far, since all *T.cr.*TS gene sequences available in Swissprot share a high degree of sequence identity. Additionally, for the TS from *T.b.br.* eight clones have been described, which differ in only nine positions. However, when expressed all these clones exhibited full SA and transfer activity [32] and may actually represent only one TS gene. This is further substantiated by the finding that the *T.con.*TS gene copies described here differ

significantly from each other. In that case, a second or multiple gene copies of *T.b.br.*TS still remain to be identified.

There is evidence for a common ancestor of the American *T.cr.* and the African *T.b.br.* living around 100 million years ago, before separation of the African and American continents, carrying the primitive TS gene [41]. However, due to their relatively distinct relationship and despite conserved motifs, the identity between the *T.cr.*TS and the *T.b.br.*TS does not exceed 44 % in the region corresponding to the catalytic domain [32]. Equally, the catalytic domain of both *T.con.*TS sequences exhibited about 43-45 % identity to *T.cr.*TS. In comparison, the SA from the closely related American trypanosome *T.r.* shares approximately 70 % identity in the amino acid sequence of the global core region with the TS from *T.cr.* [35;42]. Since *T.con.* and *T.b.br.* are both African trypanosomes, sharing a similar life cycle and expressing TS at the same developmental stage, one would assume that a close relationship between them exists. However, such a high degree of identity as reported for *T.r.*SA and *T.cr.*TS could not be detected for *T.b.br.*TS and *T.con.*TS. Nevertheless, the catalytic domain of *T.con.*TS1 and *T.con.*TS2 exhibit a significant degree of identity to *T.b.br.*TS, with the identity being 56 % and 46 %, respectively. Additionally, both *T.con.*TS sequences show 40-42 % identity to the catalytic domain of *T.r.*SA, which is again comparable to the identity between *T.b.br.*TS and *T.r.*SA (43 %) [32]. Interestingly, *T.con.*TS1 and *T.con.*TS2 possess a comparable degree of similarity (67 and 63 %, respectively) to *T.b.br.*TS. However, in comparison to *T.con.*TS1 that possesses 54 % and 53 % similarity to *T.cr.*TS and *T.r.*SA, respectively, *T.con.*TS2 shares a higher degree of similarity with the American trypanosomal enzymes *T.cr.*TS (64 %) and *T.r.*SA (63 %). This emphasises again the difference of both *T.con.*TS gene sequences.

With the detection of three Asp boxes in both *T.con.*TS sequences and, additionally the FRIP region in *T.con.*TS2, it could be shown that the primary structures of both *T.con.*TS gene sequences possess conserved motifs which are also found in bacterial and trypanosomal

SA and TS. The Asp boxes in both *T.con.*TS are located at similar positions in the fold of the protein to that reported for other bacterial SA, *T.r.*SA and other known trypanosomal TS. However, Asp boxes have been shown to have no effect on the enzymatic activity, since they are remote from the active site and site-directed mutagenesis revealed no influence on SA activity [43]. Furthermore, Asp boxes have also been identified in some other proteins with no SA/TS activity [32;44]. Nevertheless, Asp boxes may be involved in protein folding, as they are located on the surface of the protein (🗐 3.6., Fig. 5A). Additionally, two other motifs (ISRVIGNS, VPVMLITHP) were conserved in both *T.con*.TS sequences, as well as in *T.b.br.*TS. These motifs may, together with the residue P232, be species specific and present exclusively in African trypanosomal TS.

A comparison of the putative catalytic domain in both *T.con.*TS sequences revealed the conservation of critical active site residues displayed in viral, bacterial and trypanosomal SA and TS. In both *T.con.*TS sequences amino acid residues required for sialic acid binding are conserved. Furthermore, *T.con.*TS1 exhibits most of the critical active site amino acid residues found in *T.r.*SA, *T.cr.*TS and *T.b.br.*TS, with the exception of one conservative substitution (W313Y). In addition, *T.con.*TS1 possesses the same amino acids that are conserved in the two other trypanosomal TS, but differ in *T.r.*SA (🗐 3.6., Fig. 5B). More importantly, a peptide sequence derived from the native, active TS from *T.con.* was found within the *T.con.*TS1 sequence. Taken together, these findings suggest that the partial sequence of *T.con.*TS1 encodes an active enzyme with SA and transfer activity.

In contrast, in the *T.con.*TS2 sequence three critical residues, which are present in *T.cr.*TS, *T.b.br.*TS and *T.con.*TS1 are not found (🗐 3.6., Table II). One of the substitutions (Y120 in *T.cr.*TS to P87 in *T.con.*TS2) is at a position which has been defined as a distinct binding site for the acceptor. The second substitution, A180 to V149, which also occurs in *T.r.*SA, may lead to the removal of a possible acceptor binding site. The third substitution (S286 to G257) occurs in a triplet of amino acid residues (PGS 284-286) conserved in all

trypanosomal TS. However, site directed mutagenesis experiments demonstrated that S286 is probably not important for transfer activity [35].

In conclusion, these findings imply, that the protein encoded by *T.con.*TS2 either exhibits variations in its transfer activity or may even be inactive. With that in mind, the examination of changes resulting from the substitutions P87, V149 and G257 in *T.con.*TS2 *via* the site directed mutation of currently available recombinant TS proteins (*T.cr., T.b.br.*) will be required to assign a functional role for these amino acids. Additionally, it might be of interest to explore whether the substitution W313Y (observed in *T.con.*TS1) increases the specificity of TS towards α2,6 linked sialic acid. These mutagenesis studies might provide the opportunity to generate TS with different substrate specificities and transfer efficiencies, as well as being complementary to the development of high potential, structure-based TS inhibitors.

Certainly, further studies are required not only to obtain the full sequence encoding *T.con.*TS1 and *T.con.*TS2, but also to express active enzyme. However, until then, our findings will aid in the understanding of the mechanism and functionality of TS, as well as confirming or revealing essential residues required for transfer activity. So far, only two complete TS sequences, one American and one African, have been analysed. By providing sequence information on the catalytic domain of TS from an additional African trypanosoma species, conclusions can be drawn concerning the relationship of the enzymes, as well as which amino acid exchanges might be simply due to species differences.

3.6. Figures and Tables

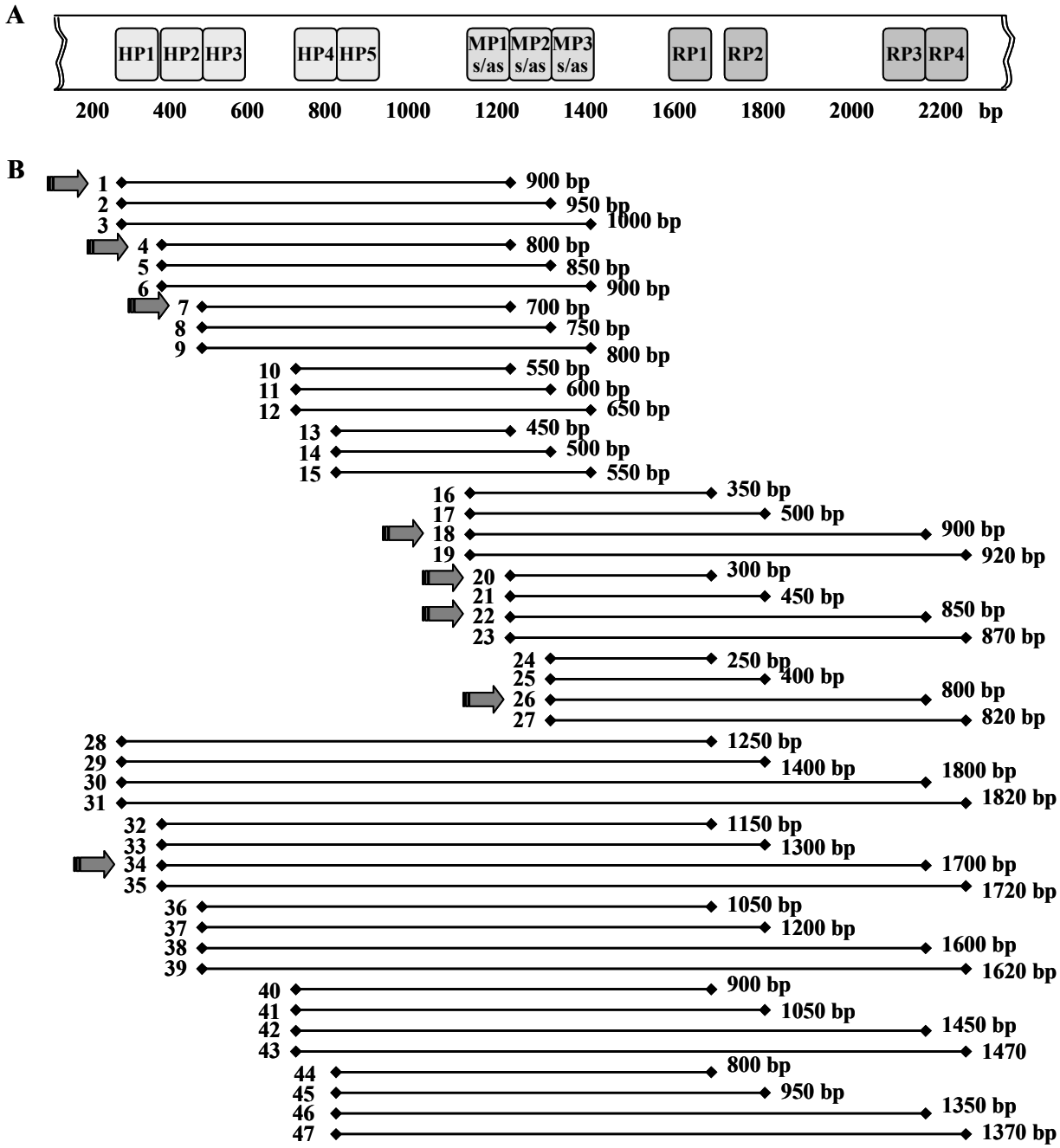

Fig. 1: Location of the deduced degenerate primers in the sequence alignment (panel A) and the proposed PCR products with their expected size (panel B). Arrows indicate the PCR products with sequence similarity to genes of *T.r.*SA, *T.cr.*TS, and *T.b.br.*TS.

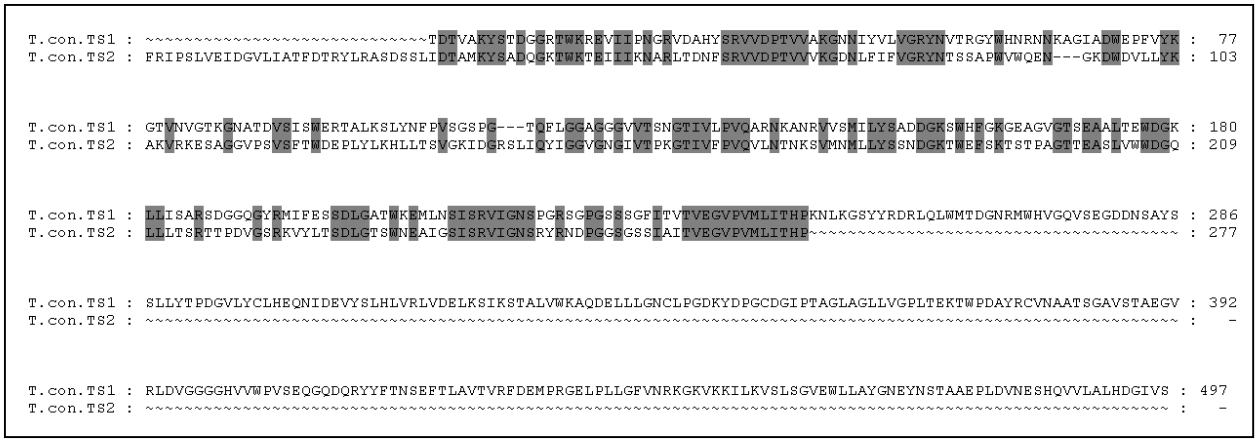

Fig. 2: Sequence comparison of the two partial *T.con.*TS genes (*T.con.*TS1, long and *T.con.*TS2, short). The sequences were aligned with CLUSTALW and, subsequently, shaded in GENEDOC. Identical residues in both sequences are shaded grey.

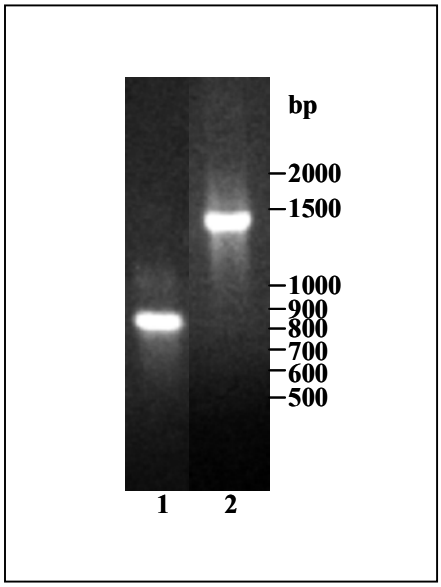

Fig. 3: Amplification of two partial TS genes from genomic DNA of *T. congolense* with specific primers. Lane 1 and 2 show the PCR products from *T.con.*TS2 (short, 830 bp) and *T.con.*TS1 (long, 1491 bp), respectively.

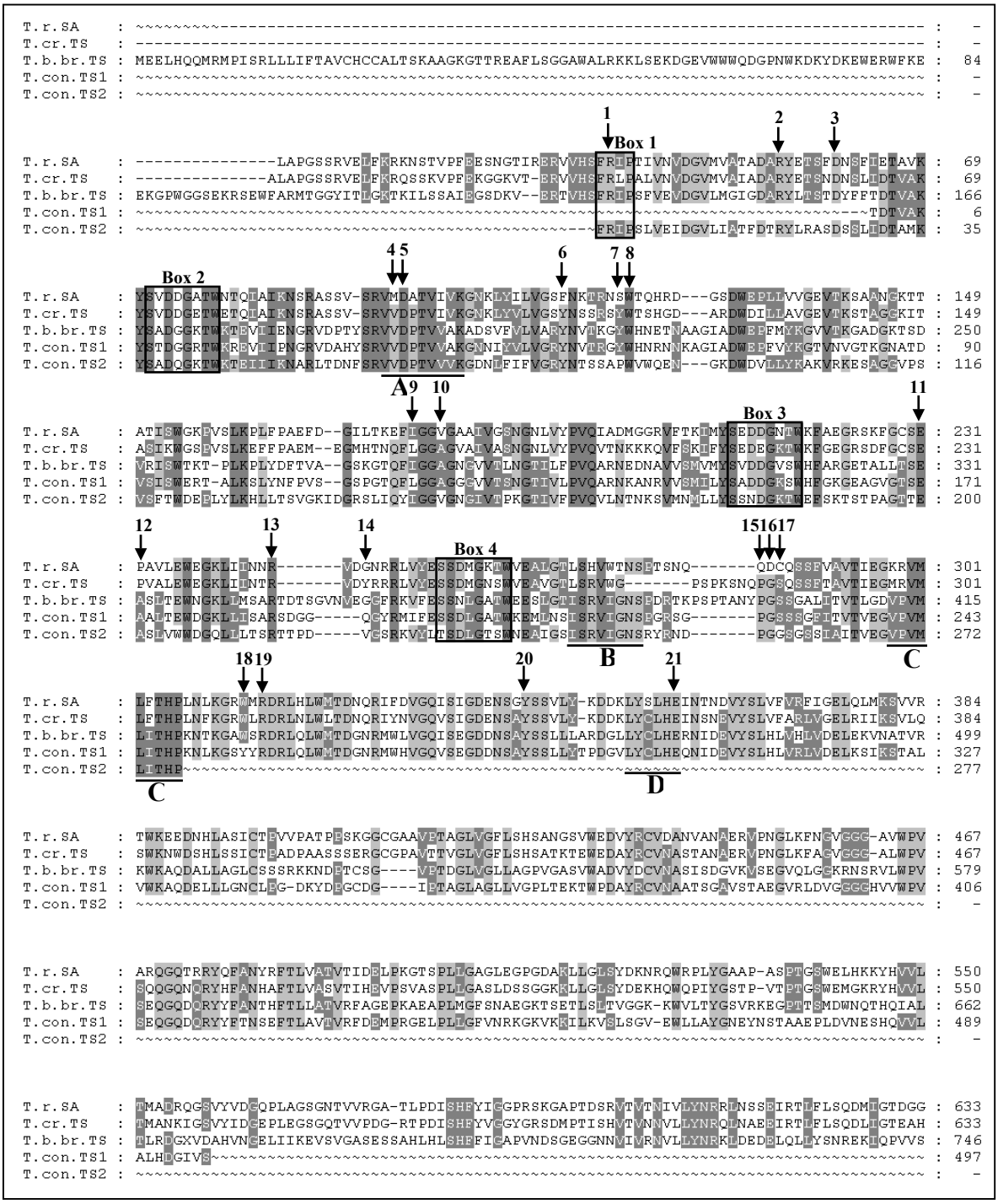

Fig. 4: Comparison of the amino acid sequences of the two partial *T.con.*TS with *T.r.*SA, *T.cr.*TS and *T.b.br.*TS. The sequences were aligned with CLUSTALW and, subsequently, shaded in GENEDOC. Residues identical in all sequences are printed black on dark grey, while residues identical in at least four of the five sequences are printed black on lighter grey. Residues identical in at least three of the five sequences are printed white on dark grey. Box 1 shows the FRIP region. Boxes 2–4 indicate the Asp boxes. Structurally relevant residues are marked with arrows (1-21). The motifs LYCLHE (D), ISRVIGNS (B), VPVMLITHP (C) and the peptide obtained from the native *T.con.*TS, VVDPTVVAK (A), are underlined. The SwissProt accession numbers of the sequences aligned are: *T.r.*SA, O44049; *T.cr.*TS, Q26964 and *T.b.br.*TS, Q9GSF0.

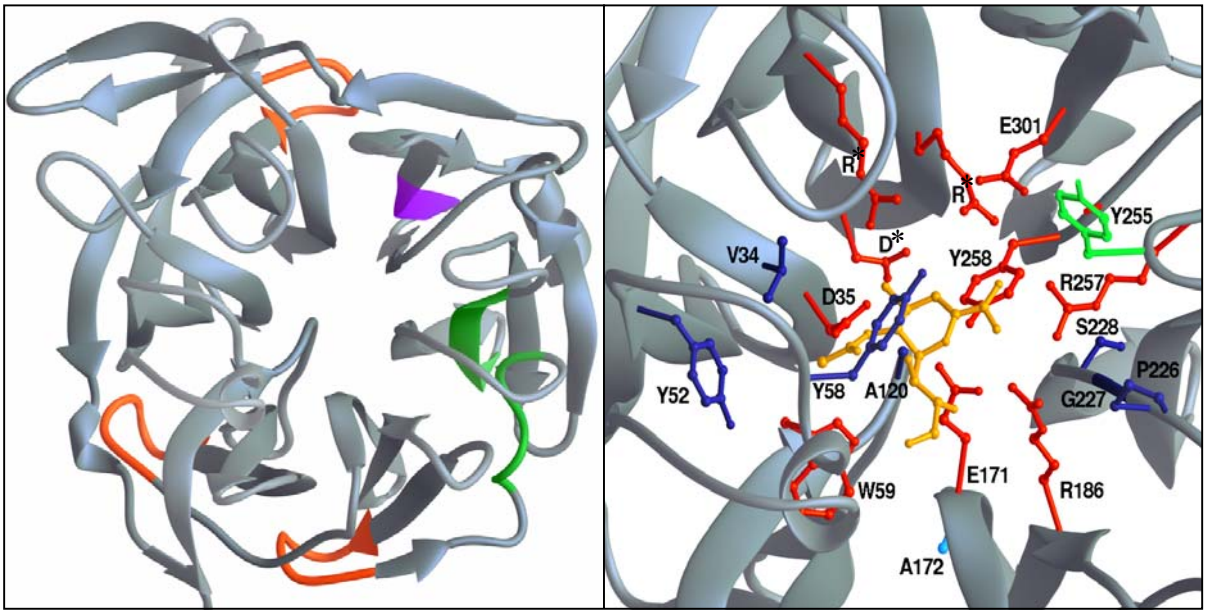

Fig. 5: Model of the N-terminal domain of *T.con.*TS1 according to the crystal structure of *T.r.*SA-Neu2en5Ac complex [13].

A: Conserved motifs in the N-terminal domain. Asp boxes, conserved motifs in bacterial and viral sialidases, are coloured in orange, the motif LYCHLE, common to all known trypanosomal trans-sialidases, is coloured in purple and the motifs ISRVIGNS and VPVMLITHP, which have now been found to occur in all African TS genes so far studied, are coloured in green.

B: Putative active site of *T.con.*TS1. The inhibitor Neu2en5Ac is illustrated in yellow. Amino acids shown in red are conserved throughout the four trypanosomal enzymes (*T.r.*SA, *T.cr.*TS, *T.b.br.*TS, *T.con.*TS1), whereas residues shown in blue are conserved in the three trypanosomal TS, but diverge in *T.r.*SA. The tyrosine shown in green is unique to *T.con.*TS1. At this position *T.r.*SA, *T.cr.*TS and *T.b.br.*TS contain a tryptophan. The alanine coloured in light blue differs between the American and African trypanosomal enzymes. Numbering of the *T.con.*TS1 model was performed according to the *T.con.*TS1 sequence shown in Fig. 4 and Table I. *Because of incomplete sequence information for *T.con.*TS1, these residues were taken from the *T.r.*SA sequence.

Table I: Degenerate primers used for the amplification of *T.con.*TS genes from genomic DNA.

Primer location	Sequence[a]	Abbreviation
N-terminus	5`-CGYRYTGTKCAYTCCTTYCG-3`	HP1
	5`-AYCGAMACCGYTGYTAAATAC-3`	HP2
	5`-TCYCGTGTTRTKGAYSCKAC-3`	HP3
	5`-GCYRRYGGTAAAAYCASYG-3`	HP4
	5`-ARTTCMTBGGWGGWGYTGG-3`	HP5
Middle	5`-GTKATGYTGWTYACCCACCCG-3`	MP1s
	5`-CGGGTGGGTRAWCARCATMAC-3`	MP1as
	5`-CGKGACCGTCTRMASCTGTGG-3`	MP2s
	5`-CCACAGSTKYAGACGGTCMCG-3`	MP2as
	5`-GGYGACGAWAACWSCGSTTAC-3`	MP3s
	5`-GTAASCGSWGTTWTCGTCRCC-3`	MP3as
C-terminus	5`-GGMSAGKAAACCAACMAGRCC-3`	RP1
	5`-GCGAASTRRTAMCKMYGGKYCTGWCC-3`	RP2
	5`-CGRTTGTACAGMASRAYRTT-3`	RP3
	5`-GTACAGMASRAYRTTKBTRACG-3`	RP4

[a] (R= A or G, Y= T or C, M= C or A, V= A, C or G; S= G or C, K= G or T, N= A, G, T or C)

Table. II: Summary of active site residues and their postulated effects displayed in trypanosomal sialidases and trans-sialidases. The residues are illustrated in Fig. 4 and Fig. 5B.

No	T.r.SA	T.cr.TS	T.b.br.TS	T.con.TS1	T.con.TS2	Postulated Effect
1	R36	R36	R133	n.d.	R2	Binds the carboxylate group of Sia[a] [28;30]
2	R54	R54	R151	n.d.	R20	Forms hydrogen bonds to O4 of Sia [13]
3	D60	D60	D157	n.d.	D26	Possible proton donor [31]
4	M96	V96	V194	V34	V63	Defines hydrophobic pocket that accommodates the *N*-acetyl of Sia [13]
5	D97	D97	D195	D35	D64	Involved in hydrogen bonding interactions [13]
6	F114	Y114	Y212	Y52	Y81	Defines hydrophobic pocket that accommodates the *N*-acetyl of Sia [13]
7	S120	Y120	Y218	Y58	P87	Distinct acceptor binding site [36]
8	W121	W121	W219	W59	W88	Defines hydrophobic pocket that accommodates the *N*-acetyl of Sia [13]
9	I177	L177	I277	L117	I146	Defines hydrophobic pocket that accommodates the *N*-acetyl of Sia [13]
10	V180	A180	A280	A120	V149	Acceptor binding site?
11	E231	E231	E331	E171	E200	Stabilises a putative sialosyl cation intermediate [29;30]
12	P232	P232	A332	A172	A201	P to A exchange in *T.cr.*TS leads to decrease of TS activity [39]
13	R246	R246	R346	R186	R215	Binds the carboxylate group of Sia [28;30]
14	G249	Y249	G356	Q191	V220	Double mutants show loss of TS activity [36]
15	Q284	P284	P398	P226	P255	Exchange of P to Q leads to decrease of TS activity [32;36]
16	D285	G285	G399	G227	G256	Acceptor binding site?
17	C286	S286	S400	S228	G257	Acceptor binding site?
18	W313	W313	W427	Y255	n.d.	Specificity for sialyl-α2,3 linkages [36]
19	R315	R315	R429	R257	n.d.	Binds the carboxylate group of Sia [28;30]
20	Y343	Y343	Y457	Y285	n.d.	Stabilises a putative sialosyl cation intermediate [29;30]
21	E358	E358	E473	E301	n.d.	Stabilises one of the triad arginine [28;30]

[a] Sia: sialic acids; n.d.: not determined

3.7. Abbreviations and Acknowledgements

Abbreviations

aa, amino acid; bp, base pair; Neu2en5Ac, 5-*N*-acetyl-2-deoxy-2,3-didehydro-neuraminic acid; SA, sialidase; *T.b.br.*, *Trypanosoma brucei brucei*; *T.con.*, *Trypanosoma congolense*; *T.cr.*, *Trypanosoma cruzi*; *T.r.*, *Trypanosoma rangeli*; TS, trans-sialidase

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CHAPTER 4

Unpublished Data

Protein chemistry

4.1. Background

Using a combination of anion exchange chromatography, isoelectric focusing and gel filtration two TS forms from *T. congolense* were separated and purified (🗐 2.4.2.). The two TS forms showed significant differences in their isoelectric points and in their ability to form oligomers. However, both TS forms showed a single 90 kDa band on SDS-PAGE, which was identified as TS *via* micro-sequencing (🗐 2.4.5.). Chapter 2 does not outline in detail how the existence of two forms was first observed, as well as it does not illustrate the various purification methods trialed using concentrated culture supernatant. These aspects will be addressed in the following sections. Furthermore, the 96-well-plate TS assay, which was mentioned in Chapter 2, will be described in more detail.

4.2. Experimental Procedures

4.2.1. Reagents and general methods

All reagents, organisms, antibodies and general methods were used as stated in Chapter 2 (🗐 2.3.). Additional methods are listed below.

4.2.2. Non-radioactive TS assay in 96-well-plates

The TS test outlined in Chapter 2 (🗐 2.3.5.) was modified by applying the general assay principle described to a 96-well-plate format. Since this novel 96-well-plate assay has a number of significant advantages in comparison to the methods generally employed to assay

for TS activity the TS test has been prepared for publication (Silke Schrader, Evelin Tiralongo, Alberto C.C. Frasch, Teruo Yoshino and Roland Schauer, A non-radioactive 96-well-plate assay for screening trans-sialidase activity, manuscript in preparation).

TS activity was monitored by incubating 25 µl of enzyme solution in 50 mM Bis/Tris buffer, pH 7.0, containing 1 mM α2,3-SL as the donor and 0.5 mM MUGal as the acceptor in a final volume of 50 µl at 37°C for 2 h in polypropylene 96-well-plates (MicroWell™ plates 0.5 ml, Nunc, Denmark) which were sealed with Nunc™ Well Caps (Nunc). The reaction was terminated by the addition of 350 µl ice cold water to each well and subsequently applied to a UNIFILTER® 800-filter-well-plate (GF/D glass fiber filter, Polyfiltronics®, Whatman, U.K.) loaded with 300 µl of Q-Sepharose FF (acetate form) that had been pre-washed 6 times with 500 µl of water.

All wash and elution steps were performed using a vacuum manifold (QIAvac 96, Qiagen, Germany). In order to avoid drying out the columns, the vacuum was applied slowly and only after the appropriate wash or elution buffers had been added to each well.

Following washing (6 times 500 µl), the sialylated product was eluted with 1 M HCl (6 times 150 µl), with the first 100 µl of eluate being discarded. The eluate was collected in a 2 ml Nunc™ 96 DeepWell Plate (Nunc) and sealed with Polyolefin sealing tape (Nunc). After acid hydrolysis of the eluted product at 95°C for 45 min in a waterbath, the sealing tape was removed and the plate cooled on ice for 15 min. One hundred and twenty µl of 6 M NaOH and 300 µl 1 M glycine/NaOH buffer, pH 10, were added and the plate sealed using Nunc™ Well Caps (Nunc, Denmark). Following mixing by inversion (2 to 3 times) 300 µl of reaction mixture was transferred into black 96-well-plates (Microfluor, Dynex, U.S.A.) and the fluorescence of MU released measured immediately at an excitation and emission wavelength of 365 nm and 450 nm, respectively. One unit of TS activity equals one µmol of MU released per minute, which is equivalent to 1 µmol of sialic acid transferred per minute.

4.2.3. Native-Polyacrylamide Gel Electrophoresis (PAGE) and electro-elution

Native-PAGE was performed at 15 °C utilising a Protean Cell (2 mm x 16 cm x 18 cm) from Biorad (Munich, Germany). The gel was prepared in the absence of SDS and reducing agents according to Laemmli [1] and consisted of a 6 % resolving gel and 3 % stacking gel. Following electrophoresis the gel was cut in two vertical pieces of which one was stained with silver [2] and the other further divided into 5 horizontal pieces. With the aid of Biotrap Electro Separation System from Schleicher & Schuell (Dassel, Germany) protein was immediately eluted from the gel pieces at 4 °C for 14 h according to manufacturer`s. In the retrieved fractions buffer was exchanged, the samples concentrated and enzymatic activity determined.

4.2.4. Affinity chromatography

Equine submandibular gland mucin (ESM) - Sepharose 4B and bovine submandibular gland mucin (BSM) - Sepharose 4B

In order to bind the recommended amount of ESM (2 µmol sialic acid/ml gel) and BSM (1 µmol sialic acid/ml gel [3]) to the activated support the sialic acid content of both mucins was determined using a micro-adaption of the orcinol/Fe^{3+}/HCl reaction [4]. Either 200 mg of ESM or 270 mg of BSM were linked to 4 ml of CNBr activated Sepharose 4B according to the method described by Corfield *et al*. (1979) [5]. Briefly, CNBr activated Sepharose 4B was suspended in 4 ml of binding buffer (0.1 M $NaHCO_3$, pH 8.0) and subsequently added to the same volume of binding buffer containing the appropriate ligand (ESM or BSM). The mixture was incubated with shaking for 12 h at 4 °C and then washed with 800 ml of water, 800 ml of 2 M NaCl and 800 ml of water. Following washing, the

matrices were resuspended in 4 ml of blocking buffer (0.1 M ethanolamine, pH 8.5), left overnight at 4 °C and washed with binding buffer. The column was equilibrated with binding buffer prior to its use.

The affinity matrices (4 ml) equilibrated in 20 mM Bis/Tris buffer, pH 7.0, were incubated with concentrated culture supernatant overnight at 4 °C. Unbound protein was removed by washing with 20 mM Bis/Tris buffer, pH 7.0. TS activity was eluted stepwise, initially with a 30 ml continuous NaCl gradient (0-0.3 M), followed by a further 30 ml of 0.5 and 1 M NaCl, respectively, at a flow rate of 0.5 ml/min. Fractions of 10 ml were collected, immediately desalted, concentrated and activity determined.

De-*O*-acetylation of the matrices was performed by incubating the mucins in 5 % ammonia for 6 h at room temperature.

Various affinity material

Affinity chromatography was performed on a number of different matrices which were obtained from the following sources. *N*-(p-aminophenyl)oxamic acid agarose, α-lactose agarose and Concanavalin A agarose were purchased from Sigma (Deisenhofen, Germany) and Galβ1,4GlcNAc-glycosorb from GlycorexAB (Lund, Sweden). The affinity material (arm-2-thio-a-D-Neu5Ac-Sepharose 4B) was a kind gift from Prof. M. von Itzstein (Griffith University, Qld., Australia). The conditions under which affinity chromatography for each material was performed are stated in Table 3 under "Results and Discussion" (🗐 4.3.4).

4.3. Results and Discussion

A variety of purification methods using concentrated culture supernatant were tested in order to develop an efficient isolation strategy for TS from the culture supernatant of

procyclic *T. congolense*. As described in Chapter 2 utilising these methods the existence of two TS forms from *T. congolense* was established, with a combination of anion exchange chromatography, isoelectric focusing (IEF), gel filtration and subsequently immunoaffinity chromatography, being employed to separate and purify the two TS forms (🗐 2.4.2.). The results of the preliminary purification experiments will be discussed here.

4.3.1. Isoelectric focusing (IEF)

Isoelectric focusing was performed using ampholytes possessing three different pH ranges. As can be seen in Fig. 1A, the usage of ampholytes with a wide pH range (pH 3-10) revealed one broad activity peak consisting of both, SA and transfer activity. In contrast, when using ampholytes with a narrow pH range (pH 4-6, Fig. 1B, pH 3-5, Fig. 1C), two peaks of activity were detected, both possessing SA and transfer activity. The isoelectric points (pI) for the two activity peaks were found to be in the range of pH 4-5 and pH 5-6.5, respectively.

4.3.2. Gel filtration

Gel filtration chromatography on Sephadex-G150 SF using concentrated culture supernatant detected two major activity peaks with a molecular weight of 350-600 kDa and 130-180 kDa (Fig. 1D). As was already seen in IEF experiments, two activity peaks consisting of SA and transfer activity were observed. However, one activity peak possessed a high transfer to SA activity ratio, whereas the other showed a low transfer to SA activity rate.

Further studies revealed that the activity peak with a high transfer to SA activity ratio and a pI of pH 4-5 consisted of a molecular weight of 350-600 kDa and was referred to as TS-form 1 (🗐 2.4.2.). On the other hand the activity peak with a low transfer to SA activity ratio

and a pI of pH 5-6.5 possessed a molecular weight of 130-180 kDa and was referred to as TS-form 2 (🗐 2.4.2.).

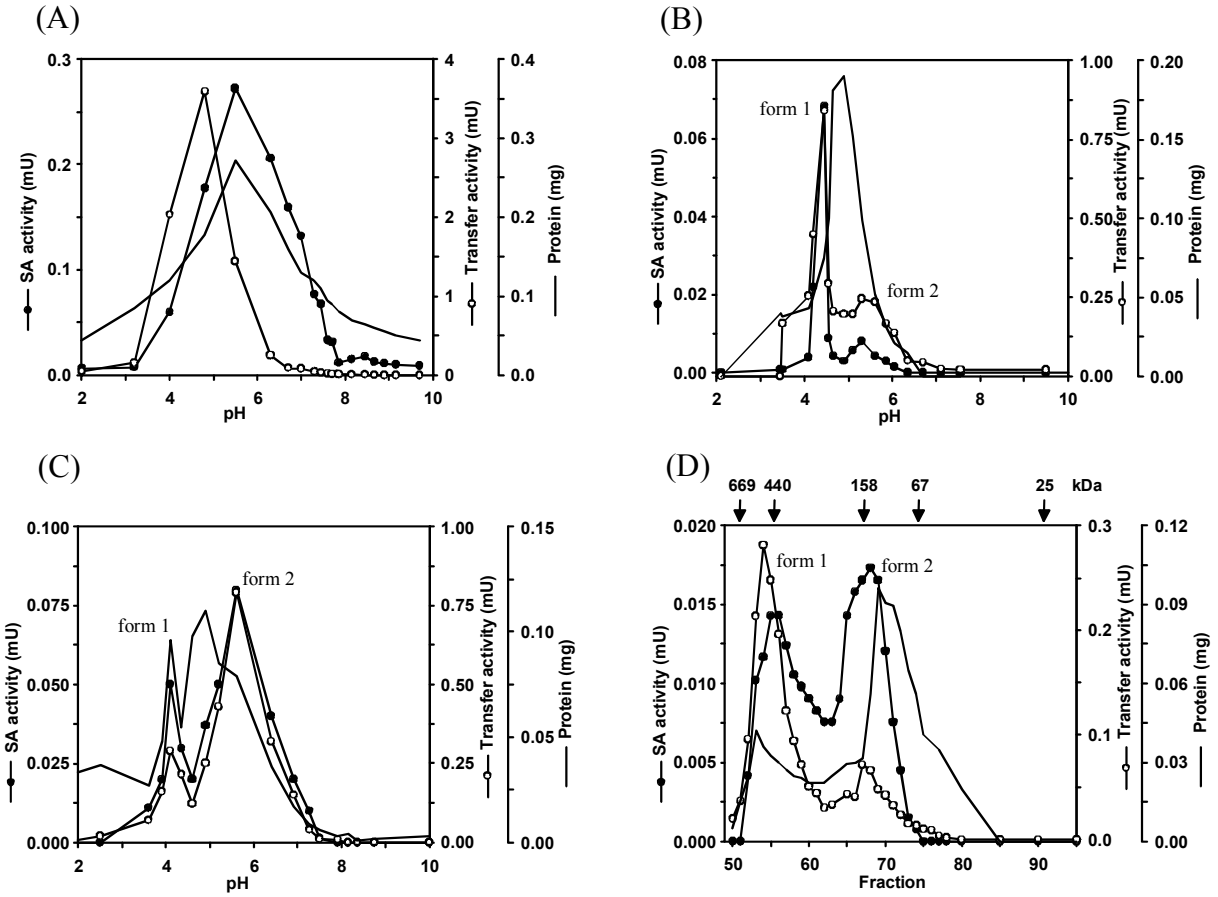

Fig. 1: Elution profiles of preliminary chromatography experiments using concentrated culture supernatant from *T. congolense*. (A) IEF chromatogram performed at a pH range 3-10. (B) IEF chromatogram performed at a pH range 4-6. (C) IEF chromatogram performed at a pH range 3-5. (D) Elution profile of gel filtration chromatography on Sephadex-G150 SF. The Sephadex-G150 SF column was calibrated with the following protein standards: thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), albumin (67 kDa) and chymotrypsinogen A (25 kDa).

The difference in the pI of both activity peaks was exploited by using ion exchange chromatography on Q-Sepharose FF as the first step in the purification cascade outlined in Chapter 2 (🗐 2.3.6.). As expected at the pH at which ion exchange chromatography was performed (pH 7.0), the activity peak with the lower pI (TS-form 1) eluted at higher a salt concentration, whereas the activity peak with the higher pI (TS-form 2) eluted at a lower salt concentration (🗐 2.4.2.) due to stronger binding to the Q-Sepharose FF.

4.3.3. Native-Polyacrylamide Gel electrophoresis (PAGE) and electro-elution

Another purification method attempted was Native-PAGE followed by electro-elution. In the silver stained native gel, illustrated in Fig. 2A, no defined protein bands were visible. Following electro-elution of the unstained portion of the native gel SA and transfer activities were detected in a number of the 5 fractions tested (Table 1). Fractions 1-3 were found to consist of a high transfer to SA activity ratio (ratio: 27), whereas fraction 4-5 revealed a low transfer to SA activity ratio (ratio: 1.4), again indicating that two TS forms from *T. congolense* exist.

Table 1: Activity determination after Native-PAGE

Sections (cm)	Protein (mg)	Total activity (mU)[1]		Specific Activity (mU/mg)		Enrichment (x-fold)		Recovery (%)	
		SA activity	Transfer activity	SA activity	Transfer activity	SA activity	Transfer activity	SA activity	Transfer activity
Fract.1 1 – 3.5	0.009	0	0.093	0	10	0	14	0	6
Fract.2 3.5 – 5	0.016	0	0.14	0	8.7	0	12	0	9
Fract.3 5 – 6.5	0.017	0.015	0.17	0.9	10	3	14	2	11
Fract.4 6.5 – 8	0.007	0	0.014	0	2	0	3	0	0.9
Fract.5 8 – 10	0.09	0.024	0.019	0.27	0.2	0.8	0.3	2	1.2

[1] One unit of activity equals one µmol of MU released per minute, which is equivalent to 1 µmol of sialic acid released or transferred per minute.

Furthermore, protein eluting from fractions 1-3, as analysed by immunoblotting, revealed a 90 kDa band which reacted with anti-*T. congolense* TS mAb 7/23 (Fig. 2B). The intensity of this protein band, which was later identified by micro-sequencing as TS (🗐 2.4.5.), was found to be proportional to the level of TS activity observed (Table 1). In

contrast, an antiserum to *T. cruzi* TS reacted with a protein band at about 70 kDa (Fig. 2C). However, the detection of this band was found not to correlate with the level of TS activity. No bands corresponding to the immuno-reactive 90 kDa band detected using anti-*T. congolense* TS mAb 7/23 were observed following SDS-PAGE and silver staining (Fig. 2D).

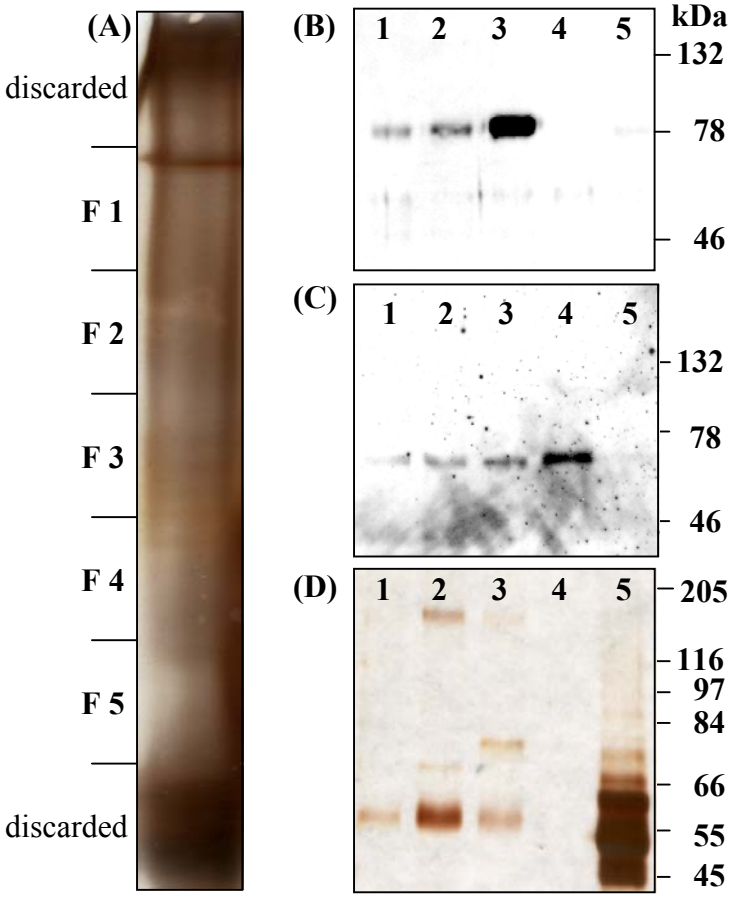

Fig. 2: Native-PAGE of concentrated culture supernatant from *T. congolense.* (A) and the analysis of the 5 fractions following electro-elution of the native gel *via* immunoblot with anti-*T. congolense* TS mAb 7/23 (B), *via* immunoblot with an antiserum to *T.cr.*TS (C) and *via* silver stained SDS-PAGE (D). Two to four µg of protein was applied to each well. Lane 1: fraction 1, lane 2: fraction 2, lane 3: fraction 3, lane 4: fraction 4; lane 5: fraction 5

4.3.4. Affinity chromatography

Equine submandibular gland mucin (ESM) and bovine submandibular gland mucin (BSM)

Mucins are glycoproteins, which are highly glycosylated. The terminal structure of ESM and BSM consist predominantly of sialic acids, α2,6 linked to GalNAc [6;7]. The sialic acid present is at least 30 % *O*-acetylated, in the case of ESM at position C-4, and in the case of BSM on the side chain (C-7, -8, -9) [8].

SA from human liver [9] and from *T. rangeli* [10] were purified by affinity chromatography on ESM. Although the employment of ESM or BSM has not been described for the purification of TS, both mucins were tested as affinity matrices for the isolation of TS. BSM and ESM were covalently linked to activated Sepharose 4B and utilised in affinity chromatography as outlined under "Experimental Procedures" (🗐 4.2.4.).

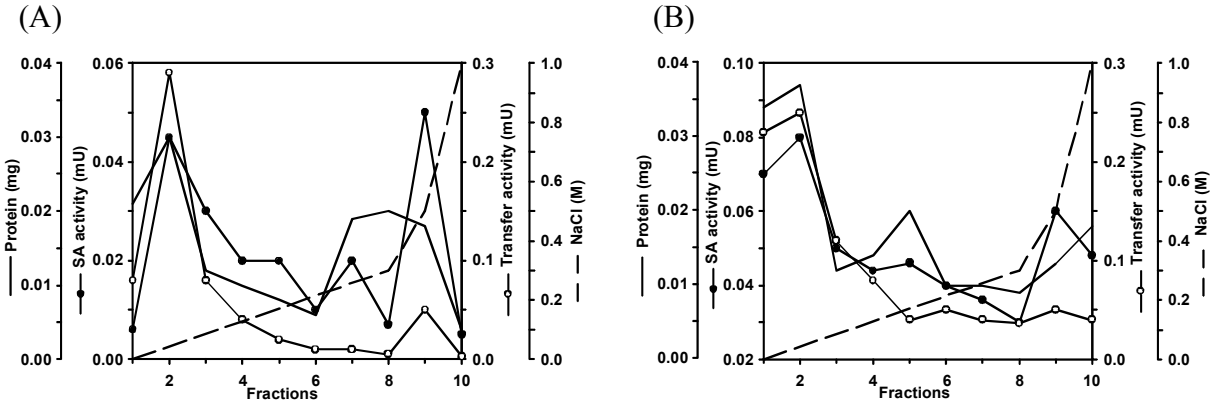

Fig. 3: Elution profiles of affinity chromatography on ESM- (A) and BSM-Sepharose (B) using concentrated culture supernatant from *T. congolense*.

As shown in Fig. 3A and 3B a separation of 2 activity peaks using both, ESM- and BSM-Sepharose was observed. At a salt concentration of less than 0.1 M an activity peak consisting of a high transfer to SA activity ratio was eluted. In contrast, with a salt concentration of greater than 0.5 M an activity peak with a low transfer to SA activity ratio

was obtained (Table 2). These findings further substantiated the results obtained using IEF, gel filtration and Native-PAGE which indicated the existence of two TS forms.

Table 2: Activity determination following affinity chromatography of concentrated culture supernatant from *T. congolense* on ESM- and BSM-Sepharose.

Elueted Fractions (NaCl)	Protein (mg)	Total activity (mU)[1]		Specific Activity (mU/mg)		Enrichment (x-fold)		Recovery (%)	
		SA activity	Transfer activity	SA activity	Transfer activity	SA activity	Transfer activity	SA activity	Transfer activity
ESM-Seph.									
0.075 M	0.03	0.045	0.3	1.5	10	3	5	4	6
0.5 M	0.018	0.05	0.05	2.8	2.8	6	2	4	1
BSM-Seph.									
0.075 M	0.034	0.08	0.23	2.3	6.8	5	6	5	6
0.5 M	0.013	0.06	0.05	4.6	3.8	10	3	4	1

[1] One unit of activity equals one µmol of MU released per minute, which is equivalent to 1 µmol of sialic acid released or transferred per minute.

Earlier reports described that *O*-acetylated sialic acids hinder the action of TS [11]. In order to elucidate whether *O*-acetylated sialic acids on ESM and BSM influence the binding behaviour of *T. congolense* TS, ESM and BSM were de-*O*-acetylated prior to affinity chromatography. However, de-*O*-acetylation had no effect on the binding of *T. congolense* TS, with similar purification results to the one using *O*-acetylated ESM and BSM being obtained.

Affinity chromatography on ESM- and BSM-Sepharose was, however, not utilised for the isolation of the two TS forms from *T. congolense* because of low recovery and the insufficient enrichment of TS activity. The weak binding of TS from *T. congolense* to ESM- and BSM-Sepharose may be due to the enzyme`s preference for α2,3 linked sialic acid. However, *T. rangeli* SA, which also prefers α2,3 linked sialic acid, was purified using ESM-Sepharose [10].

Various affinity matrices

A variety of affinity matrices, most of them mimicking either donor or acceptor substrates, were also trialed for their ability to purify TS from *T. congolense*. The conditions under which affinity chromatography was performed are outlined in Table 3.

Table 3: Affinity chromatography on various matrices using concentrated culture supernatant from *T. congolense*.

Matrices	Binding Conditions	Elution Conditions	Wash TS activity	Eluted TS activity	Comments
Neu5Ac-Sepharose 4B [1]	4 °C, overnight, 20 mM Bis/Tris, pH 7.0	0-1 M continuous NaCl gradient	70 %	3 %	Remaining activity not recovered
Neu5Ac-Sepharose 4B [1]	4 °C, overnight, 20 mM Bis/Tris, pH 7.0 and 0.5 mM lactose	0-1 M continuous NaCl gradient	65 %	4 %	Remaining activity not recovered
α Lactose-agarose [1]	4 °C, overnight, 20 mM Bis/Tris, pH 7.0	0-1 M continuous NaCl gradient	60 %	0 %	Remaining activity not recovered
N-(p-aminophenyl) oxamic acid-agarose [1]	4 °C, overnight, 20 mM Bis/Tris, pH 7.0	1 mM α2,3-SL	40 %	10 %	Remaining activity not recovered
		0.5 and 1 M NaCl	40 %	10 %	
N-acetyllactosamine-glycosorb [2]	4 °C, overnight, 20 mM Bis/Tris, pH 7.0	0.5 M and 1 M NaCl	75 %	0 %	Remaining activity not recovered
Concanavalin A-agarose [2]	4 °C, overnight, 20 mM Bis/Tris, pH 7.0	0-2 M discontinuous NaCl gradient	0 %	0 %	Activity remained bound
		0.5 and 1 M Methyl-α-D-mannopyranoside	0 %	0 %	
		0.1 M borate buffer, pH 8.0	0 %	0 %	
		0.5 M phosphate buffer, pH 6.8	0 %	0 %	

[1] Chromatography was performed on a 3 ml column at a flow rate of 1 ml/min. Binding and elution conditions are as stated. [2] Experiments were performed in a batch method. Binding and elution conditions are as stated.

As can be seen in Table 3, most of affinity materials tested were unable to bind TS activity, even if the complementary acceptor substrate was added to the binding buffer. For instance, no binding of TS activity to Neu5Ac-Sepharose was observed, even though reports showed the successful use of this affinity matrix for the purification of *Vibrio cholerae* SA and the partial purification of TS from *T. cruzi*. However, in the latter case very low recovery of TS activity was obtained [12]. In contrast, the synthetic SA inhibitor *N*-(4-nitrophenyl)oxamic acid coupled to agarose was able to bind TS activity, although insufficiently. Furthermore, Concanavalin A bound enzymatic activity very strongly, however, TS activity could not be eluted using either salt or methyl-α-D-mannopyranoside which should provide specific elution. These findings are similar to those reported for the isolation of TS from *T. b. brucei* [13], as well as for bovine erythrocyte acetylcholinesterase [14]. Due to the above stated inability of the matrices tested to bind or elute TS activity, as well as the low recovery rates, these affinity matrices were not employed for the purification of TS from *T. congolense*.

The experiments performed using concentrated culture supernatant showed the existence of two TS forms from *T. congolense*. Both TS forms were found to possess SA and transfer activity, but consisted of either a low or a high transfer to SA ratio. The preliminary purification experiments using concentrated culture supernatant from *T. congolense* described in this chapter allowed for the development of optimal isolation methods for both TS forms to be established (🗐 2.4.2.). Furthermore, the partial purification of both TS forms gave the opportunity to raise anti-*T. congolense* TS mAb. With the aid of these antibodies an efficient affinity chromatography was established which was subsequently used in the final purification step of both TS forms from *T. congolense* (🗐 2.4.4.).

Molecular biology

4.4. Background

Utilising a PCR-based approach two TS gene copies from *T. congolense* (*T. congolense* TS1 and *T. congolense* TS2), which share only 50 % identity with each other, but show significant similarity with known viral, bacterial and trypanosomal SA and TS (🗐 Chapter 3) were identified. However, both TS gene sequences obtained were incomplete. In order to obtain the entire gene sequence for both *T. congolense* TS Southern blot analyses and cloning experiments utilising both partial TS sequences from *T. congolense* as probes were performed.

4.5. Experimental Procedures

4.5.1. Reagents and general methods

All reagents, trypanosomes and general methods were used as already stated in Chapter 3 (🗐 3.3.). Additionally, the PCR DIG Probe synthesis Kit, the DIG Quantification Teststrips, the DIG Control Teststrips and the DIG Luminescent Detection Kit were purchased from Roche Molecular Biochemicals (Mannheim, Germany) and used as described in the Handbook "The DIG System User´s Guide for Filter Hybridisation".

4.5.2. DNA probe labelling

Both partial TS gene sequences from *T. congolense, T. congolense* TS1 and *T. congolense* TS2, were obtained by PCR using specific primers and conditions as stated in

section 3.3.6.. Following purification using QIAquick PCR purification kit (Qiagen, Hilden, Germany) probes were labelled with digoxigenin (DIG) by PCR. A reaction mixture containing 1 µl of template (28 ng of *T. congolense* TS1 or *T. congolense* TS2), 25 pmol of each primer (TconTS1Ps and TconTS1Pas or TconTS2Ps and TconTS2Pas, 🗐 3.3.6.), 5 µl of PCR DIG Probe Synthesis Mix (containing DIG labelled dUTP) and 5 µl reaction buffer in a final volume of 49.5 µl was prepared. Subsequently, a hot-start PCR in a "T Gradient Thermocycler" (Biometra GmbH, Göttingen) was performed and after the addition of 0.5 µl of *Taq* DNA polymerase at 72 °C the following program was applied: 30 x (30 s 94 °C, 45 s 52 °C, 2 min 72 °C), 7 min 72 °C. PCR reactions were analysed using a 0.7 % agarose gel stained with ethidium bromide. DIG labelled PCR products were extracted and purified from the gel employing QIAquick extraction kit (Qiagen), quantified using the DIG Quantification Teststrips and used as a probe for hybridisation experiments.

4.5.3. Southern blotting and hybridisation

Genomic DNA from procyclic *T. congolense* was digested with different combinations of restriction enzymes (🗐 4.6.2., Fig. 2) for 2 h at 37 °C. The samples were electrophoresed in a 0.7 % agarose gel and transferred overnight to Hybond N^+ nylon membranes (Amersham, Freiburg, Germany) as described in the DIG user´s guide. Following denaturation and immobilistion of DNA the membrane was prehybridised for at least 1 h in 5 x standard saline citrate buffer containing 0.1 % sodium lauroylsarcosine, 0.02 % SDS, 2 % blocking reagent and 50 % formamide (standard buffer, see DIG user´s guide) at 39 °C and subsequently hybridised with the DIG-labelled probe (30 ng/ml in standard buffer) for 14-16 h at 39 °C. Following hybridisation the membrane was washed twice for 5 min at 30 °C in 2 x standard saline citrate buffer containing 0.1 % SDS and twice for 20 min at 64 °C in 0.5 x standard saline citrate buffer containing 0.1 % SDS. The DIG labelled probe was detected using an

anti-DIG antibody and the chemiluminescent alkaline phosphatase substrate CSPD® as described in DIG user`s guide.

4.5.4. Cloning of DNA fragments

Genomic DNA from procyclic *T. congolense* was digested with BamH I and Sph I for 2 h at 37 °C and separated on a 0.7 % preparative agarose gel. Following electrophoresis one vertical lane was cut from the gel, blotted, hybridised and the exact size of DNA fragments detected with DIG labelled probe as outlined in section 4.5.3.. According to their size, gel portions containing DNA fragments detected with the DIG labelled probe were excised, extracted and purified from the gel using the QIAquick extraction kit (Qiagen) and introduced into the appropriate restriction sites of pUC19 (Biolabs, Frankfurt, Germany) to generate pUC19/insert. *E. coli* XL-1 blue MRF´ from Stratagene (Amsterdam, The Netherlands) were transformed with pUC19/insert by electroporation in a Gene-Pulser™ from Biorad (Munich, Germany) and, subsequently, plated onto LB agar plates containing 0.5 mM IPTG, 20 mg/ml X-Gal and 50 µg/ml ampicillin. The resulting colonies were transferred to Hybond N^+ nylon membranes (Amersham) and hybridised with DIG-labelled probe (see DIG user`s guide). DIG labelled probe detection was performed as described above. Positive colonies were used to prepare 4 ml overnight cultures. Following plasmid isolation the clones were sequenced by MWG Biotech (Ebersberg, Germany).

4.6. Results and Discussion

4.6.1. DNA probe labelling

The partial sequences *T. congolense* TS1 (1491 bp) and *T. congolense* TS2 (830 bp) were labelled with DIG *via* PCR and used as probes in Southern blotting and hybridisation experiments in order to obtain full sequence information for both partial *T. congolense* TS gene sequences. Due to multiple incorporation of DIG-dUTP following the PCR process the molecular weight of the PCR product increased significantly compared to the unlabelled product (Fig. 1). The quantification of the DIG labelled probes revealed a concentration of 3 ng/µl for *T. congolense* TS1-DIG and 1 ng/µl for *T. congolense* TS2-DIG. The yield of the labelling reaction was low with 150 ng/µl of *T. congolense* TS1-DIG and 50 ng/µl of *T. congolense* TS2-DIG per PCR reaction being obtained.

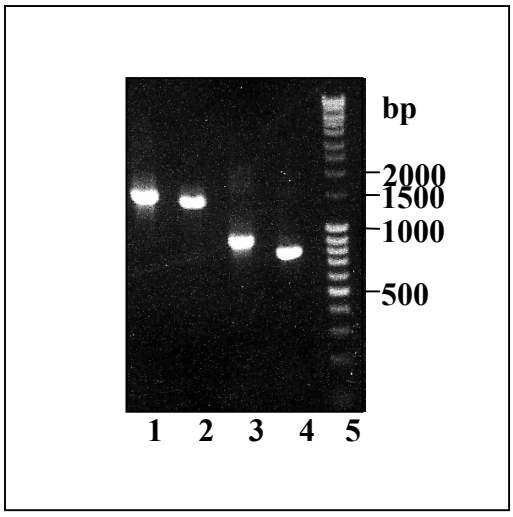

Fig. 1: Labelling of *T. congolense* TS1 and *T. congolense* TS2 with DIG-dUTP during PCR. Lane 1: labelled *T. congolense* TS1, lane 2: unlabelled *T. congolense* TS1 (1491 bp), lane 3: labelled *T. congolense* TS2 (830 bp), lane 4: unlabelled *T. congolense* TS2 (830 bp), lane 5: molecular weight marker.

4.6.2. Southern blotting and hybridisation

Genomic DNA was digested with a variety of different restriction enzymes. The choice of enzymes was made according to the following criteria: The enzymes should not cut within the known sequences of *T. congolense* TS1 and *T. congolense* TS2 and therefore not within the labelled probe. The enzymes chosen, however should be able to cut in the multiple cloning site of pUC19. As can be seen in Fig. 2 the restriction enzymes BamH I, Sph I, Hind III, Sal I, Acc I and Hinc II were used in single or double digests in various combinations. The hybridisation with *T. congolense* TS1-DIG (Fig. 2A) and *T. congolense* TS2-DIG (Fig. 2B) led to the detection of different DNA fragments (Table 1).

Table 1: Detected DNA fragments (kbp) after hybridisation with DIG-labelled *T. congolense* TS1 and *T. congolense* TS2

Restriction enzymes	TS1-DIG	TS2-DIG
BamH I + Sph I	4.9; 4.6; 3.2	4.8; 3.6; 3.5
Hind III + Sal I	15.0; 4.8	4.6
Sph I	14.0; 13; 3.8	14.0; 13.0
Sal I	22; 17.0	5.0; 4.6
Acc I + BamH I	16.0	14; 5.2; 5.0
Hind III + Hinc II	1.7	1.1

The different hybridisation pattern observed supports the hypothesis that two different TS genes from *T. congolense* exist (🗐 3.4.3. and 3.5.). Depending on the restriction enzyme used, one to three fragments of different sizes for each probe were detected (Table 1, Fig. 2A and 2B).

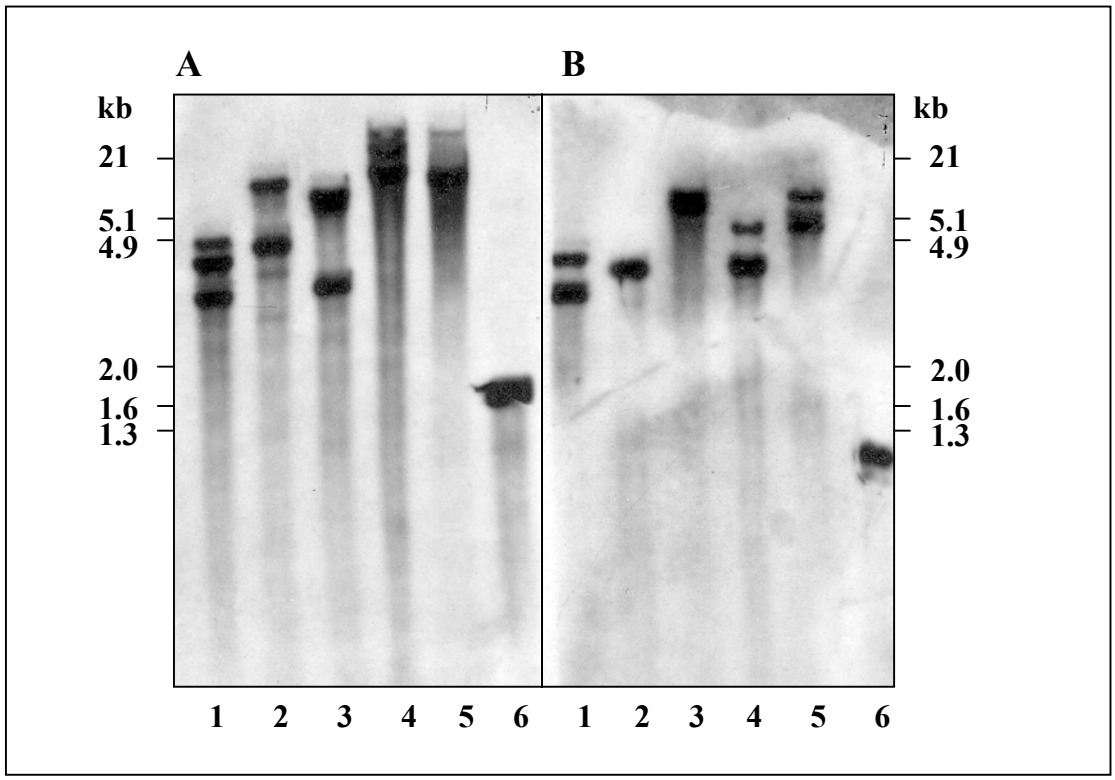

Fig. 2: Southern Blot analysis of genomic DNA from *T. congolense* after restriction enzyme digest. Hybridisation was performed with DIG labelled *T. congolense* TS1 (A) and *T. congolense* TS2 (B). Digests performed were: lane 1, BamH I and Sph I; lane 2, Hind III and Sal I; lane 3, Sph I; lane 4, Sal I; lane 5, Acc I and BamH I, lane 6, Hind III and Hinc II.

Since the enzymes were chosen so as not to cut within the probe one would conclude that for each of the identified TS genes from *T. congolense* up to three copies exist. These copies may be identical or very similar to each other, as they were detected in hybridisation experiments under specific conditions using a single probe. The exact number of gene copies can not be stated with certainty, since some of the enzymes may have cut in the so far unknown *T. congolense* TS gene sequences. However, the findings suggest that two genes with a identity of 50 % exist (*T. congolense* TS1 and *T. congolense* TS2), which may have up to three copies with a higher identity. This would explain why the gene copies of *T. cruzi* TS and *T. b. brucei* TS so far identified share high identity with each other (Fig. 3 and [15], respectively), whereas the two *T. congolense* TS genes identified exhibit just 50 % identity (🗐 3.6., Fig. 2). Furthermore, the number of TS gene copies found for *T. congolense* is

similar to that described for the African *T. b. brucei* TS [15], but different to the American trypanosome *T. cruzi*, where the TS family comprises at least 140 members [16].

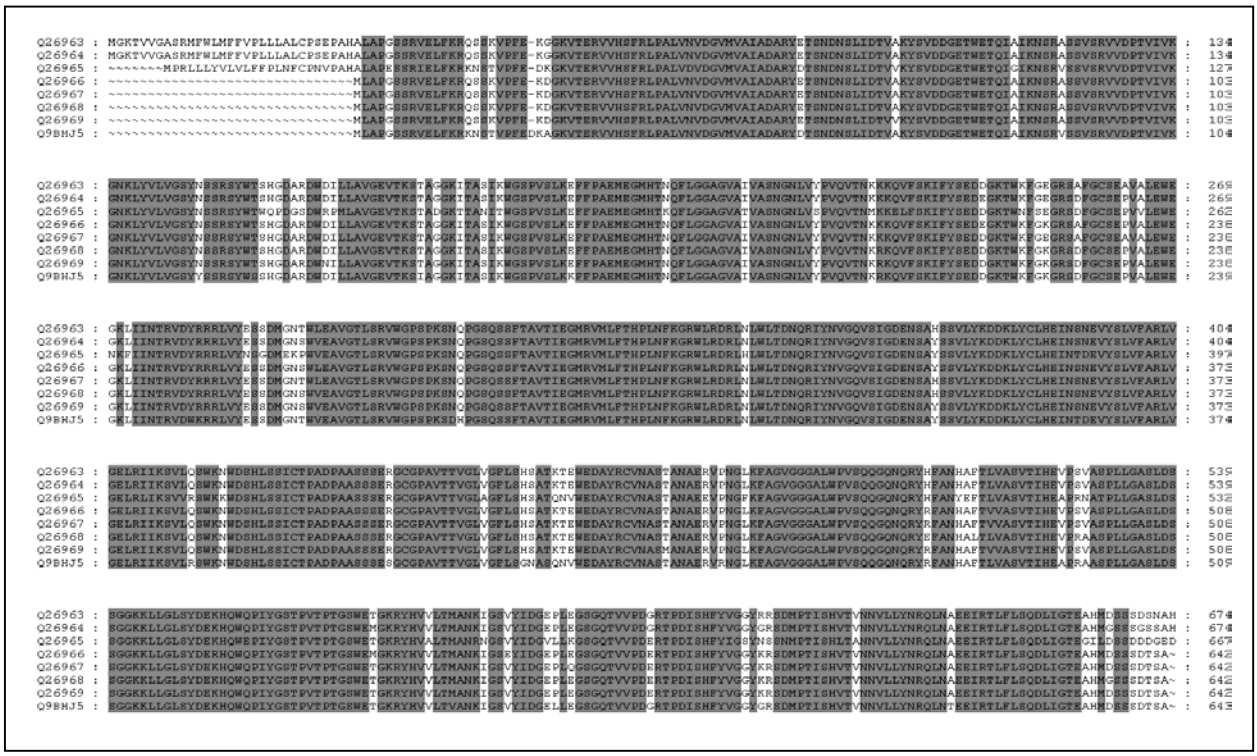

Fig. 3: Sequence comparison of *T. cr*uzi TS genes. The sequences were aligned with CLUSTALW and subsequently shaded using GENEDOC. Identical residues in all sequences are shaded grey. The sequences were taken from Swissprot and their accession numbers are stated at the start of each sequence. Some sequences have been shortened.

4.6.3. Cloning of DNA fragments

Restriction enzymes BamH I and Sph I were chosen for the preparative digestion of genomic DNA, since DNA fragments detected (Table 1) possessed sizes suitable for ligation into pUC19. Additionally, the fragments detected with the two different probes were of similar size, which would increase the chance of obtaining both *T. congolense* TS genes (*T. congolense* TS1 and *T. congolense* TS2). As can be seen in Fig. 4 six DNA samples were isolated (🗐 4.5.4.) varying in sizes from 2.5 to 5.0 kbp, each of them containing a mixture of DNA fragments.

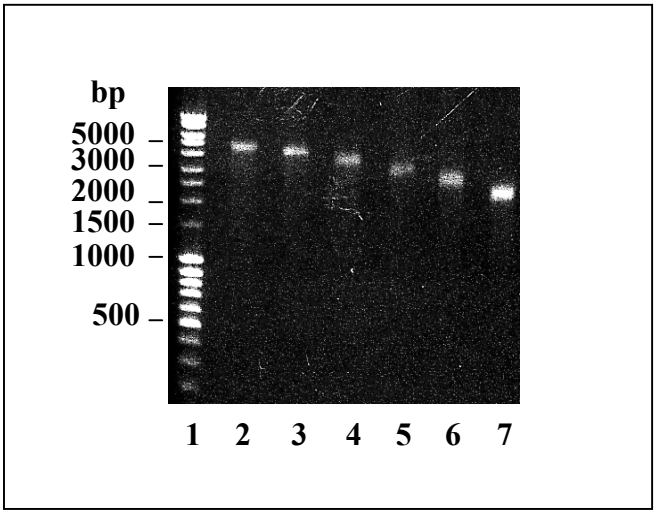

Fig. 4: Isolated and purified DNA samples which were detected through hybridisation with *T. congolense* TS1 and *T. congolense* TS2 and were subsequently used for cloning experiments.

Following the introduction of these fragments into pUC19 and transformation into *E. coli*, the resulting colonies were screened with both of the two DIG labelled probes. All positive clones (13) were purified and subsequently sequenced, however, the clones contained no TS sequence information. Instead, other sequences were identified which shared a 30-40 % similarity to the RHS2a proteins and Delta tubulin of *T. b. brucei*, as well as some sequence identified showed 78 % similarity to the glycosomal malate dehydrogenase of *T. b. brucei.*

It is unclear at this stage why none of the hybridising clones contained TS sequence information. Further studies are required to optimise the conditions for colony hybridisation, in order to detect TS positive clones with certainty. Since trypanosome genes do not contain introns [17], it seemed sensible to utilise genomic DNA, as well as to employ it in a similar approach to that undertaken for the TS from *T. b. brucei* [15]. However, if Southern blot analyses do not lead to full gene sequence information of *T. congolense* TS the isolation of mRNA should be considered. This would enable techniques like RT-PCR (reverse transcription PCR) and RACE (Rapid Amplification of cDNA ends), otherwise known as anchored PCR, to be performed. Another approach would be to construct a cDNA library

which would be screened by PCR using gene specific primers, or *via* colony hybridisation. The availability of full sequence information for both *T. congolense* TS and related genes will hopefully allow the recombinant expression of active enzyme.

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CHAPTER 5

Summaries and Conclusions

5.1. Summary (English)

Trypanosoma congolense is the agent of Nagana, the trypanosomiasis in African ruminants. During its insect stage the parasite expresses an enzyme called trans-sialidase (TS), which sialylates the major cell surface glycoprotein (GARP) of the parasites in the vector. Thus, a negatively charged glycocalyx is formed which is believed to protect the parasites from digestive conditions in the fly gut and may enable them to interact with epithelial cells [1;2].

Unlike typical sialyltransferases, which require CMP-activated sialic acid as the monosaccharide donor [3], TS catalyses the transfer of, preferably, α2,3-carbohydrate-linked sialic acids to another carbohydrate forming a new α2,3-glycosidic linkage to galactose or *N*-acetylgalactosamine. In the absence of an appropriate acceptor TS acts like a sialidase (SA), similar to viral, bacterial, mammalian and trypanosomal SA, hydrolysing glycosidically linked sialic acids [4;5].

Thus far, the native TS of the American *T. cruzi* [6-8] and the African *T. b. brucei* [1] trypanosome have been studied in detail. In the case of *T. cruzi* [5] the primary sequence has been known for quite some time, whereas the sequence of the African trypanosome *T. b. brucei* was only reported recently [9].

This study describes the purification and characterisation of two TS forms from the African trypanosome *T. congolense.* The purification of these forms using a combination of anion exchange chromatography, isoelectric focusing, gel filtration and, subsequently, antibody affinity chromatography resulted, in both cases, in the isolation of a 90 kDa monomer on SDS-PAGE which was identified as TS using micro-sequencing. Monoclonal antibody 7/23, which bound and partially inhibited TS activity, was found in both cases to react with a 90 kDa protein. Both *T. congolense* TS forms possessed SA and transfer activity, but markedly differed in their activity ratios. The TS form with a high transfer to SA activity

ratio, referred to as TS-form 1, exhibited a pI of pH 4-5. In contrast, the form with a low transfer to SA activity ratio, referred to as TS-form 2, possessed a pI of pH 5-6.5. TS-form 2, as observed by gel filtration, seems to form homodimers (~180 kDa), whereas TS-form 1 probably exists as oligomers (tetramer or higher), resulting in the high molecular weight observed by gel filtration (~350-600 kDa).

Both TS forms from *T. congolense* were not significantly inhibited by known SA inhibitors and were found to have the same donor and acceptor substrate preferences. However, TS-form 1 was able to utilise the acceptor substrates more efficiently than TS-form 2. The donor substrates tested, on the other hand, were utilised with similar efficiencies by both TS forms. Furthermore, SA activity was predominately found in TS-form 2, whereas TS-form 1 possessed significantly less SA activity and higher transfer activity. The results suggest that the transfer activity associated with TS-form 2 is decreased due to reduced acceptor binding capacity. The reduced acceptor binding capacity of TS-form 2 might be also the reason for the described result that only TS-form 1 can interact with GARP, the major cell surface glycoprotein of *T. congolense* in the vector. This interaction with GARP could even mediate or facilitate the formation of oligomers of TS-form 1.

This study also describes the identification of two different partial TS gene sequences from *T. congolense* utilising a PCR-based approach. Both *T. congolense* TS genes, TS1 and TS2, which share only 50 % identity with each other, show significant similarity with known viral, bacterial and trypanosomal SA and TS. Interestingly, *T. congolense* TS1 and *T. congolense* TS2 possess a comparable degree of similarity (67 and 63 %, respectively) to *T. b. brucei* TS. However, in comparison to *T. congolense* TS1 that possesses 54 % and 53 % similarity to *T. cruzi* TS and *T. rangeli* SA, respectively, *T. congolense* TS2 shares a higher degree of similarity with the American trypanosomal enzymes *T. cruzi* TS (64 %) and *T. rangeli* SA (63 %).

A comparison of the catalytic domain in both *T. congolense* TS sequences revealed the conservation of conserved motifs (e.g. Asp boxes, FRIP), as well as critical active site residues displayed in viral, bacterial and trypanosomal SA and TS. Furthermore, in both *T. congolense* TS sequences amino acid residues required for sialic acid binding are conserved.

T. congolense TS1 exhibits most of the critical active site amino acid residues found in *T. rangeli* SA, *T. cruzi* TS and *T. b. brucei* TS, with the exception of one conservative substitution, W313 in *T. cruzi* TS to Y255 in *T. congolense* TS1. In addition, *T. congolense* TS1 possesses the same amino acids that are conserved in the two trypanosomal TS, but differ in *T. rangeli* SA. More importantly, a peptide sequence (VVDPTVVAK) derived from the native, active TS-form 1 was found within the *T. congolense* TS1 sequence. Taken together, these findings suggest that the partial sequence of *T. congolense* TS1 encodes an active enzyme with SA and transfer activity.

In contrast, in the *T. congolense* TS2 sequence three critical residues of the active site, which are present in *T. cruzi* TS, *T. b. brucei* TS and *T. congolense* TS1 (Y120, A180, S286 *T. cruzi* TS numbering), and have been postulated to be important for acceptor binding, are substituted by the amino acids P, V and G, respectively. These findings suggest that the protein encoded by *T. congolense* TS2 exhibits variations in its transfer activity due to reduced acceptor binding capacity.

To this point, no crystal structure of TS exists. Even though a comparison of the crystal structure of *T. rangeli* SA with the model of *T. cruzi* TS has been carried out [10], neither the exact mechanism of the transfer reaction nor the reasons why TS is more efficient in transferring than hydrolysing terminal sialic acid are understood. The results in this study will aid in the understanding of the mechanism and functionality of TS, and with that will boost its biotechnological applications. Furthermore, findings reported here will enhance the opportunity to develop high potential, structure-based TS inhibitors. Given that trypanosomiasis has reached epidemic magnitude in some countries, one should consider

methods to control not only the disease, but also its transmission stage inside the vector [11]. TS inhibitors could serve not only in combating trypanosomes inside the host, in the case of *T. cruzi,* but also inside the vector, in the case of *T. b. brucei* and *T. congolense*.

5.2. Summary (German)

Trypanosoma congolense ist der Erreger der Nagana, einer in Afrika vorkommenden Rinderseuche. Während des Insektenstadiums exprimiert der Parasit das Enzym Trans-sialidase (TS), welches das Hauptoberflächenglykoprotein des Parasiten im Insektenstadium, GARP, sialyliert. Dadurch entsteht eine negativ geladene Glykoproteinschicht, von der angenommen wird, dass sie den Parasiten vor der Verdauung im Fliegenmagen schützt und zusätzlich eine Interaktion mit Epithelzellen ermöglicht [1].

Im Gegensatz zu den typischen Sialyltransferasen, welche CMP aktivierte Sialinsäure als Donor benötigen [3], ist die TS in der Lage, den Transfer von vornehmlich α2,3-glykosidisch gebundenen Sialinsäuren von einem Kohlenhydrat auf ein anderes zu übertragen, wobei haupsächlich Galaktose und *N*-Acetylgalaktosamin als Akzeptoren dienen. In der Abwesenheit eines geeigneten Akzeptors verhält sich die TS ähnlich wie die bereits bekannten viralen, bakteriellen und trypanosomalen Sialidasen (SA) und hydrolysiert glykosidisch gebundene Sialinsäuren [4].

Bisher wurden die TS des amerikanischen Trypanosomen *T. cruzi* [6] und des afrikanischen Trypanosomen *T. b. brucei* [1] im Detail beschrieben. Im Falle von *T. cruzi* ist die Gensequenz bereits seit einiger Zeit bekannt [5], während die Sequenz des afrikanischen Trypanosomen *T. b. brucei* erst kürzlich veröffentlicht wurde [9].

In der vorliegenden Arbeit wird die Aufreinigung und Charakterisierung zweier TS Formen des afrikanischen Trypanosomen *T. congolense* beschrieben. Die Aufreinigung der zwei Formen gelang mittels einer Kombination aus Ionenaustauschchromatographie, Isoelektrischer Fokussierung, Gelfiltration und anschließender Antikörper-Affinitäts-chromatographie. In beiden Fällen wurde nach SDS-PAGE eine Proteinbande von 90 kDa als Monomer erhalten, das mit Hilfe der Mikrosequenzierung als TS identifiziert werden konnte. Eigens produzierte monoklonale Antikörper, mAb 7/23, die TS binden und teilweise

inhibieren konnten, zeigten eine Reaktion mit dem 90 kDa Protein beider TS Formen. Beide Formen besitzen SA- und Transferaktivität, allerdings mit unterschiedlichem relativen Verhältnis zueinander. Die TS Form mit dem höheren Transfer-/SA-Aktivitätsverhältnis, welche als TS-Form 1 bezeichnet wurde, wies einen pI Wert von pH 4-5 auf. Im Gegensatz dazu präsentierte die Form mit einem niedrigeren Transfer-/SA-Aktivitätsverhältnis einen pI von pH 5-6,5 und wurde als TS-Form 2 bezeichnet. Während TS-Form 2, wie bei der Gelfiltration beobachtet, Homodimere zu formen scheint (~ 180 kDa), bildet TS-Form 1 möglicherweise Oligomere, womit das bei der Gelfiltration beobachtete hohe Molekulargewicht (~ 350-600 kDa) zu erklären wäre.

Beide *T. congolense* TS Formen werden durch bekannte Sialidaseinhibitoren nicht signifikant gehemmt und bevorzugen ähnliche Donor- und Akzeptorsubstanzen. Allerdings konnte TS-Form 1 die Akzeptorsubstrate mit höherer Effizienz nutzen, während beide Formen die getesteten Donorsubstanzen mit gleicher Effizienz umsetzten. Interessanterweise wies TS-Form 2 vor allem SA-Aktivität auf, während TS-Form 1 weniger SA-Aktivität zeigte, dafür aber eine höhere Transferaktivität. Die Ergebnisse lassen vermuten, dass bei TS-Form 2 die Akzeptorbindungskapazität reduziert ist, was zu einer verringerten Transferaktivität führt. Dies könnte auch der Grund dafür sein, dass, wie in der Arbeit gezeigt, nur TS-Form 1 mit GARP, dem Hauptoberflächenglykoprotein von *T. congolense* im Insektenstadium, interagiert. Dadurch könnte die Bildung von Oligomeren der TS-Form 1 begünstigt oder eventuell erst ermöglicht werden.

Diese Arbeit beschreibt außerdem die Identifizierung zweier partieller TS Gensequenzen von *T. congolense* mittels eines auf der PCR basierenden molekularbiologischen Versuchsansatzes. Beide TS Gensequenzen sind sich nur zu 50 % identisch, zeigen aber signifikante Übereinstimmungen mit bereits bekannten viralen, bakteriellen und trypanosomalen SA und TS. Interessanterweise besitzen sowohl die längere Sequenz (*T. congolense* TS1) als auch die kürzere Sequenz (*T. congolense* TS2) ein

vergleichbares Maß an Ähnlichkeit zur Primärsequenz von *T. b. brucei* TS. Allerdings, im Vergleich zur längeren Sequenz *T. congolense* TS1, welche 54 % Ähnlichkeit zur *T. cruzi* TS und 53 % Ähnlichkeit zur *T. rangeli* SA besitzt, weist die kürzere Sequenz *T. congolense* TS2 einen höheren Grad an Ähnlichkeit zu den amerikanischen trypanosomalen Enzymen, *T. cruzi* TS (64 %) und *T. rangeli* SA (63 %), auf.

In der katalytischen Domäne beider *T. congolense* TS Sequenzen finden sich sowohl homologe Bereiche (z. B. Asp Boxen, FRIP-Region) als auch wichtige Aminosäuren des aktiven Zentrums, die in viralen, bakteriellen und trypanosomalen SA und TS konserviert sind. So sind in beiden *T. congolense* TS Sequenzen Aminosäuren, die für die Sialinsäurebindung notwendig sind, konserviert.

T. congolense TS1 besitzt fast alle Aminosäuren des aktiven Zentrums, welche auch in der *T. rangeli* SA, *T. cruzi* TS und der *T. b. brucei* TS vorhanden sind, mit einer Ausnahme, den konservativen Austausch W313 in *T. cruzi* TS zu Y255 in *T. congolense* TS1. Zusätzlich besitzt *T. congolense* TS1 die gleichen Aminosäuren, die auch in den beiden trypanosomalen TS konserviert, aber in *T. rangeli* SA nicht vorhanden sind. Bemerkenswert ist auch, dass die mittels Mikrosequenzierung der TS-Form 1 gewonnene Peptidsequenz, VVDPTVVAK, in der Sequenz von *T. congolense* TS1 wiedergefunden wurde. Die Ergebnisse lassen erwarten, dass die *T. congolense* TS1 Sequenz ein Enzym kodiert, welches SA- und Transferaktivität besitzt.

Im Gegensatz dazu sind in der *T. congolense* TS2 Sequenz drei kritische Reste, die in der *T. cruzi* TS, *T. b. brucei* TS und der *T. congolense* TS1 vorhanden sind (Y120, A180, S286 *T. cruzi* TS Nummerierung) und wahrscheinlich für die Akzeptorbindung eine bedeutende Rolle spielen, gegen die Aminosäuren P, V und G ausgetauscht. Diese Ergebnisse lassen vermuten, dass *T. congolense* TS2 für ein Protein kodiert, das Variationen in der Transferaktivität aufweist, was möglicherweise auf eine verminderte Akzeptorbindung zurückzuführen wäre.

Bisher konnte noch keine Kristallstruktur einer TS erstellt werden. Obwohl ein Vergleich der Kristallstruktur von *T. rangeli* SA mit dem Modell der *T. cruzi* TS durchgeführt wurde [10], sind weder der exakte Mechanismus der Transferreaktion noch der Grund aufgeklärt, warum die TS effizienter im Transfer als in der Hydrolyse von Sialinsäuren ist. Die Ergebnisse dieser Arbeit werden helfen, den Mechanismus und die Funktionalität der TS zu verstehen und damit ihre biotechnologische Anwendung zu erweitern. Außerdem ist zu erwarten, dass die hier gewonnenen Erkenntnisse dazu beitragen werden, auf der Proteinstruktur basierende TS Inhibitoren zu entwickeln. Auf Grund der epidemischen Ausmaße der Trypanosomiasis in einigen Ländern wäre nicht nur eine Bekämpfung der Krankheit, sondern auch die Kontrolle ihrer Übertragung wichtig [11]. TS Inhibitoren könnten also nicht nur der Bekämpfung der Trypanosomen im Wirt, wie im Falle von *T. cruzi*, sondern auch im Vektor, wie im Falle von *T. b. brucei* und *T. congolense,* dienen.

5.3. References

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CHAPTER 6

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